CYCLIC NUCLEOTIDE METABOLISM IN THE
STREPTOZOTOCIN DIABETIC RAT CORPUS CAVERNOSUM;
RELEVANCE TO HUMAN DIABETIC ERECTILE DYSFUNCTION

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BY

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Diabetes mellitus is an important risk factor for the development of male erectile dysfunction. Notwithstanding the effects of diabetes in terms of angiopathy and neuropathy which have been thought to be the principal causes for erectile dysfunction there are well described alterations in the transduction mechanisms that are responsible for normal penile erection. Much attention has focused recently on the role of nitric oxide as the main neurotransmitter responsible for the mediation of penile erection. Using the streptozotocin rat model of diabetes mellitus the effects of diabetes upon nitric oxide mediated transduction mechanisms was studied using a variety of experimental techniques. Nitric oxide synthase autoradiography demonstrated that at two and six months duration of diabetes there was an increased nitric oxide synthase binding compared to control rats. Further investigations demonstrated the effects of experimental diabetes in the same model upon guanylate and adenylate cyclase, the enzymes responsible for the synthesis of 3’5’ cyclic guanosine monophosphate and 3’5’ adenosine monophosphate respectively. These two important second messengers are crucial for the control of smooth muscle relaxation and hence penile erection. It was found that the activity of these cyclases as measured by generation of the cyclic nucleotides determined by radioimmunoassay were significantly increased in the diabetic model. Further experiments were performed to elucidate changes in the activity of the phosphodiesterase enzymes which act specifically to hydrolyse the cyclic nucleotides, it was found that diabetes resulted in a reduction of phosphodiesterase activity. Taken together, these findings would seem to support the hypothesis that penile tissues adapt to the diabetic environment in a manner which attempts to maintain cyclic nucleotide bioavailability. Further studies were carried out to examine the effects of various vascular risk factors on cyclic nucleotide metabolism in an acute experimental setting. A clinical study was also undertaken to examine the possible role of fibrinogen as a risk factor in the development of erectile dysfunction, it is our impression from this preliminary study that fibrinogen is indeed a novel risk factor for erectile dysfunction. The relevance of our findings in the streptozotocin diabetic rat model to human erectile dysfunction are discussed, as is the validity of the model, possibilities for future investigations and areas for novel drug interventions.
To Charlotte, David, Harry, Michael
and my father
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ABBREVIATIONS

ACh  acetylcholine
Ad   adrenalin
AS   atherosclerosis
ATP  adenosine triphosphate
AVP  arginine vasopressin
BCR  bulbocavernosus reflex
cAMP adenosine-3’5’ cyclic monophosphate
cGMP guanosine-3’5’ cyclic monophosphate
CGRP calcitonin gene related peptide
DM   diabetes mellitus
DNA  deoxyribonucleic acid
ED   erectile dysfunction
EDRF endothelium-derived relaxing factor
EFS  electrical field stimulation
ET   endothelin
HDL  high density lipoprotein
5-HT 5-hydroxytryptamine
IBMX isobutylmethylxanthine
IDDM insulin dependent diabetes mellitus
IIDM ischaemic heart disease
LDL  low density lipoprotein
MEM  minimum essential medium
mRNA messenger ribonucleic acid
NAd noradrenalin
NANC nonadrenergic noncholinergic
NaNP sodium nitroprusside
NIDDM non-insulin dependent diabetes mellitus
NO   nitric oxide
NOS  nitric oxide synthase
NSAID non-steroidal antiinflammatory drug
NPT  nocturnal penile tumescence
NPY  neuropeptide Y
NZW New Zealand White (rabbit)
oxLDL oxidised low density lipoprotein
PAP  papaverine
PDE(I) phosphodiesterase (inhibitor)
PEI  polyethylimine
PG   prostaglandin
RIA  radioimmunoassay
SP   substance P
STZ  streptozotocin
SPACE single potential analysis of cavernous activity
TG   triglycerides
TLC  thin layer chromatography
VIP  vasoactive intestinal polypeptide
VSMC vascular smooth muscle cell
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CHAPTER 1

THE PHYSIOLOGY OF PENILE ERECTION

1.1 Introduction

Erectile dysfunction (ED) is increasingly recognised as being a common and often distressing condition. The problem of failing male sexual potency is probably as old as Man himself. Although the Bible relates that men would father children well into their dotage, these must have been rather exceptional individuals. Over the centuries there have been many theories about the nature of male potency and sexual function; similarly there is a long history of treatments for failing sexual powers. Folklore remedies, such as the eating of oysters, rhinoceros horn or tiger penis, persist to this day although many are of dubious scientific value.

Attitudes to human sexual behaviour have varied over the years almost as much as to what constitutes an acceptable sexual practice. However, in the twentieth century workers such as Freud, Masters and Johnson, and Shere Hite have all contributed to the liberalisation of sexual thinking which culminated in the ‘sexual revolution’ of the 1960s. Nevertheless, it is only now that it is becoming acceptable to talk openly about the subject of male impotence as the stigma associated with this topic is being decreased. This change has perhaps been brought about not only by an increased media interest but also because of the availability of effective treatments. Furthermore, there has been a veritable explosion in the amount of basic science research effort devoted to this subject. Knowledge of the physiological and biochemical events underpinning
erection advances daily bringing with it the possibility of formulating novel therapeutic interventions, if not also the advent of preventative measures in certain subpopulations of impotent men. Certainly the demand for the provision of Andrological services will continue to increase especially as we have a population which is becoming ever older. With an informed public it is also inevitable that we will witness increased patient expectations. However, it is also important to remember that not all patients seeking such services will necessarily be old, for example, many diabetics and those with spinal injuries may be relatively young men.

The mechanisms of penile erection are complicated and despite intensive research effort remain incompletely understood. The male sexual response and erection is dependent upon the integrated action of anatomical, vascular, neural, psychological and endocrinological factors. Major advances have taken place over the past decade not only in our understanding of the physiology of normal erection and the pathogenesis of ED, but also in its effective management. It is the purpose of the two introductory chapters to review these aspects and to put the aims of this thesis into perspective. The present thesis is primarily concerned with the effects of experimental diabetes mellitus on penile cyclic nucleotide metabolism, in addition, the possible contribution of other metabolic abnormalities to the pathogenesis of erectile dysfunction are also considered.

1.2 Penile anatomy

The erectile tissues of the penis are found in the paired corpora cavernosa and the single corpus spongiosum (Fig. 1.1). The corpus spongiosum is a ventrally situated structure that surrounds the urethra and dilates distally to form the glans penis. The paired corpora cavernosa are dorsally situated structures that are joined by a thin fibrous midline septum. The corpora cavernosa separate proximally to form the crura of the penis which are attached to the inferior aspect of each ipsilateral ischiopubic ramus. The crura are covered inferiorly by the ischiocavernosus muscle.
Fig. 1.1 Diagram to show the gross anatomy of the erectile chambers of the penis.

(Reproduced with permission from Hinman, 1993)
The bulb of the penis is situated medially between the crura and is traversed by the urethra. The bulb is firmly connected to the perineal membrane and is covered on its inferior aspect by the bulbocavernosus muscle. Each cavernosal body terminates within the glans penis which thus forms a cap overlying them.

Each corpus is ensheathed by a thick, two-layered fibrous sheath, the tunica albuginea (Fig. 1.2) which is composed of bundles of collagen and elastin fibres. The deep fibres of the tunica albuginea pass circularly around each corpus cavernosum to unite in the midline thus forming the pectiniform septum of the penis. The septum is incomplete distally in that it is fenestrated, such an arrangement is presumably important in allowing for the free communication of blood flow between the paired erectile chambers. The outermost fibres of the tunica albuginea are longitudinally arranged and form a covering around both corpora cavernosa. In addition, all three corporeal bodies are covered by deep and superficial fascial layers that are continuous with the fascia of the perineum. The deep layer is known as Buck’s fascia and is attached to the undersurface of the urogenital diaphragm. The more superficial layer is known as Colle’s fascia and is continuous with Scarpa’s fascia of the lower abdominal wall as well as the dartos fascia of the scrotum.

Two ligaments blend with the fascia of the penis to support its body. The suspensory ligament is attached to the symphysis pubis and fans out as it blends with the fascia of the penis. The fundiform ligament consists of fibres which arise from the lower part of the linea alba and pass on both sides of the penis, blending with its fascia. The two wings of this fascia meet inferiorly and blend to form part of the scrotal septum. That part of the tunica surrounding the corpus spongiosum is thinner and more elastic than it is elsewhere. The corporeal parenchyma consists of a trabecular meshwork formed by smooth muscle fibre bundles, endothelial cells, fibroblasts and a collagenous extracellular matrix. It is this criss-crossing pattern which creates the vascular spaces.
Fig. 1.2 Diagram of the structural layers of the penis.
(Reproduced with permission from Hinman, 1993)
known as lacunar spaces or sinusoids which are lined by endothelial cells and surrounded in turn by smooth muscle cells. It is this arrangement which accounts for the spongy nature of the penile tissues (Fig. 1.3). Immunohistochemical studies have provided some insight into the nature of the corporeal extracellular matrix. There is an abundance of type I and IV collagen with a decreased amount of type III (Luangkhot et al, 1992). The abundance of type IV collagen is related to the prevalence of endothelial cells within the trabeculae of the corpus cavernosum. Endothelial cells are known to secrete type IV collagen which forms the basement membrane of blood vessels (Krane and Neer, 1985). These histological observations suggest that human erectile tissue may be considered as being a specialised vascular tissue (Bossart et al, 1980; Conti and Virag, 1989; Heaton, 1989).

Electron microscopy studies investigating the ultrastructure of the erectile tissues have demonstrated the presence of gap junctions in the human corpus cavernosum (Campos de Carvalho et al, 1990). It has subsequently been postulated that these gap junctions are responsible for the rapid transmission of neural or hormonal stimulation which enables the human corpus cavernosum to behave as a co-ordinated functional syncytium (Christ et al, 1991). Furthermore, electrophysiological investigations have demonstrated that human corporeal smooth muscle cells in culture are coupled by gap junctions (Moreno et al, 1990). These findings have added another layer of complexity to the understanding of the physiology of human penile erection (Fig. 1.4).

1.3 Penile arterial supply

The blood supply to the erectile tissues is derived from the penile artery, a branch of the internal pudendal artery, which in turn is a branch of the anterior division of the internal iliac artery. From its origin the internal pudendal artery runs in a curve on the dorsolateral pelvic wall. It enters the lesser pelvis with the nerve of the same name via the lesser sciatic notch.
Fig. 1.3 Diagram of the trabecular structure of the corpus cavernosum.

(Reproduced with permission from Hinman, 1993)
Fig. 1.4 Transmembrane channels believed to be present in the corporeal smooth muscle. (Reproduced with permission from Christ et al, 1993)
Having reached the ischioanal fossa it then passes in Alcock's canal. At the
dorsal aspect of the urogenital triangle it gives rise to the superficial perineal artery
which supplies the scrotum, and thereafter it is called the penile artery. The penile
artery pierces the urogenital diaphragm and continues along the medial margin of the
inferior ramus of the pubis. In the anterior perineum the penile artery gives rise to a
number of branches on each side: the spongiosal, bulbar or bulbourethral, the dorsal,
and the deep or cavernous arteries (Fig. 1.5). It is not uncommon for there to be
anatomical variation and there may be accessory internal pudendal arteries from the
obturator, external iliac or other arteries giving supplemental branches to the dorsal or
cavernous artery. In some instances the accessory pudendal arteries may be the only
blood supply to the corpus cavernosum (Breza et al, 1989). Each cavernous artery
penetrates the tunica albuginea and gives off multiple terminal helicine arteries which
open directly into the sinusoidal spaces. The cavernous or deep artery frequently varies
in number, origin and communication with other penile arteries (Valji and Bookstein,
1988; Bahren et al, 1988). Within the corpus cavernosum, the branches of these vessels
are supported by the fibrous trabeculae.

The dorsal artery runs between the tunica albuginea and Buck's fascia with the
deep dorsal vein. More distally the dorsal artery anastomoses with the spongiosal artery
to form a rich arch which gives supply to the glans. It gives circumflex branches to the
mid-dorsal corpora cavernosa (Flanigan et al, 1985; Bookstein, 1988) and, for this
reason, bypass into a proximally occluded dorsal penile artery can improve flow into the
corpora cavernosa. The penile skin also receives an arterial blood supply, but this is
from the external iliac system, a fact which needs to be remembered in the interpretation
of blood flow studies which use radioisotopes. This blood supply does not contribute to
the process of erection.
Fig. 1.5 Arterial supply and venous drainage of the penis.
(Reproduced with permission from Batra and Lue, 1991)
1.4 Penile venous drainage

The penis has a rich venous drainage (Fig. 1.5) which has been described in terms of deep, intermediate, and superficial systems (Newman and Northup, 1981) although it has to be remembered that there is often considerable anatomical variation as well as a rich anastomotic network. The subcutaneous tissues are drained by the superficial subcutaneous veins that drain into the superficial dorsal vein, this vessel usually drains into the left saphenous vein as a single vessel. However, there may be multiple veins and drainage may be via the right saphenous, epigastric or femoral veins.

The peripheral sinusoidal spaces of the corpora cavernosa are drained by small venules that coalesce to form venous plexuses beneath the tunica albuginea (Fig. 1.6). A number of these subtunical plexuses unite and drain into the short emissary veins which pass through the tunica albuginea (Lue and Tanagho, 1988). In the distal, pendulous part of the penis the blood draining via the emissary veins drains laterally into circumflex veins, dorsally into dorsal veins and ventrally into urethral veins. Most of these then run into the deep dorsal vein to drain to the vesicoprostatic plexus of Santorini and the internal pudendal veins. The glans penis is drained by the deep dorsal vein. The corpus spongiosum drains into both the deep dorsal vein and the internal pudendal vein. In the proximal part, or root of the penis, emissary veins drain into the cavernous and crural veins, which drain into the cavernous and crural veins, and these drain in turn into the internal pudendal veins.

1.5 Penile nerve supply

The sacral autonomic innervation and higher centres which modulate the basic sexual reflexes are both essential for normal penile erection. For a fuller discussion of the central mechanisms of penile erection the reader is referred elsewhere (Andersson and Wagner, 1995). Penile erection depends on the integrated action of the sympathetic
Fig. 1.6 Diagram of penile venous drainage in the flaccid state. The arteries, arterioles and sinusoids are contracted. The venous plexuses are open with free flow to the emissary veins. (Reproduced with permission from Lue and Tanagho, 1988)
and parasympathetic systems, (the anatomy of the penile nerve supply is shown diagrammatically in Fig. 1.7).

1.5a Afferent pathways

Sensory information from the penis is carried in afferents in the dorsal nerve of the penis which pierces the pelvic diaphragm and then continues as the pudendal nerve to enter the sacral cord via the dorsal roots of the second and fourth segments. Sensory information is then transmitted to the cerebral cortex and thalamus in ascending tracts. Afferent input via this pathway is important in eliciting reflexogenic erections (Weiss, 1972; Nunez et al, 1986). Recent studies have shown that in rats the cavernous nerves also contain somatic afferent fibres (Steers et al, 1988).

1.5b Efferent pathways

Somatic efferents to the bulbocavernosus and ischiocavernosus muscles originate within the cerebral cortex, these are conveyed in the corticospinal tracts to the ventral horn of the sacral spinal cord. They synapse in the ventral horn, and large myelinated fibres travel via the anterior sacral roots (S2-S4) to join the pudendal nerve which supplies the pelvic floor muscles. The autonomic pathways supplying the penis originate in the hippocampus, anterior cingulate gyrus and the thalamus. The fibres then pass down to the spinal erectile centres. A sacral parasympathetic erection centre lies at S2-S4 and a thoraco-lumbar sympathetic erection centre at T12-L3. From both these centres, fibres pass to the hypogastric and pelvic plexi, before fusing to form the cavernous nerves. Parasympathetic preganglionic input to the human penis originates from spinal segments S2-S4, with the cell bodies located within the intermediolateral cell column. The dendritic projections of these neurones are such that they can receive sensory input from both visceral and somatic structures.
Fig. 1.7 Diagram of the peripheral neural pathways involved in the control of penile erection. (Reproduced with permission from Giuliano et al, 1995)
The cavernous nerves travel along the posterolateral surface of the prostate (Walsh and Donker, 1982) and curve anteriorly to join the membranous urethra, pierce the urogenital diaphragm to pass through the tunica albuginea to supply the corpora. The cavernous nerves are the final common pathway for both vasodilator and vasoconstrictor neural input to the cavernous spaces in man (Lepor et al, 1985).

1.6 The physiology of erection

Penile erection is predominantly a vascular event. Investigations in human and animal models have shown that both erection and detumescence are haemodynamic events regulated by the state of relaxation or contraction of penile smooth muscle. This in turn is controlled by the autonomic nervous system. Most researchers now agree that trabecular smooth muscle and arteriolar relaxation are the key events that initiate and control erection (Newman and Northup, 1981; Aboseif and Lue, 1988); the precise mechanisms by which this occurs remain to be fully elucidated and much research effort is currently being invested into understanding the control of penile smooth muscle tone.

1.6a Arterial haemodynamics

During flaccidity (and detumescence) adrenergic sympathetic tone dominates and the terminal arterioles and sinusoidal smooth muscles are contracted (Saenz de Tejada et al, 1988b). Nevertheless, a small amount of arterial blood flows via the sinusoidal spaces place to provide the penile nutritional requirements. This flow has been established to be of the order of 2.5-5.0 ml/min/100g of tissue (Wagner and Uhrenholdt, 1980) and over the past two decades it has become clear that the key event in penile erection is a parasympathetically-mediated (S2-4) relaxation of the blood vessels and sinusoids of the smooth muscle of the corpora cavernosa, which produces a dilation and engorgement of the sinusoids because of an increased arterial inflow (De
Groat and Booth, 1980; Saenz de Tejada et al, 1985). The muscle relaxation increases sinusoidal compliance and is thus able to cause elongation, expansion and erection of the penis. At erection the flow in the cavernous artery decreases and intracavernosal pressure rises to some 10-20mmHg below systolic. In contrast, blood flow continues to be higher in the glans penis and corpus spongiosum (Newman and Northup, 1981). There is normally some arterial inflow during systole and an absence of diastolic flow. However, during full rigidity the intracavernous pressure rises above systolic and consequently there is no inflow of blood.

1.6b The veno-occlusive mechanism

Recent dynamic vascular studies have shown that the restriction of penile venous outflow is an essential component for the initiation and maintenance of a rigid erection. These venous events have been studied both in humans and in animal models (Fournier, Jr. et al, 1987). It is now thought that veno-occlusion is a passive, mechanical event which is dependent on an adequate arterial inflow, an intact tunica albuginea and a sufficient degree of smooth muscle relaxation (Lue and Tanagho, 1988). As mentioned above, during flaccidity the contracted trabecular smooth allows for unhindered venous drainage via the subtunical venous plexus and emissary veins. During tumescence there is smooth muscle relaxation and the lacunar spaces fill with blood (Wespes and Schulman, 1986; Lue and Tanagho, 1988). An instantaneously increased arterial inflow, dilation of the arterial tree and sinusoidal filling results in the compression of the subtunical venous plexus, hence increasing the resistance to blood flow through these vessels and stopping flow in the emissary veins (Fig. 1.8). Erection is maintained by this greatly decreased venous outflow and rigidity is imparted by the subsequent rise in intracavernosal pressure, this is further aided by the contraction of the bulbocavernosus and ischiocavernosus muscles (Wespes and Schulman, 1986). Thus, the veno-occlusive mechanism depends largely on penile smooth muscle relaxation which is mediated by both neurogenic and endothelial derived elements. With the advent of rigidity, the extra-
Fig. 1.8 Diagram of the penis in the erect state showing the relaxed trabeculae and arterioles which allow for the blood flow into the sinusoidal spaces. The larger venules are compressed between the sinusoidal wall and the tunica albuginea. (Reproduced with permission from Lue and Tanagho, 1988)
tunical deep venous system is compressed by Buck's fascia. Defects in any of the components of this mechanism can lead to the inability of the penis to 'trap' blood effectively, this idea forms the basis of the concept of 'venous leakage' a subject which is discussed more fully in chapter 2.

1.7 Neurophysiology and pharmacology of erection

As has been discussed above, penile erection is a haemodynamic event under the control of the autonomic nervous system. It has become clear that this process can no longer be regarded as the result of a simple balance between pro-erectile parasympathetic activity and anti-erectile sympathetic innervation: NAd release from sympathetic nerve fibres maintaining flaccidity and erection being the result of an antagonistic parasympathetic release of ACh. In addition to NAd and ACh a large number of other substances have been demonstrated in perivascular nerves and are now considered as putative neurotransmitters.

The serendipitous discovery of the effects of intracavernosal papaverine injection (Michal et al, 1977) and subsequent pioneering work (Virag, 1982b; Brindley, 1983) has resulted in a revolution not only in the evaluation and treatment of impotence, but also in the understanding of the pharmacology of erection. Papaverine is a smooth muscle relaxant which increases arterial blood flow, venous resistance and sinusoidal relaxation. Papaverine has been widely used by itself and in combination with phentolamine, an alpha-blocker, with an observed effect that is synergistic rather than additive.

The biochemical and neurophysiological events underpinning the erectile process have been an area of intense research activity over the past decade. The search for a single relaxant neurotransmitter in erection has been long and not particularly successful; it seems that erection is a very complicated event with much interplay and
modulation between a number of neurotransmitters. While major advances in our understanding have taken place, we remain far from an integrated physiological model and it would seem that a complex multi-transmitter system is responsible. Following advances in vascular physiology (Furchgott and Zawadski, 1980; Furchgott, 1990; Palmer et al, 1987) it is perhaps not surprising that nitric oxide (NO) has been implicated as being the final determinant in the erectile process (Burnett et al, 1992). Nevertheless, a considerable effort continues into the elucidation of the contribution of other substances such as vasoactive intestinal polypeptide (VIP), substance P (SP), acetylcholine (ACh), neuropeptide Y (NY) and the prostaglandins (PGs) to erectile physiology.

1.8 Adrenergic mechanisms

It has long been known that the sympathetic division of the autonomic nervous system has an anti-erectile effect on the penis. It has been shown in rabbits that the electrical stimulation of the lowest parts of the sympathetic trunk will cause the penis to shrink, even if all connections of these sympathetic trunks with the CNS are cut (Sjostrand and Klinge, 1979). In man, the evidence for such an anti-erectile nervous pathway comes from experiments on the actions of drugs given intracavernosally (Brindley, 1986). This evidence makes it almost certain that there is an anti-erectile pathway which is continuously active when the penis is flaccid (Brindley, 1986). The peripheral endings of this pathway release NA, however, little is known about the anatomy of the pathway but it is likely to be roughly similar to its counterpart in the rabbit.

More recently it has been proposed that there exists a pro-erectile sympathetic pathway. Evidence has been presented that in the rabbit, cat and man the hypogastric plexus contains efferent nerve fibres that cause erection and tumescence. It has been shown conclusively that these fibres exist in the rabbit, and that their electrical
stimulation will cause erection (Sjostrand and Klinge, 1979). In man, the electrical stimulation of the intact hypogastric plexus caused full erection in two out of nine patients and a conspicuous tumescence in the other seven (Brindley et al, 1989). Lesions of the hypogastric plexus are known to cause ejaculatory failure but do not usually cause complete erectile dysfunction. Thus, the parasympathetic system itself is sufficient to cause erection. Loss of the parasympathetic erectile pathway with survival of the sympathetic is seen in men with complete lesions of the cauda equina or conus medullaris - a proportion of these men, though not all, are capable of full erection.

The presence of adrenergic nerves has been demonstrated in trabecular smooth muscle as well as in the cavernosal and helicine arteries (Shirai et al, 1972; Benson et al, 1980; Gu et al, 1983). As mentioned above it is generally accepted that in the flaccid state penile smooth muscle is kept contracted by NAd release acting via postjunctional alpha-adrenoceptors on the cavernous and helicine arteries as well as on trabecular smooth muscle (Hedlund et al, 1984). Furthermore, NAd release is modulated by presynaptic alpha2-adrenoceptors (Molderings et al, 1989). Modulation of adrenergic activity seems to be one of the most important means by which the contractile state of the smooth muscle of the corpus cavernosum and the penile vasculature is influenced.

It has been shown by a large number of workers that NAd and phenylephrine will produce concentration-dependent contraction in isolated human corpus cavernosum, corpus spongiosum, penile arteries and veins, in vivo (Benson et al, 1980; Adaikan and Karim, 1981; Andersson et al, 1983; McConnell and Benson, 1982; Imagawa et al, 1991). Both alpha1 and alpha2 agonists will contract trabecular tissue, however alpha2 (clonidine) was less potent in this respect (Hedlund and Andersson, 1985b; Kimura et al, 1989; Christ et al, 1990). In cavernosal artery segments, clonidine was a much more powerful contractile agent (Hedlund and Andersson, 1985b). It was also shown that the alpha1 blocker prazosin but not the alpha2 blockers yohimbine or rauwolscine relaxed NAd precontracted trabecular tissue (Hedlund and Andersson, 1985b; Christ et al, 1990). Prazosin and rauwolscine relaxed NAd induced contractions equally in
cavernosal artery segments. Prazosin was more potent than rauwolscine in inhibiting contractions evoked by electrical nerve stimulation of trabecular tissue (Saenz de Tejada et al, 1989c; Hedlund and Andersson, 1985b). In arterial segments, rauwolscine was more potent than prazosin (Saenz de Tejada et al, 1989c). These findings suggest that alpha\textsubscript{1} adrenoceptors predominate in the human corpus cavernosum, while alpha\textsubscript{2} predominate in the cavernosal artery. It is also known that both alpha\textsubscript{1} and alpha\textsubscript{2} adrenoceptor functions can be demonstrated in circumflex veins and the deep dorsal penile vein (Kirkeby et al, 1989a; Fontaine et al, 1986). More recently, three subtypes of alpha\textsubscript{1} adrenergic receptors in mRNA (alpha\textsubscript{1A}, alpha\textsubscript{1B}, alpha\textsubscript{1C}) have been identified in human corporeal tissue. The alpha\textsubscript{1A} and alpha\textsubscript{1C} receptors were found to be the predominant subtypes expressed in this tissue at an mRNA level (Price et al, 1993).

The in vivo results have been confirmed by the intracavernosal injection of alpha-adrenoceptor antagonists - thus, injection of phenoxybenzamine, phentolamine and thymoxamine produced erection and tumescence (Brindley, 1983; Brindley, 1986) and injection of the alpha-adrenoceptor agonists metaraminol and NAd caused detumescence (Brindley, 1984; De Meyer and De Sy, 1986). It was further shown that the injection of the selective alpha\textsubscript{2} adrenoceptor blocker, idazoxan, had no effect therefore supporting the view that it is the alpha\textsubscript{1} adrenoceptor that is the functionally dominant subtype.

Drugs such as trazodone and ketanserin have been reported to influence, at least partly, penile erectile tissues by the blockade of alpha-adrenoceptors. Trazodone is a non-tricyclic antidepressant which was shown to have marked alpha-adrenoceptor blocking activity (Abber et al, 1987; Azadoi et al, 1990) and was reported to cause priapism during treatment for depressive disorders (Andersson et al, 1991b). Ketanserin, a selective 5HT receptor blocker, which also blocks alpha-adrenoceptors in human corpus cavernosum tissue, produced erection following its intracavernosal injection in man (Adaikan et al, 1991). Thus from the above it is clear that alpha antagonism causes tumescence and erection, while alpha agonism maintains flaccidity. It has been postulated that in some cases impotence may be secondary to changes in alpha-
adrenoceptor function. A small, but significant, difference was found between cavernous tissue from diabetic and non-diabetic patients with impotence. Diabetic patients had both increased and reduced sensitivity to phenylephrine (Christ et al, 1990). In contrast, no differences were found in sensitivity to phenylephrine between cavernous tissue preparations taken from impotent men with DM, alcoholism, or Peyronie's disease and men with no obvious condition causing impotence (Creed et al, 1989).

No difference in the alpha-adrenoceptor function of isolated penile circumflex veins between potent and impotent men due to venous leakage was found (Kirkeby et al, 1989a). In a study of the kinetics of alpha\textsubscript{1} adrenoceptor-mediated contractions at a steady state, age and pathology-dependent alterations that could result in heightened corporeal tissue tone and may contribute to the pathophysiology of erectile dysfunction were found in some patients (Christ et al, 1992).

1.9 Cholinergic mechanisms

For many years the parasympathetic autonomic nervous system has been believed to be the sole effector of physiological erections. The results of early experiments showed that electrical stimulation of the nervi erigentes of animals brought about penile erection (Eckhardt, 1863; Langley and Anderson, 1895; Nikolsky, 1879). This response was not abolished by the administration of atropine, it was later suggested that that a balance exists between adrenergic anti-erectile and cholinergic pro-erectile activity (Klinge and Sjostrand, 1977b). The function of cholinergic activity being to inhibit via muscarinic receptors the adrenergically-induced penile flaccidity (Hedlund and Andersson, 1985b; Saenz de Tejada et al, 1988a; Godec and Bates, 1984). However, the exact roles of cholinergic mechanisms are unclear; indeed, a considerable body of evidence exists to suggest that vasodilatation and hence tumescence and penile erection are not accounted for by a simple mechanism of ACh release which then acts via muscarinic receptors.
Muscarinic receptors have been clearly identified in the human corpus cavernosum in ligand binding studies, these have been suggested to be of M₂ and M₃ type and the radioligand bound to both cavernosal tissue and to endothelium (Godec and Bates, 1984; Shirai et al, 1972). Furthermore, acetylcholinesterase containing nerves have been demonstrated in the human and rat penile tissue as has choline uptake, ACh synthesis and release following electrical stimulation of nerves in the cavernous smooth muscle (Blanco et al, 1988; Godec and Bates, 1984; Blanco et al, 1990; Traish et al, 1990). However, there have been many conflicting findings in experiments on penile corpus cavernosum in vitro. Some investigators have reported contraction of penile tissue by ACh (Adaikan et al, 1983). In contrast, others have found a relaxation of corpus cavernosum and corpus spongiosum produced by ACh when the tissue had been precontracted with NAd, a response which was blocked by scopolamine and thus presumably mediated via muscarinic receptors (Hedlund et al, 1984; Saenz de Tejada et al, 1988a; Andersson et al, 1983). Muscarinic receptor blockade in dogs with atropine caused no effect or significant decrease in blood flow response to pelvic nerve stimulation, however it did however curtail the erectile response (Andersson et al, 1984). Similarly, a reduced erectile response was found in response to neurostimulation after the injection of ACh, but it is unclear if this was mediated by muscarinic or alpha-adrenoceptor blockade (Larsson et al, 1984). The intracavernous injection of ACh in monkeys will produce a triphasic response, including full erection (Stief et al, 1989).

The differences in all these observations may be explained on the basis of species variation in part, but there remains a general agreement that atropine will only partially block erectile responses. Furthermore, it is also known that atropine injection fails to block penile erection in humans this led to the suggestion that muscarinic transmission plays no significant part in penile erection (Brindley, 1986; Wagner and Brindley, 1980). ACh release and synthesis were found to be significantly reduced in corporeal tissue from men with diabetes (Saenz de Tejada et al, 1988a). The destruction of the endothelium with detergent effectively eliminated/attenuated the relaxation produced by ACh (Trigo-Rocha et al, 1993), a finding which suggests that, as in other vascular preparations, the effect of ACh is endothelium-dependent (Furchgott, 1990).
It should be remembered in the present context that parasympathetic activity does not equate to the sole actions of ACh; clearly other transmitters may be released from cholinergic nerves. There are three distinct mechanisms by which parasympathetic activity may affect erection and tumescence: (1) the release of NAd may be inhibited by stimulation of muscarinic receptors on adrenergic nerve terminals, (2) the postjunctional effects of NAd may be counteracted by muscarinic receptor-mediated release of relaxant factor(s) from the endothelium, and (3) the postjunctional effects of NAd may be counteracted by relaxant factors, such as NO and VIP, released from the parasympathetic nerves.

Overall, the findings suggest that parasympathetic activity may contribute to penile tumescence and erection by mechanisms which antagonise the anti-erectile effects of NAd. This is likely to include the muscarinic receptor mediated inhibition of adrenergic activity as well as the generation of NO by the endothelium. Thus, much of the current research effort has focused on the role of other putative neurotransmitter substances and the term non-adrenergic non-cholinergic (NANC) transmission is used to describe these other processes that are responsible for the control of smooth muscle relaxation.

1.10 Non-adrenergic non-cholinergic mechanisms

There are a large number of substances included in the term NANC and the reader is referred to the comprehensive review by Andersson and Wagner (Andersson and Wagner, 1995). The role of the PGs and NO in particular are discussed below as these substances are of import and of direct relevance to the experimental sections of this thesis. The role and importance of VIP is introduced here and is subsequently dealt with in chapter four, the other mechanisms are briefly discussed for completeness.
1.10a Nitric oxide and the role of the endothelium

The effects of exogenously administered nitrates on the vasculature have been studied for decades; however, that endothelial cells are able to release a substance which can cause vasodilatation was only discovered relatively recently. Furchgott and Zawadaski found that the relaxation of aortic sections in response to agonists would only occur in the presence of an intact endothelium (Furchgott and Zawadski, 1980). They proposed that endothelial cells produce an endothelial derived relaxing factor (EDRF) responsible for the relaxation of vascular smooth muscle; EDRF was thus implicated in the control of vascular tone (Furchgott and Zawadski, 1980). EDRF was subsequently characterised as being nitric oxide (Palmer et al, 1987). This finding opened a new area of intense biological research endeavour and shortly afterwards it was demonstrated that nitric oxide (NO) is formed by the action of an enzyme, nitric oxide synthase (NOS), present in the vascular endothelium but not in vascular smooth muscle cells (Palmer et al, 1987). It was very quickly realised that NO was a secretory product of mammalian cells of considerable import. Since then it has become apparent that NO is a truly remarkable molecule with a remarkably wide range of biological activities which include neurotransmission, vasodilatation and cytotoxicity (Lowenstein et al, 1994).

NO is synthesised from L-arginine and oxygen in a reaction under the control of NOS; although the precise details of the reaction are unclear the products of this reaction are NO and citrulline. NOS exists in two distinct forms: a constitutive and an inducible isoform. The constitutive form is cytosolic, calcium-calmodulin dependent and generates/releases NO for short periods in response to physical or receptor stimulation. In this context, NO has a number of properties which make it ideal as a transmitter substance: it is uncharged and can easily diffuse into and out of cells; it has an unpaired electron and is thus a radical with a short half-life of between 2-30 seconds; it subsequently undergoes a spontaneous degradation to nitrite. The inducible form is induced following the activation of macrophages, endothelial and other cell types. This
isoform brings about the synthesis of NO for much longer periods; it is calcium-independent, requires a number of other cofactors and is inhibited by glucocorticoids. The NO produced by this isoform acts primarily as a cytotoxic molecule, however, its release may have other biological consequences including pathological vasodilatation, tissue damage and its generation has been implicated in the pathogenesis of septicaemic shock (Jeremy et al, 1994b).

The walls of the sinusoidal spaces and penile vessels are lined with endothelial cells (Benson et al, 1980; Saenz de Tejada et al, 1988b); given the above context the role of the endothelium in penile erection has perhaps been somewhat neglected. It has been shown that ACh-induced relaxation depends on endothelial integrity and it was therefore argued that the endothelium may play a role in the regulation of local penile haemodynamics (Saenz de Tejada et al, 1988b). Furthermore, it was thought that cavernosal endothelial cells may be directly innervated (Schmalbruch and Wagner, 1989). This has led to speculation that after the initiation of vasodilatation and increased blood flow, an additional mechanism - mediated by the endothelium - is required to maintain tumescence. It is possible that the increased flow and shear forces provide the stimulus for such a mechanism to come into operation. An increase in flow has been shown to induce endothelial-dependent relaxation in a variety of vessels (Holtz et al, 1984; Hull et al, 1986; Rubanyi et al, 1986). In addition, SP, 5HT, ACh, and ATP are all released from vascular beds and/or endothelial cells in culture under hypoxia-induced vasodilatation or shear stress (Burnstock, 1988). Clearly the role of the endothelium and its stores of vasoactive agents in erectile vasodilatation cannot be ignored and warrant careful consideration.

It has become increasingly recognised that the endothelium itself plays a major role in the modulation of vascular smooth muscle tone, and may indeed have the ability to autoregulate blood flow through vessels and vascular beds. Furthermore, it has been established that in disease states such as atherosclerosis, hyperlipidaemia and diabetes mellitus there is endothelial cell dysfunction which may contribute to the pathogenesis.
of ED (Lowenstein et al, 1994; Saenz de Tejada et al, 1989b; Azadzoi and Saenz de Tejada, 1992). Thus, in cavernosal tissue from impotent diabetic patients, a decreased relaxation was found in response to electrical stimulation, this was associated with a lack of NO production as measured by the ability to form nitrites, and not as the inability of the smooth muscle to relax (Pickard et al, 1992). In isolated corpus cavernosum from diabetic patients with erectile dysfunction, both neurogenic and endothelium-dependent relaxation was impaired (Saenz de Tejada et al, 1989b), a finding which was replicated in rabbits with alloxan-induced diabetes (Azadzoi and Saenz de Tejada, 1992). Similarly, hypercholesterolaemia was reported to impair endothelium-mediated relaxation of rabbit corpus cavernosum smooth muscle (Azadzoi and Saenz de Tejada, 1991).

In addition to its effects on cavernosal smooth muscle cells to produce relaxation and vasodilatation, it is widely accepted that NO also acts as both a central and peripheral neurotransmitter. Thus, penile erection is an interesting example of NO functioning as both a neurotransmitter and a vasodilator. It is now thought that most penile NO is actually of neuronal origin (Keast, 1992) and a number of lines of evidence show that neuronal NO is both implicated and important in mediating penile erection:


(ii) Immunohistochemical studies have shown that NOS is localised in the nerves of the pelvic plexus, the cavernous nerve and the processes extending into the corpora cavernosa to innervate the adventitial layers of penile arteries (Burnett et al, 1992).
Using an antiserum to constitutive NOS it was demonstrated to be present in the pelvic plexus and axonal processes of the cavernosal nerve (Burnett et al, 1992). Furthermore, the same workers demonstrated NOS in human urogenital tissue: NOS was present in the cavernous nerves and the terminals ending in the corpus cavernosum as well as in the branches of the dorsal penile nerve (Burnett et al, 1993).

(iii) In vivo electrical stimulation of the pelvic nerves of rats causes a 'physiological' erection which can again be completely blocked by the intravenous administration of NOS inhibitors (Burnett et al, 1992).

Nitric oxide diffuses freely into cells and has its intracellular effects via its ability to activate soluble guanylate cyclase, the enzyme responsible for the conversion of guanosine triphosphate to cGMP, NO thus has its intracellular actions by raising the tissue levels of cGMP (Bush et al, 1992a; Bush et al, 1992b; Heaton, 1989; Holmquist et al, 1992a). This was first demonstrated in rabbit corpus cavernosum where it was further shown that a selective inhibition of the cGMP specific phosphodiesterase (PDE) enhanced the relaxant effect of electrical stimulation (Bush et al, 1992a; Bush et al, 1992b; Holmquist et al, 1993; Ignarro et al, 1990). Thus, the intracellular levels of this important second messenger molecule are regulated by its generation via guanylate cyclase and its subsequent degradation by PDE. The guanylate cyclase stimulating effects of NO are inhibited by methylene blue (Martin et al, 1985; Waldman and Murad, 1987). Thus, it was reported that relaxation via EFS in the rabbit corpus cavernosum was inhibited by the addition of methylene blue, a finding which was also reported in human tissues (Holmquist et al, 1992a; Rajfer et al, 1992; Pickard et al, 1991). The available in vitro results obtained in isolated penile tissues suggest that the penile L-arginine/NO system is essential for normal erection and there is accumulating in vivo evidence to support this.

It is somewhat surprising that given its pivotal role in the mediation of penile erection it is not known if abnormalities of NO and NOS are involved in the
pathogenesis of erectile dysfunction (Lowenstein et al, 1994). It has been postulated that a lack of NOS, and therefore a lack of NO, might cause erectile dysfunction while an excess might cause priapism (Lowenstein et al, 1994). It is interesting to note that NOS activity, and hence NO production, depends on \( O_2 \) tension, hence in the flaccid state where contraction of the helicine vessels and corporeal tissues is maintained a lowered \( O_2 \) tension might lead to lowered NOS activity, and vice versa during erection (Kim et al, 1992; Kim et al, 1993). The intracavernosal injection of NO in vivo (including humans) has been performed and at present has rather limited utility, for example the intracavernosal injection of linsidomine (a NO donor) does result in penile erection although it seems not to be a particularly powerful erectogen (Porst, 1993; Stief et al, 1992; Stief et al, 1991b).

In summary, there is good evidence that NO is one of the most important neurotransmitters responsible for penile erection; in this respect it is interesting that mice which lack neuronal NOS are not only viable but that they are also fertile (Huang et al, 1993), this clearly means that there are other pathways in existence which can mediate erection. However, these mice have had the opportunity to develop powerful compensatory mechanisms which is clearly not the situation in individuals who develop an acquired defect of NO pathways in adult life.

1.10b Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) is a potent vasodilator consisting of 28 amino acid residues which inhibits contractile activity in many types of smooth muscle (Said, 1981). It interacts with a specific receptor and its mode of action appears to be dependent upon the production of cAMP (Ganz et al, 1986) and/or cGMP (Chakder and Rattan, 1993) as well as the modulation of NO release (Grider et al, 1992) although data is not available relating to the penis. VIP has been found to be present in high concentrations in the erectile tissues of the human penis, in autonomic nerve fibres
which have their endings around cavernous smooth muscle, penile arteries, arterioles and also circumflex veins (Polak et al, 1981; Gu et al, 1983; Kirkeby et al, 1992a). It has also been demonstrated in the penis of many other species (Juenemann et al, 1987; Keast and De Groat, 1989; Schmalbruch and Wagner, 1989). VIP has long been a putative neurotransmitter in the mechanism of erection the evidence for such a role is reviewed in chapter four.

1.10c Prostaglandins

As for other putative neurotransmitters, a variety of experimental approaches have been used in the attempt to elucidate the role of PGs in normal penile erection. When interpreting the results of these studies, it is important to remember which species the tissue comes from, which part of the penile anatomy is under study, and whether or not we are witnessing physiological or pharmacological phenomena.

A number of studies have now established that the human penis has the ability to both produce and to subsequently metabolise PGs (Saenz de Tejada et al, 1988b; Roy et al, 1984; Roy et al, 1989; Jeremy et al, 1986b). It is known that homogenates of human corpora cavernosa can produce PGE$_2$, PGF$_{2\alpha}$, PGD$_2$, and 6-keto-PGF$_{1\alpha}$ in vitro (Roy et al, 1984). Furthermore in the human penis it was shown that the production of PGI$_2$ was under, at least in part, muscarinic control (Jeremy et al, 1986b). Other investigators demonstrated that human corpus cavernosal endothelial cells in culture also have the ability to produce 6-keto-PGF$_{1\alpha}$, PGE$_2$, and PGF$_{2\alpha}$ (Saenz de Tejada et al, 1988b) thus confirming the findings of previous studies. The prostanoids so produced are broken down by PG 15-hydroxydehydrogenase (Roy et al, 1989). That penile tissues possess the capability to synthesise and degrade PGs is perhaps not surprising as the sinusoidal spaces are lined with endothelium and smooth muscle cells (Saenz de Tejada et al, 1988b).
A role for PGs in erection was first indicated by Klinge and Sjostrand when they reported that PGF$_{2\text{-alpha}}$ contracted the corpus cavernosum and penile artery of bulls (Klinge and Sjostrand, 1977a). Experiments with in vitro tissue strips have elucidated the ability of the various prostanoids to either contract or relax penile tissue in organ bath preparations. Thus, PGF$_{2\text{-alpha}}$, PGI$_2$, PGE$_2$ and TXA$_2$ analogues were all able to contract corpus cavernosum and arteries (Hedlund and Andersson, 1985a). It was suggested that the contraction-mediating prostanoid receptor in the human corpora cavernosa is a TXA$_2$ sensitive receptor (Hedlund et al, 1989a; Hedlund et al, 1989b).

Jeremy et al showed that muscarinic receptor stimulation caused PGI$_2$ production by the human corpora cavernosa (Jeremy et al, 1986b). However, a major role for PGI$_2$ in the erectile response seems unlikely because although PGI$_2$ is able to relax segments of penile vessels it was unable to relax trabecular tissue which was actually contracted (Hedlund and Andersson, 1985a). It may be argued that PGI$_2$ is important in the initial phases of erection to facilitate an increased blood flow as well as acting to prevent platelet aggregation in a situation of relative stasis (Hedlund and Andersson, 1985a), but one must remember that this is also probably a function of NO (Mellion et al, 1983).

In organ bath experiments PGE$_1$ and PGE$_2$ were both found to be effective in relaxing human trabecular tissue as well as precontracted segments of cavernous artery (Hedlund and Andersson, 1985a). PGE$_1$ was found to also inhibit NAd release from penile adrenergic nerves (Molderings et al, 1989) and it had been previously shown that this PG will affect cAMP formation (Bhargava et al, 1990), however the precise intracellular mode of action and signal transduction mechanism for PGE$_1$ remains unclear. Furthermore, there is recent evidence that PGE$_1$ also affects intracellular calcium ion fluxes (Derouet et al, 1994). In isolated human corpus cavernosal cells patch-clamp analysis and monitoring of the intracellular calcium concentration suggest that PGE$_1$ induces smooth muscle relaxation by an inhibition of voltage-dependent calcium channels and a subsequent reduction in intracellular calcium concentration (Derouet et al, 1994). In vitro, PGs may be responsible for the spontaneous activity of corpus cavernosum strips (Christ et al, 1990). In addition, cyclo-oxygenase products are
released simultaneously with NO and partially counteract its actions when the endothelium is stimulated by ACh (Azadzoi et al, 1992), despite the observation that some PGs have relaxant effects (Hedlund and Andersson, 1985a).

That PGI\textsubscript{2} is unlikely to be a major relaxant mediator was further supported in light of the finding that the intracavernosal injection of PGI\textsubscript{2} in monkeys in vivo, at a dose of 100-200mg, didn't increase the arterial blood flow and indeed the resultant smooth muscle contraction produced a large reduction in cavernosal compliance (Bosch et al, 1989). This is in contrast to the observations of increased cavernosal arterial blood flow and cavernous smooth muscle relaxation when PGE\textsubscript{1} was injected in monkeys in vivo (Bosch et al, 1989); intracavernosal injection of PGE\textsubscript{1} has also been shown to cause an increase in intracavernosal pressure in rats (Chen et al, 1992). Thus, the role of the endogenous penile prostaglandins in normal physiological erection and erectile dysfunction remains uncertain and was recently specifically addressed in a review article (Miller and Morgan, 1994). However, of the PGs, PGE\textsubscript{1} would appear to have the most appropriate profile of action for an erectogen; it is therefore somewhat surprising that there remains a lack of information of the ability of the human corpora cavernosa to produce PGE\textsubscript{1} (Mikhailidis et al, 1990). Nevertheless, cavernosal PGE\textsubscript{1} receptors have recently been demonstrated in a variety of species, including man, using a radioligand binding technique (Aboseif et al, 1993).

1.10d Neuropeptide Y

Neuropeptide Y (NPY) has been co-localised with NAd in adrenergic postganglionic neurones and may therefore participate in vasoconstriction with NAd in blood vessels (Grundemar and Hakansson, 1993; Wahlestedt and Reis, 1993). It has been clearly demonstrated both in the penile vasculature and in the corpus cavernosum of several species (Schmalbruch and Wagner, 1989; Wespes et al, 1988; Adrian et al, 1984; Cocchia et al, 1990; Crowe et al, 1991; Kirkeby et al, 1990b). Interestingly, it
has been found to be co-localised with VIP in the cavernous tissue and helicine arteries of the monkey (Schmalbruch and Wagner, 1989). NPY has been found in reasonably high concentration in the human corpus cavernosum and has therefore been thought to be involved in the control of erection (Adrian et al., 1984). Wespes studied the distribution of NPY-containing nerves in the human penis, he found NPY to be present in the fibres of the adventitia of both arterial and venous vessels and among smooth muscle cells (Wespès et al., 1988). It is thought that this peptide may be acting as a neurotransmitter or neuromodulator. Crowe speculated that it may contribute to veno-occlusion as it was found in the media of the deep dorsal vein of the penis (Crowe et al., 1991). It was also suggested that it may play a role in detumescence, although without any supporting experimental evidence (Crowe et al., 1991).

However, there are no effects of NPY on contraction of the corpus cavernosum or cavernosal artery to support its role in detumescence (these findings were at both basal and precontracted levels) (Hedlund and Andersson, 1985c). Furthermore, the reported effects on in vitro contraction are inconsistent both within and between species. Thus, the experimental evidence supporting the notion that there is a contractile mechanism involving NPY is clearly insufficient.

1.10e Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) immunoreactivity has been shown to be present in the nerves of the corpus cavernosum in a variety of different species, including man (Stief et al., 1991c). CGRP binding sites are numerous in rat penile tissue extracts (Wimalawansa et al., 1987). CGRP has been shown to act as a potent vasodilator in a variety of human blood vessels in vitro and is believed to act via an endothelium-dependent mechanism (Crossman et al., 1987). In the bull, the penile artery is relaxed via a direct action on the smooth muscle cells (Alaranta et al., 1991).
When injected via the intracavernosal route in an in vivo monkey preparation, CGRP increased the flow in the cavernosal artery as well as relaxing the cavernosal smooth muscle (Stief et al, 1993). In humans, erectile responses have been recorded after the intracavernosal injection of CGRP (Stief et al, 1991c). This is in contrast to the effects observed in vitro where CGRP has little relaxant effect on human corpus cavernosal strips (Andersson and Wagner, 1995). If CGRP does indeed have a role in normal penile erectile physiology then this role remains to be fully established.

1.10f Substance P

Substance P (SP)-containing nerves innervating the human corpus cavernosum and penile vasculature are not dense (Andersson et al, 1983; Gu et al, 1983; Lincoln et al, 1991), the SP immunoreactivity was concentrated mainly in groups of nerve fibres underneath the glans epithelium (Gu et al, 1983). Similarly, in the rat penis SP immunoreactivity was dense in the dermis of the glans (Carvalho et al, 1986). SP has been shown to contract the retractor penis muscle of the bull, and in human corpus cavernosum strips SP had contractile effects at basal tension (Klinge and Sjostrand, 1974), however, no such effects were seen in the cavernosal artery (Andersson et al, 1983; Hedlund and Andersson, 1985c). In precontracted strips, SP produced a transient moderate relaxation; similar to that found in rabbit tissues (Andersson et al, 1983).

It is thought that the relaxation of contracted corpus cavernosum and the vasodilatation produced when injected intra-arterially may be the result of SP releasing NO and relaxation-mediating cyclooxygenase products from the endothelium (Andersson and Wagner, 1995). Overall, SP is not thought to play an important role in erection but is much more likely to play an important part in penile sensory innervation (Keast and De Groat, 1989; Keast and De Groat, 1992; Carvalho et al, 1986).
1.10g Histamine

Histamine is present in arteries and veins and is synthesised by endothelial cells, its presence has been demonstrated in rabbit erectile tissues (Pentilla and Vartiainen, 1964). It is also present in mast cells (and platelets) and has been demonstrated in the human corpus cavernosum (Sathananthan et al, 1991). Histamine has been shown to contract the retractor penis muscle in a number of species (Ambache et al, 1975; Klinge, 1970; Luduena and Grigas, 1966). However, variable responses have been observed in human tissues in vitro in response to histamine administration, these include both contraction and relaxation (Adaikan and Karim, 1977). H₁ receptor stimulation by 2-methylhistamine led to contraction, in contrast to 4-methylhistamine which stimulates H₂ receptors and leads to relaxation. Blocking the H₁ receptor would therefore enhance relaxation by histamine and blocking H₂ receptor will enhance contraction (Adaikan and Karim, 1977). The in vivo results from a primate preparation were similar and therefore supported the idea that H₁ receptor stimulation leads to contraction and that H₂ receptor stimulation leads to relaxation and hence erection (Adaikan et al, 1991). It was reported that in the guinea pig histamine was able to dilate coronary arteries via H₁ receptors and the subsequent release of endothelium-derived NO (Kelm et al, 1993).

In experiments on the human corpus cavernosum and the circumflex vein, histamine had no effects on basal tension; but relaxed both tissues when they had been precontracted with NAD (Kirkeby et al, 1989b). These observations were not significantly affected by either H₁ receptor (mepyramine) or H₂ receptor (cimetidine) blockade. Nevertheless, the intracavernosal injection of histamine in humans was reported to lead to erection (Adaikan et al, 1991). Whether this response is a result of the stimulation of H₂ receptors which mediate relaxation and/or endothelial NO release is unknown. Furthermore, histamine may also act via an inhibition of the release of noradrenaline by the stimulation of H₂ receptors (McGrath and Shepherd, 1976).
1.10h Endothelins

It is now widely accepted that sympathetic adrenergic nerves are responsible for the maintenance of penile flaccidity; NAd may do this alone but it is likely that a number of other factors also play a role in mediating the contraction of corporeal smooth muscle and hence penile flaccidity. Amongst the most important of these factors are the endothelins (ETs).

The endothelins constitute a family of peptides; ET-1 is known to be a potent, long-lasting vasoconstrictor and may therefore maintain penile flaccidity. Cultured endothelial cells from the human corpus cavernosum have been shown to express ET-1 mRNA (Saenz de Tejada et al, 1991; Saenz de Tejada et al, 1989a). Significant ET-1 activity has been measured with RIA in the supernatant of these cultured endothelial cells (Saenz de Tejada et al, 1991). Furthermore, using an ET-1 monoclonal antibody, ET-like immunoreactivity was strongly localised to the endothelium as well to human cavernosal smooth muscle, although binding to the latter was less strong (Saenz de Tejada et al, 1991). Such an ability to both synthesise and release ET appears to be a relatively specific function of the endothelial cells (Saenz de Tejada et al, 1991). Using autoradiographic binding techniques, ET-1 binding sites have been shown in the deep penile artery, the circumflex veins and in the cavernous tissues (Holmquist et al, 1992b). Further binding experiments have shown that at least two different ET receptors, with varying affinities, are present on corporeal membranes (Saenz de Tejada et al, 1991).

A number of in vitro studies have shown that ET-1 induces slowly developing, but prolonged, contractions in the penile smooth muscles, the corpus cavernosum, cavernous artery, deep dorsal and penile circumflex veins (Holmquist et al, 1992b; Saenz de Tejada et al, 1991; Saenz de Tejada et al, 1989a). ET-1 was the most potent contractor when compared to both ET-2 and ET-3 (Saenz de Tejada et al, 1991). The precise intracellular mechanisms for ET-induced contraction are unclear. In a
preparation of human circumflex vein ET-1 appeared to act independently of calcium influx (Holmquist et al, 1992b). In the human corpus cavernosum and circumflex vein the observed ET-induced contractions were greatly reduced by a calcium free medium. Those contractions observed in a calcium free medium were abolished by the addition of a protein kinase C inhibitor (Holmquist et al, 1990). In the rabbit corpus cavernosum, ET-1 induced phosphoinositide hydrolysis (Holmquist et al, 1992c), however, whether ET-1 similarly activates phospholipase C in the human corpus cavernosum remains unknown.

Muscarinic receptor stimulation and VIP both counteract the ET-1 induced contractions of rabbit and human tissues (Holmquist et al, 1990). In addition, the ET-1 induced contraction of human tissues were reversed by ACh and sodium nitroprusside (Saenz de Tejada et al, 1991). These findings are of importance because ACh and VIP are found in the same nerves (Dail et al, 1986; Lincoln et al, 1987) and so have VIP and NOS (Junemann et al, 1993). Furthermore, these findings may provide evidence of an important anti-contractile action of VIP and NO in addition to the well-described pro-relaxant effects of these putative erectogens.

While one may speculate that high ET concentrations significantly contribute to both physiological flaccidity and erectile dysfunction, the role of the ETs remains to be fully established but they probably constitute another component of the complex control mechanism of corporeal smooth muscle tone. In particular, ET may play a part in the events of detumescence. In this context, it is interesting that an increased ET binding was reported in the STZ diabetic rat (Bell et al, 1995).

1.10i Arginine vasopressin

Arginine vasopressin (AVP)-like activity is widely distributed in the mammalian sympathetic nervous system, both in the ganglionic neurones as well as in peripheral
nerve fibres (Hanley et al, 1984). AVP has also been demonstrated by radioimmunoassay studies to be present in human cavernous tissue in high concentrations (Andersson et al, 1986).

However, bull retractor penis muscle strips were not affected by AVP, in vitro (Klinge and Sjostrand, 1974). In a rabbit preparation AVP was found to suppress erectile responses, perhaps due to the peptide producing a high resistance in the penile vascular bed (Sjostrand and Klinge, 1979). Similarly, AVP contracted human corpus cavernosum, corpus spongiosum, as well as cavernosal artery preparations, observations which could be inhibited by AVP antagonists (Andersson et al, 1986). These AVP antagonists were without effect on electrically induced contractions (Andersson et al, 1986), therefore AVP is not released by this means to affect penile smooth muscle and its physiological significance remains uncertain.

1.10j 5-Hydroxytryptamine

5-hydroxytryptamine causes contraction of the retractor penis muscle of the bull (Klinge and Sjostrand, 1974). In an in vivo rat preparation the increase of intracavernosal pressure induced by the stimulation of the sacral part of the spinal cord was inhibited by the administration of 5-HT (Finberg and Vardi, 1990). It was concluded from these observations that 5-HT exerts an inhibitory action on penile erection by a peripheral mechanism, an effect that may be mediated by vasoconstriction of the cavernosal vessels or an inhibition of the release of a vasodilator substance.

1.10k Adenosine 5'-triphosphate and adenosine

Both ATP and adenosine contract the retractor penis muscle of the bull (Klinge and Sjostrand, 1974). ATP and other purines were shown to decrease both basal tension
and phenylephrine-stimulated tension in the rabbit corpus cavernosum (Broderick et al, 1991; Tong et al, 1992; Wu et al, 1993). ATP was found to produce relaxation in the canine penile artery (Bowman and Gillespie, 1983). The response was found to be independent of the endothelium (Broderick et al, 1991; Tong et al, 1992). It was initially proposed that ATP is a NANC transmitter in the corpora cavernosa and that purinergic transmission may be an important component involved in the initiation and maintenance of penile erection (Tong et al, 1992). However, other experiments failed to demonstrate the facilitation or inhibition of the corpora to electrical field stimulation, therefore their role may be in neuromodulation rather than neurotransmission (Wu et al, 1993).

The intracavernosal injection of ATP in dogs produced increases in intracavernosal pressure and erection, an effect unaffected by atropine or hexamethonium (Takahashi et al, 1992b). Interestingly adenosine produced full erection when injected intracavernosally in dogs (Takahashi et al, 1992c). The roles of ATP and adenosine in the mediation of erection remain to be fully established.

1.11 The role of second messengers

From the preceding sections it should be apparent that corporeal smooth muscle tone is now considered to be one of the most important factors which determine the state of penile tumescence. A number of intracellular second messengers are responsible for the control of this tone (Christ et al, 1991). The most important of these are cAMP, cGMP, calcium, potassium, and inositol trisphosphate. The mechanisms influencing the state of penile smooth muscle tone have their effects via complex intracellular second messengers systems.

Cyclic AMP and cGMP were discovered more than 30 years ago. In the last ten years much has been learned about receptor-cyclic nucleotide interactions and how
GTP-binding proteins couple cAMP synthesis to receptor occupation. We have seen from the above that NO is active via the soluble guanylate cyclase. The importance of the PDEs in the control of cyclic nucleotide levels has been realised for a number of years, but it is only recently that definitive work on their structure, function and regulation has been performed. It has become clear that different isozymes catalyse the synthetic and degradative processes and that more than one isozyme of adenylate cyclase can act as a catalyst. Furthermore, many different receptors can couple with cyclases with differing effects and end results - a wide variety of different mechanisms for controlling cAMP synthesis in a cell can now be visualised. The role of the cyclic nucleotides is discussed further in chapter four.

Intracellular calcium concentration and bioavailability are among the most important determinants in the control of smooth muscle tone. Free intracellular calcium results in the events of contraction-coupling, therefore anything that will free this ion from intracellular stores will result in contraction. Membrane active compounds which increase calcium concentration will increase tone and lead to penile flaccidity, while those which extrude calcium ions and therefore lead to a decrease of calcium concentration will decrease muscle tone and therefore lead to penile erection.

The importance of potassium channels in the process of smooth muscle relaxation has only been recognised relatively recently (Hamilton and Weston, 1989; Quast and Cook, 1989). There is now much evidence that potassium channel openers relax vascular, tracheal, and urinary bladder smooth muscle cells from a number of species. This is accomplished by the opening of the 86Rb-permeable potassium channels and the subsequent hyperpolarisation of the cells. Hyperpolarisation in turn leads to muscle relaxation by preventing the opening of voltage dependent calcium-channels (Longman and Hamilton, 1992; Cook, 1988). The mechanisms that regulate corporeal smooth muscle tone are shown in Fig. 1.9.
Fig. 1.9 Diagram of the major mechanisms regulating corporal smooth muscle tone. Shown are two corporal smooth muscle cells, interconnected by a gap junction at their lateral border. The left cell depicts the series of intracellular events linked to corporal smooth muscle contraction - an elevation of intracellular calcium levels. The cell on the right depicts the events linked to corporal smooth muscle relaxation - a diminution of transmembrane calcium flux, sequestration of intracellular calcium, membrane hyperpolarisation and hence smooth muscle relaxation. The effects of PKA, PKC andPKG on gap junctions, potassium and calcium channels are probably mediated by phosphorylation of specific amino acid residues on target proteins. (Reproduced with permission from Christ, 1995)
1.9 Effects of sexual hormones

Androgens and testosterone in particular are necessary (although not sufficient) for sexual desire (libido) in men (Everitt and Bancroft, 1991). The relationship between circulating androgen levels, libido and erectile function are incompletely understood. The peripheral effects of androgens on the erectile tissues are similarly not fully understood. It has been reported that hormonal treatment (with oestrogens) does not qualitatively change the response observed to EFS stimulation and drugs (Adaikan and Karim, 1981; Hedlund and Andersson, 1985b). In contrast, in vivo work on castrated dogs suggested that the androgen deficiency so produced had direct effects on the functioning of erectile tissues (Muller et al, 1988). In a study using human tissues in an in vitro preparation, testosterone was without significant effect on contraction or relaxation (Kimura et al, 1990). Interestingly, in castrated rabbits NANC-mediated relaxation of erectile tissues was enhanced (Andersson et al, 1992; Holmquist et al, 1994). It was thought that the responsiveness to NO (linsidomine) was not altered in this preparation. It was noted however that an observed reduction of noradrenaline release from adrenergic nerves might be the mechanisms underpinning the changes (Holmquist et al, 1994). In a rat study it was shown that castration reduced the erectile responses and that this was reversible by testosterone, the authors concluded that the effect of testosterone is to enhance the erectile response to cavernous nerve stimulation at a site peripheral to the spinal cord, and more specifically that it is the postganglionic parasympathetic neurones that are the target for androgen action (Giuliano et al, 1993). The roles of both testosterone and prolactin in human sexual function were assessed in a recent study which suggested that both sexual behaviour and nocturnal erections were androgen-dependent, but that different thresholds of serum testosterone concentration applied to these different aspects of sexual function (Carani et al, 1996). The role of testosterone supplementation is more fully considered in the next chapter which is concerned with the pathophysiology and management of erectile dysfunction.
CHAPTER 2

ERECTILE DYSFUNCTION: AETIOLOGY AND MANAGEMENT

2.1 Definition of erectile dysfunction

The term impotence is imprecise and has also become stigmatised, therefore erectile dysfunction (ED) is the term currently used to describe erectile impotence.

The term impotence....has traditionally been used to signify the inability of the male to attain and maintain erection of the penis sufficient to permit satisfactory sexual intercourse. However, this use has often led to confusing and uninterpretable results in clinical and basic science investigations. This, together with its pejorative implications, suggests that the more precise term erectile dysfunction be used instead to signify an inability of the male to achieve an erect penis as part of the overall multifaceted process of male sexual function.

(NIH Consensus Conference, 1993)

Furthermore, it should be remembered that ED is a symptom complex which is often multifactorial in origin and does not in itself constitute a diagnosis. It should also be remembered that such a definition limits itself to a narrow aspect of male sexuality and does not concern itself with disorders of libido, ejaculation or orgasm.
2.2 Epidemiology of erectile dysfunction

ED is a common disorder but its exact incidence is difficult to determine; it has been estimated that some 10M men in the USA are affected and that one out of ten men will suffer with erectile failure at some time (Padma-Nathan et al, 1987b). The precise incidence will remain unknown not only because of methodological difficulties but also as many men do not actively seek medical advice. It has recently been demonstrated that the prevalence of complete impotence tripled from 5 to 15% between the ages of 40 and 70 years (Feldman et al, 1994). It is known that the frequency and intensity of sexual activity declines with increasing age, for example, 65% of men aged 60-70 report a continued sexual interest, but this falls to 20% in those over the age of 70 (Rousseau, 1986). Estimates of the prevalence of ED in the over 70s varies according to the definition of ED used, but estimates range from 15 to 55% (Feldman et al, 1994). While ED is a frequent accompaniment of old age, it is important to remember that many older men have an altered erectile physiology rather than impotence and still have adequate erections for sexual intercourse (Pentimone and Del Corso, 1994). It has been argued that while ED is common in the later years it shouldn't be considered as part of normal ageing (Morley and Kaiser, 1992). Nevertheless it is likely that many men simply accept erectile impotence as part of the normal ageing process. However, it is equally likely that many do not seek treatment because they think that no effective treatment is available, or because simple embarrassment prevents them seeking help. Nonetheless, the number of men seeking treatment continues to increase; an increasing demand that is sure to continue with the growing public and media awareness of effective treatments, factors which will be compounded in turn by an increasingly aged population.

The incidence of erectile dysfunction in particular groups of patients is especially high, the elderly have already been mentioned, another example is that of diabetes mellitus where it is estimated that between 35-50% of male diabetics are impotent (McCulloch et al, 1980). However, while the importance of such patient
subpopulations cannot be denied one must remain aware that a large number of impotent men are neither diabetic nor old and that Andrological services must be provided for men regardless of the aetiology of their erectile problems (Miller et al, 1993).

2.3 Aetiology of erectile dysfunction

For much of this century it was thought that ED was predominantly the result of psychological factors or psychogenic in aetiology; over the past two decades it has become increasingly recognised that there are a large number of organic causes of ED. In a recent study of men over 70 with erectile dysfunction it was estimated that 80% of cases were actually due to organic causes (Carroll et al, 1992). However, it is important to remember that patients with an obvious organic cause for ED may nevertheless have a secondary psychological component, and vice-versa, indeed to categorise patients as having either pure psychogenic or organic ED may be highly spurious. Nevertheless, the aetiology of ED is still divided into psychogenic or organic causes, a distinction usually made on the basis of the clinical history and nocturnal penile tumescence (NPT) recording. Following the finding that nocturnal erections are associated with periods of rapid eye movement (REM) sleep and occur from infancy to senescence, an absence or diminution of nocturnal tumescence was thought to identify patients with "organic impotence" (Fisher et al, 1965). The current role of NPT recording in the assessment of ED is discussed later. There are many organic causes of ED (table 2.1) and an ever-increasing number of drugs have also been implicated in the pathoaetiology of erectile dysfunction (table 2.2).

Physical disease may affect sexual function and erectile capacity in a number of ways. It may have direct effects by interfering with the substrates necessary for erection, e.g. by producing a vascular or neurological deficit, but the non-specific effects of disease such as pain and fatigue may also affect sexual function.
Table 2.1 The organic causes of erectile dysfunction

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<td>Autonomic neuropathy</td>
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<td>Spinal and pelvic trauma</td>
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<td>Spinal cord lesions (e.g. MS)</td>
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<td>Abnormal anatomy</td>
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<td>Renal transplantation</td>
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<td>Drugs</td>
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Table 2.2 Drugs implicated in erectile dysfunction

<table>
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<tr>
<th>Drug Class</th>
<th>Example Drugs</th>
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<td>Barbiturates</td>
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Thus, diseases such as rheumatoid arthritis may make the individual unable to perform because of limited mobility and pain. Physical illness may also result in significant secondary psychological effects, such as a loss of self-esteem and depression. These will affect the patient’s relationship and its normal dynamics. Furthermore, there may be concern about the effects of sexual activity on health (e.g. in men with a history of ischaemic heart disease or recent myocardial infarction).

Finally, the effects of treatment itself may compromise the sexual and erectile functions of the individual: as already mentioned many drugs are implicated in the pathogenesis of ED (table 2.2) and may have their effects via a number of different mechanisms. A large number of surgical procedures, such as pelvic surgery or prostatectomy, may directly produce ED. Other procedures, such as colostomy, may produce a physical disfigurement which results in a psychological component contributing to ED. The diseases which contribute significantly to ED and are of direct relevance to this thesis are discussed in detail below, the remainder are mentioned en passant. In considering the pathogenesis of erectile failure, it is apparent that abnormalities in isolation or in combination can result in erectile dysfunction.

2.4 Vasculogenic erectile dysfunction

Vasculogenic impotence may be the result of a lack of arterial supply due to occlusive vascular disease (arteriogenic impotence), or to the inability of the penis to trap blood by the veno-occlusive mechanism (so-called “venous leak”), or a combination of both. Occlusive vascular disease (viz. atherosclerosis) may result in: (1) a narrowing of the penile and/or pelvic vessels, (2) vascular damage to the erectile and endothelial tissues, and (3) a secondary venous leak. ED due to occlusive vascular disease may therefore be associated with conditions such as ischaemic heart disease, diabetes mellitus, hypertension, peripheral vascular disease and hyperlipidaemia. This
has been substantiated in a number of studies which have linked vascular risk factors with ED (Virag et al, 1985; Shabsigh et al, 1991; Feldman et al, 1994).

Diabetes mellitus is a risk factor for both atherosclerosis (AS) and ED. Notwithstanding the importance and contribution of diabetic neuropathy to the development of ED, it is AS and the associated cavernosal/endothelial dysfunction that are of paramount importance with respect to the subject matter of this thesis. In this context, a review of atherogenesis and vascular defence mechanisms is warranted.

2.4a The mechanism of atherosclerosis

Atherogenesis involves a complex pathological interaction between the arteries, the cellular components of blood (platelets and leucocytes) and their release substances, as well as with other circulating factors (lipids, fibrinogen, catecholamines). Blood vessels possess endogenous defence mechanisms (e.g. PGI$_2$, NO and adenosine) which are reported to play a role in the prevention of atherogenesis (Bartecchi et al, 1994; Wiseman et al, 1990).

Vascular lesions begin in childhood with the accumulation of lipid in smooth muscle cells of the intima to form fatty streaks (McGill Jr, 1977). In later life fibrous plaques develop; these are elevated lesions with lipid and necrotic debris and caps of smooth muscle and connective tissue. These lesions appear only rarely before the age of 20, but are more prevalent between the ages of 20 to 30. In subsequent years these fibrous plaques increase in size and undergo vascularisation, haemorrhage and ulceration. Thrombosis may occur which leads directly to arterial occlusion and infarction of the affected tissues. (The aetiology of atherosclerosis is summarised in Fig. 2.1.)
Fig. 2.1 Aetiology of atherosclerosis: 1) A healthy artery (1st decade of life); 2) First manifestation of atherosclerosis (AS) is the fatty streak. In early atheroma (2nd decade), monocytes begin to invade this area and transform into macrophages; 3) As AS progresses (3rd-4th decade), macrophages transform into foam cells which actively accumulate cholesterol. Surrounding smooth muscle cells proliferate; 4) An occluded artery due to the fully formed atherosclerotic plaque (4th decade onwards). This often ruptures precipitating platelet aggregation, thrombus formation and ultimately ischaemia.
Key events in the initiation and growth of the atherosclerotic plaque are the proliferation of vascular smooth muscle cells (VSMCs), the deposition of lipid and an accumulation of collagen, elastic fibres and proteoglycans (Ross, 1993). Atherogenesis is initiated, in part, by endothelial cell injury which results in the adherence of platelets and subsequent local release of platelet substances: TXA$_2$, serotonin, platelet activating factor, histamine and platelet derived growth factor. These substances are pro-mitogenic, promote further adhesion and aggregation of platelets and attract monocytes and neutrophils (Ross, 1993; Jeremy and Mikhailidis, 1990). Invasion of monocytes into vascular tissue and their transformation into macrophages is now accepted as a principal progenitor of the atherosclerotic plaque (Ross, 1993). Macrophages, in turn, transform into foam cells which appear at the edges of most advanced atherosclerotic lesions and accumulate lipids, in particular cholesterol (Gerrity, 1981). Macrophages also release many mitogenic and inflammatory substances (histamine, serotonin, leukotrienes, interleukins, tumour necrosis factor, prostaglandins, TXA$_2$, PDGF-like substance, toxic free radicals and neutral proteases (Ross, 1993; Jeremy and Mikhailidis, 1990). Neutrophils are also involved in atherogenesis. Apart from releasing pro-atherogenic cytokines, neutrophils generate large amounts of superoxide which elicit lipid peroxidation of fatty acids both in the plasma membrane and in low density lipoprotein (LDL), which enhance the atherogenicity of this lipoprotein (Witzum and Steinberg, 1991). Recent studies have also demonstrated that leucocyte activation can lead to platelet activation via leucocyte release substances (Nystrom et al, 1993).

Notwithstanding the mechanisms that initiate atherosclerosis, platelets certainly play a role in the end stage pathological events that lead to thrombus formation. During this stage, the atherosclerotic lesion becomes calcified and denuded of endothelium and the plaque ruptures. The vascular lumen is also markedly stenosed which leads to abnormalities of blood flow (Karino and Goldsmith, 1987). Clearly, such a pathologically damaged site constitutes a prime target for platelet adhesion, aggregation and clot formation. The shear stresses caused by stenosis have also been
implicated as a further component in platelet activation in advanced AS (Karino and Goldsmith, 1987). (Fig. 2.2 shows how these various components interact in the pathogenesis of AS.)

2.4b Vascular defence mechanisms

Blood vessels possess the capacity to counteract the pro-atherogenic effects of platelets and leucocytes. Principal amongst these defence mechanisms are PGI$_2$, NO and adenosine, all of which are synthesised by the vascular endothelium (Flavahan, 1992; Bath et al, 1991; Libby et al, 1988). PGI$_2$, NO and adenosine all inhibit the adhesion and aggregation of platelets as well as the infiltration of monocytes into blood vessels, the adhesion of neutrophils and the release of pro-atherogenic substances from these cells (Flavahan, 1992; Bath et al, 1991). Certainly impairment of PGI$_2$ and NO synthesis in vascular tissue is known to occur in AS (Flavahan, 1992; Bath et al, 1991; Libby et al, 1988). PGI$_2$ and other PGs have also been shown to inhibit in vitro VSMC and fibroblast proliferation (Bath et al, 1991). Libby et al demonstrated that the cytokine, IL-1β, stimulated VSMCs and fibroblast proliferation, an effect blocked by both PGE$_1$ and PGE$_2$ (Libby et al, 1988). In turn, both platelets and leucocytes release substances that have been shown to stimulate the release of PGI$_2$ and other PGs from endothelial cells, VSMCs and whole vessels (Jeremy et al, 1988a), indicating that there is a complex interaction and communication between these cells in which the release of substances with opposing actions are the means by which cell proliferation may be controlled in blood vessels (Fig. 2.3).

Prostaglandins and NO have other properties relevant to the aetiology of AS. For example, PGI$_2$ has been shown to stimulate (via cAMP) the activity of cholesteryl ester hydrolase (Pomerantz and Hajjar, 1989). In the vascular cell, this enzyme converts cholesterol esters (impermeable to cell membranes) to free cholesterol (which
**Fig. 2.2** Pathogenesis of atherosclerosis. This diagram illustrates some of the possible interrelationships between blood vessels, platelets and leucocytes in atherogenesis. Damage to the endothelium results in platelet and neutrophil adhesion and monocyte infiltration where they transform into macrophages (progenitors of foam cells). All these cells release substances which are mitogenic, increase vascular permeability and further enhance leucocyte and platelet accumulation. However, these release substances also stimulate vascular PG synthesis which counteract these effects (i.e. diminish platelet and leucocyte adhesion, release reactions and inhibit cell proliferation). PG release thus constitutes a defence mechanism by which atherogenic events are endogenously counteracted by blood vessels. In turn, any impairment of normal PG release may render a blood vessel susceptible to these atherogenic events.
Fig. 2.3 Control of cellular proliferation. This figure illustrates the possible involvement of PGs in the control of VSMC proliferation (the pathognomonic lesion of atherosclerosis). Mitogens elicit several early events, in particular, phosphatidyl inositol 4,5-bis-phosphate (PIP$_2$) hydrolysis which in turn results in protein phosphorylation by kinase C and calcium/calmodulin-dependent kinases (caM-PK). These events induce expression of two genes: c-fos and c-myc, thus initiating DNA synthesis and cell division. Elevation of cAMP is associated with inhibition of mitosis. cAMP activates PKA which phosphorylates proteins that counter the effects of mitogens. Thus, the antimitogenic effect of certain endogenous PGs, is mediated by activation of adenylate cyclase.
is permeable to cell membranes), which is then liberated into the circulation. Impairment of cholesteryl ester hydrolase (possibly involving impairment of PGI$_2$ synthesis) may lead to accumulation of cholesteryl esters, an accepted contributing factor in atherogenesis (Krauss, 1991). Another important means by which blood vessels are protected from AS is by the presence of ectonucleotidases, which are discussed in chapter five.

### 2.4c Hypertension

The incidence of hypertension increases with ageing and it is known that sexual dysfunction is common in elderly hypertensive men. It seems that hypertension is a risk factor for ED (Bulpitt et al, 1976; Shabsigh et al, 1991; Feldman et al, 1994), it should be remembered that it is also an important risk factor in the development of AS. Unfortunately, the drug treatment of hypertension is often itself complicated by ED. Antihypertensive drugs may have either central or peripheral effects, or both e.g. beta-blockers. However, there is evidence that hypertension by itself may be a cause of ED. As a general principle it is usually good practice to evaluate the presence of ED before prescribing for hypertension, if it is already present then patients should be carefully counselled that it may be made worse. Attempts to modify antihypertensive medication to improve the ED do not usually meet with success.

### 2.4d Failure of the veno-occlusive mechanism

As discussed in section 1.6b, anatomical and more dynamic vascular studies have shown that venous outflow restriction is an essential requirement for the initiation and maintenance of penile erection (Saenz de Tejada et al, 1988b). A failure of the penis to trap blood has been termed "venous leak". Such an inability to trap
arterial blood within the penis is a frequent abnormality seen in ED; it has been estimated that up to 30% of men suffering with ED will have venous incompetence as a contributing cause for their impotence (Virag, 1984). ED which is caused by pure venous leakage has been described as a syndrome characterised by a 2-3 year history of a progressive difficulty in obtaining an erection, rapid detumescence without ejaculation, or the ability to achieve only a partial erection (Williams, 1991).

It is appropriate in this section to discuss the mechanism of veno-occlusion in more detail to facilitate a logical understanding of the investigation and treatment of its failure. A number of studies have demonstrated that this mechanism depends on an adequate arterial inflow, sinusoidal relaxation, the compression of subalbugineal venous plexi against the tunica albuginea and intermediate veins against Buck's fascia (Wespes and Schulman, 1986; Fournier, Jr. et al, 1987; Lue and Tanagho, 1987). Corporeal smooth muscle relaxation is brought about by autonomic efferent nerves and is a critical event in veno-occlusion (Saenz de Tejada et al, 1988b). Thus, it is argued that veno-occlusion is essentially a passive mechanism depending on neurogenically and endothelially mediated penile smooth muscle relaxation (Saenz de Tejada et al, 1988b; Wespes and Schulman, 1986). In view of this central role of the penile smooth muscle the term veno-corporeal incompetence is preferable to venous leak.

Despite the available evidence, the mechanism of veno-occlusion remains somewhat controversial and therefore it is not surprising that the precise cause of venous leakage remains unknown. Venous leakage increasingly appears to result primarily from alterations of intracorporeal structures. The failure to trap arterial blood within the penis may result from a number of abnormalities. Thus, it may be the result of anatomical abnormalities such as fistulae between the corpora cavernosa and the corpus spongiosum caused by trauma allowing for abnormal venous drainage. Shunts created for the treatment of priapism may also be responsible for continued abnormal drainage. Structural abnormalities such as Peyronie's disease can cause leak
by interrupting the closure of emissary veins. At an ultrastructural level cellular and intracellular abnormalities may contribute significantly to veno-corporeal incompetence. Thus, any fibrosis or smooth muscle degeneration will interfere with its ability to relax and thus alter corporeal compliance. Such damage to the penile smooth muscle component may be the result of a number of factors: ischaemia, toxic particles (such as found in cigarette smoke), degeneration secondary to neuropathic or myopathic abnormalities, hypercholesterolaemia and hypertension. In addition many of these factors are known to cause endothelial cell dysfunction and thus less pro-relaxant factors (e.g. NO and PG) may be produced and more pro-contractile factors (e.g. ET-1, TXA₂) may be produced thus bringing about an alteration in the normal functional balance of corporeal smooth muscle. Incomplete or absent relaxation of penile smooth muscle as a result of such processes can clearly result in veno-corporeal incompetence. Furthermore, all these factors may be compounded by a secondary psychological component.

2.5 Diabetes Mellitus

It is known that erectile impotence occurs more commonly in diabetes than in the general population (McCulloch et al, 1980; Feldman et al, 1994). ED in diabetes is caused by a combination of physiological and psychological factors. Jensen showed that many diabetic men have a marked superimposed psychological component (Jensen, 1981). The ED associated with DM is normally of gradual onset. An increasing prevalence of vascular and neurological complications of diabetes with age explains the higher incidence of impotence in the older diabetic man. The neurogenic components are usually a combination of autonomic and peripheral neuropathy. The vascular component is due to both small and large vessel disease as well as microangiopathy. Furthermore, it should not be forgotten that DM has wide-ranging effects on vasodilator mechanisms and results in endothelial dysfunction, factors which are discussed in more detail elsewhere. Evidence has been presented recently
that tight glycaemic control may delay and prevent the onset of some of the diabetic complications that can contribute to the aetiology of ED, unfortunately data for this specific complication are not available (Reichard et al, 1993; The Diabetes Control And Complications Trial Research Group, 1993). In addition, diabetic patients also often have disturbances of lipid metabolism and therefore have a secondary hyperlipidaemia.

2.6 Medication-induced erectile dysfunction

A large number of drugs have been implicated in the aetiology of ED (table 2.2). However, drug-related effects may sometimes be difficult to separate from the effects of chronic disease. Drugs may affect erectile performance at a number of levels including central, peripheral and target organ.

Antihypertensive agents are the most commonly cited drugs in causing ED. Hypertension is common and as a result antihypertensives are probably the most frequently used drugs in medical practice. As alluded to previously, beta-blockers have their effects centrally, where they act to lower libido, peripherally by reducing the penile perfusion pressure and at the endothelial level by causing decreased PG production (Campbell et al, 1981; Mikhailidis et al, 1987; Andersson et al, 1991a). The thiazide diuretics cause ED in some 9 percent of patients who take them (Hogan et al, 1980). The drugs that have the lowest incidence of ED as a side-effect of the treatment of hypertension are prazosin, calcium channel blockers and the angiotensin converting enzyme (ACE) inhibitors (Kochar et al, 1979; Pitts, 1975). The major tranquilisers, the phenothiazines, monoamine oxidase inhibitors and the tricyclic antidepressants are all in common use. The sedation which they produce as well as their anti-cholinergic and sympatholytic effects are thought to be the responsible factors. The phenothiazines can also raise prolactin levels which can interfere with luteinising hormone and testosterone secretion. As can be seen from the table a large
number of other drugs can cause ED, a definite site or mode of action cannot be determined for all of them.

2.7 Renal failure

Chronic renal failure is a well-documented cause of ED, which is present to a variable extent in between 38 to 80% of dialysis patients, some 20 to 55% are completely impotent (Abram et al, 1975). The condition is often exacerbated after the beginning of dialysis and of those men who have a renal transplant, 10% will have post surgical ED, this figure rises to 30% after a second transplant (Gittes and Waters, 1979).

2.8 Iatrogenic causes of erectile dysfunction

ED may often be the result of a number of iatrogenic causes. Not only do surgical procedures cause ED but other interventions such as dialysis, radiotherapy and endocrine manipulations may result in ED. The reported incidence of ED after a transurethral resection of the prostate is some 4 to 12% (Hanbury and Sethia, 1995). It is important in this respect to assess any pre-existing ED and to counsel the patient carefully about retrograde ejaculation. Following the anatomical studies of Walsh et al it is now reasonable to expect ED rates in the order of 15% following radical prostatectomy (Walsh and Donker, 1982).

Any surgical procedure involving the lower abdomen or pelvis can result in damage or destruction of the nerves and vessels which subserve erection. Erectile function will often be impaired only temporarily after a surgical procedure and a period of reassurance and careful counselling is of great benefit in these cases. If the ED persists then the available treatment options should be explored.
2.9 Neurogenic erectile dysfunction

It can be appreciated from the discussion in chapter one that normal physiological erection depends on the integrity of complex neural substrates. As a consequence a large number of neurological disorders can cause ED. Defects in both the peripheral and central nervous system can result in erectile impotence. Some of these patients may be relatively young and their care can place a considerable burden on Andrological services. Not only can neurological disease alter the mechanisms of erection but concomitant disorders of mobility can restrict the ability to have sexual intercourse. Furthermore, the psychological effects of such a chronic neurological illness may be considerable.

Amongst central nervous system disorders, spinal cord injury is a frequent cause of ED. However, if the lesion is below T9 then psychogenic erections can still occur, whereas reflexogenic erections will occur if the sacral roots are intact (Bors and Comarr, 1960; Comarr, 1970). It has been estimated that up to 60% of men with multiple sclerosis are impotent and most commonly this is thought to be due to cervical demyelination, however, the higher centres can also be affected (Ivers and Goldstein, 1963).

The commonest peripheral nervous system causes of ED are those seen in diabetic neuropathy, which can affect both the autonomic and somatic fibres. Cord compression from a prolapsed intervertebral disc or cauda equina tumours can also result in ED. A common iatrogenic cause of nerve damage is that due to radical pelvic surgery where the cavernous nerves are particularly at risk. In this context the recent advances such as the development of the technique of nerve-sparing radical prostatectomy have reduced the incidence of ED associated with such procedures (Walsh and Donker, 1982).
2.10 Endocrinological causes of erectile dysfunction

Endocrine disturbances are an important and potentially treatable cause of ED; these include disorders of the hypothalamo-pituitary-testicular axis, hyperprolactinaemia and DM. Disturbances of thyroid, adrenal and calcium metabolism may also result in disturbances of erectile function. However, the commonest endocrinopathy is DM and is discussed elsewhere.

2.11 Smoking and erectile dysfunction

Smoking has been clearly linked to erectile dysfunction as a risk factor for its development (Shabsigh et al, 1991; Feldman et al, 1994). Experimental data has implicated disturbances of prostaglandin metabolism in the aetiology of erectile failure, for example, rats which have been rendered diabetic have decreased penile PGI\textsubscript{2} synthesis (Jeremy et al, 1985b), and similarly, cigarette smoke extracts (and not nicotine) inhibit PGI\textsubscript{2} production in rat penile tissue (Jeremy et al, 1986a). It may be that an interference with penile prostaglandin metabolism may account, at least in part, for the impotence that is associated with certain drugs.

Notwithstanding the effects on prostaglandin synthesis, smoking has recently been demonstrated to affect the contractile activity of penile arteries and data has been published which supports the notion that smoking may further compromise penile physiology in men who are experiencing difficulty in maintaining erections long enough for satisfactory intercourse (Hirshkowitz et al, 1992).
2.12 Investigation of erectile dysfunction

2.12a History and examination

A carefully taken medical, surgical, sexual and social history is a most important aspect of the initial consultation when not only is information gleaned about the aetiology of the patient's ED but at which a working relationship is established. Time spent gauging the patient's expectations of treatment may help to identify those with unrealistic hopes. It should be established whether the onset of erectile failure was gradual or sudden, as ED with an organic aetiology is usually of gradual onset. An ability to perform with other partners or attaining a normal erection with masturbation is a clear indicator of psychogenic ED.

One should ask about the patient's libido and the occurrence of early morning erections or spontaneous erections. If erections are still obtained, their firmness and duration should be established. Patients should be asked if there is any abnormality of ejaculation. An attempt should be made at probing the partner's attitudes, and it may be that the marital history should be fully explored. A full drug history is always taken, as a large number of drugs are implicated in the aetiology of impotence and it is possible that drug therapy can be modified with good effect. Alcohol consumption should be quantified and patients should be questioned about tobacco-smoking and other drugs of abuse.

Examination of the patient should include a general examination, with attention to build and hair distribution. The cardiovascular system should be carefully examined with respect to the blood pressure and peripheral pulses. The lower limbs should be examined for neurological abnormalities, the anal and bulbocavernosus reflexes and sensation in the saddle area should be assessed. The penis should be examined for evidence of fibrosis or the plaques of Peyronie's disease which are
normally found on the dorsal aspect, but sometimes in the septum, and thus the organ should be carefully palpated. A note should be made of testicular size or atrophy.

2.12b The assessment of erectile dysfunction

The assessment of ED has two main aims. Firstly, it is useful to be able to objectively assess the capacity that a patient has for penile erection. Secondly, investigations may be directed towards establishing the specific cause of the ED. Erectile capacity may be assessed by a variety of methods the most popular of which have been the response to an intracavernosal injection of a vasoactive agent and nocturnal penile tumescence (NPT) monitoring.

Patients may be pragmatically divided according to their response to the intracavernosal injection of a vasoactive "erectogen". Those with psychogenic or neurogenic impotence will normally respond with an erection, as will those without a significant degree of vascular impairment. However, the failure to respond to such an injection may be the result of powerful situational factors and significantly different responses may be observed in a more stimulating environment. Those men who respond with an erection of sufficient quality for intercourse may then be taught to self-inject, whilst those who don't will usually undergo further assessment. The use of an intracavernosal injection of a vasoactive agent as an outpatient screen of significant vascular dysfunction has gained wide acceptance, and within limits, it does provide a convenient, cheap, and simple assessment of penile vasculature. The injected agent may be papaverine alone, or in combination with phentolamine, and PGE₁ has been increasingly used in this context. Patients with psychogenic or neurogenic impotence will normally respond to an injection of vasoactive substance with an erection as will those without a significant degree of vascular impairment. Patients failing to respond with an erection may be considered to have significant vascular dysfunction, although "psychic inhibition" may be a powerful factor in some non-responders.
Nocturnal penile tumescence monitoring has long been generally accepted as the gold standard investigation which differentiates psychogenic from organic ED. NPT occurs from infancy to senescence and is related to periods of REM sleep (Fisher et al, 1965; Karacan et al, 1966). The assumption underlying NPT recording is that men with psychogenic impotence will have normal NPT, while those with an organic aetiology will have abnormal or absent NPT. The hypothesis followed from the work of Jovancovic who found disturbances of NPT in a number of impotent patients and noted that these were more pronounced in men with organic ED than in those with psychogenic ED (Jovanovic, 1972).

The intuitive appeal of this hypothesis was so strong that it resulted in a wide acceptance of NPT recording as a valid method in the differential diagnosis of ED, indeed it is still considered by many to be a fundamental aspect of assessment. Although objections have been raised to the hypothesis on methodological grounds, more recent work with improved experimental designs still supports the original premise (Marshall et al, 1981). The nature of the relationship between NPT and erectile potential in an erotic or sexual context has been questioned and has been investigated by monitoring both penile tumescence and rigidity with a RigiScan (Dacomed Corp., Minneapolis, Minnesota) during exposure to videotaped erotic material (Condra et al, 1991). (The RigiScan is one of the newer methods available for NPT recording, which provides the investigator with rigidity data which is accomplished by measuring rigidity during radial loading of the penis). The use of visual sexual stimulation has of course intuitive appeal as it mimics an erotic setting, however, it has a number of advantages and disadvantages but in particular it should be remembered that the use of this technique has not been properly validated and any results need to be interpreted with caution (Condra et al, 1991). The importance attributed to nocturnal penile tumescence assessment has declined over the years, and its current place in the investigation of impotence has been the subject of a comprehensive review (Morales et al, 1990). Morales concluded that NPT recording only demonstrates erectile failure during sleep and it remains simplistic to believe that this invariably implies an organic aetiology - for example, it has been
shown that depression and high-anxiety content of dreams will also affect NPT (Karacan et al, 1966). It would seem sensible not to interpret the results of NPT recording in isolation, but to consider them as only part of a comprehensive assessment of the impotent patient. Abnormal NPT should never be interpreted as providing conclusive evidence of organic impotence. A major problem is that if the test is to be performed properly then it requires the use of a formal sleep laboratory and should be conducted on more than one occasion which makes it an expensive investigation, more recently it has been reported that useful data may be obtained from the use of a somewhat abbreviated study performed in a 'snooze' rather than a sleep laboratory (Morales et al, 1995). Schiavi et al recently reported their findings of abnormal NPT recording in men with diabetes but an absence of reported erectile dysfunction and thus counselled that caution should be exercised in the interpretation of abnormal NPT recording (Schiavi et al, 1993), it was argued that this might perhaps represent a potential use of NPT monitoring as a screening instrument in patients at risk of ED (Miller et al, 1994c).

2.12c Endocrinological investigation

The initial investigation of ED and more specifically whether this need include an endocrine profile has been the subject of some controversy. It is known that low testosterone, high prolactin and luteinising hormone levels are associated with decreased sexual activity (Perryman and Thorner, 1981). However, the prevalence of endocrinopathy in men with ED is low, thus financial constraints and common sense may make the rationalisation of such investigations necessary. Indeed, it has previously been argued that unless there are strong clinical indications of endocrinopathy such investigation should not be undertaken.

A recent study addressed the question of whether it was feasible to identify a particular subpopulation at high risk of endocrinopathy, thus making it a more
reasonable group to screen. Seven out of 330 patients (i.e. 2.1%) were found to have a repeatedly low testosterone, five of these had testicular atrophy, six a decreased libido and all seven had either testicular atrophy or a decreased libido. The authors concluded that in order to rationalise the investigation of impotence, only men with clinical signs of hypogonadism and poor libido should be screened (Johnson and Jarow, 1992).

Nevertheless, it remains possible to have a normal libido and examination in the presence of abnormal hormonal profiles and it is noteworthy that a proportion of men maintain normal sexual function even after medical or surgical castration (Ellis and Grayhack, 1963; Heim, 1981). Much less controversial and inexpensive is the exclusion of diabetes by urine testing which should be done routinely on all patients at their first outpatient appointment.

2.12d Radiological investigation

An intracavernosal injection of a vasoactive erectogen may be combined with duplex ultrasonography or more recently with colour-coded ultrasound scanning, which is proving to be a most useful adjunct. Following such an intracavernosal injection, the penile arterial inflow is determined by observing both the diameter of the superficial and deep penile arteries and changes therein (Lue et al, 1985; Shabsigh et al, 1989), furthermore the peak systolic and end diastolic velocities can also be determined (Fitzgerald et al, 1992). Should such an investigation reveal a significantly decreased arterial inflow then one can proceed to formal arteriography in order to delineate accurately the sites of stenosis, as well as assessing the feasibility of operative revascularisation. The success of revascularisation procedures depends largely upon methods of imaging the penile arterial vasculature and both penile and pelvic arteriography is mandatory for the localisation of lesions in the arterial tree.
However, arteriography provides little physiological or functional information and there in little or no control data from elderly potent men (Lue, 1990).

Ultrasonography also provides detailed information on penile anatomy and is able to identify penile fibrosis and the plaques of Peyronie's disease. Ultrasonography may be used to identify patients with no erectile response to an erectogen despite the presence of a normal arterial tree; impotence might then be ascribed to a venous origin, however, this methodology is of limited value in evaluating veno-occlusive dysfunction. Further studies need to be undertaken if surgical intervention is contemplated to correct a significant venous leak which normally takes the form of dynamic infusion cavernosometry and cavernosography or DICC (Virag, 1982c). Whilst considerable controversy persists about the mechanism of failure of veno-occlusion, whether or not it can be determined by the technique of pharmaco-cavernosometry is perhaps equally controversial (Ralph and Pryor, 1996). In the original description of pharmaco-cavernosometry saline was infused into the corpus cavernosum via a butterfly needle and the pressure in the contralateral corpora was monitored by another needle (Virag et al, 1979). The flow required to attain and then to maintain an erection by such an infusion is monitored. Clearly this is not a physiological study and the original technique was subsequently modified to include the administration of a vasoactive agent prior to the test (Lue et al, 1986). The addition of sufficient erectogen has been argued by many to be extremely important in order to produce full smooth muscle relaxation (Lue et al, 1986). It has been argued that if veno-occlusive dysfunction is present then there will be a low venous-outflow resistance as manifested by either an elevated infusion flow rate, or alternatively by a rapid fall in intra-corporal pressure (Wespes and Schulman, 1985). It has, however, also been argued that in the absence of sufficient standardised data, the cavernosometric criteria for the diagnosis of corporovenous dysfunction may have previously been too strict (Vickers et al, 1992).
Following cavernosometry, pharmacocavernosography is the technique which has been used to identify veins which provide abnormal drainage of the corporal bodies in erection, thus it provides information which is essential for surgical intervention (Lue et al, 1986; Dickinson and Pryor, 1989). As in the previously described investigation pharmacocavernosography is ideally performed with the administration of sufficient smooth muscle relaxant to achieve complete relaxation of the cavernosal smooth muscle, although how this state is accurately fulfilled and monitored is not clear. Following the injection of vasoactive erectogen contrast material is infused into the erect penis. When corporal veno-occlusion function is normal then little or no venous drainage is seen radiographically (Puyau and Lewis, 1983).

In the absence of a vasoactive agent, if the flow rate required to maintain erection is greater than 15 to 20ml min\(^{-1}\) the patient is considered to have a cavernovenous leak (Wespes and Schulman, 1993). However, such studies should always be combined with the injection of a vasoactive agent to ensure full smooth muscle relaxation to make the study as "physiological" as possible and the patients may require redosing (Hatzichristou et al, 1995; Lue et al, 1986). Leakage may also be diagnosed by analysing the pressure decay when infusion is stopped. There may be a role for smooth muscle EMG in the diagnosis of smooth muscle dysfunction which would perhaps be a useful adjunct; in order to be useful it would need however to be able to differentiate between myogenic and neurogenic pathologies and smooth muscle biopsy might be another useful investigation in this respect. Normal men will have an erection induced by a flow of less than 120ml min\(^{-1}\) and in the absence of an erectogen a flow of less than 50ml min\(^{-1}\) will be required to maintain the erection (Wespes and Schulman, 1993). With the use of a vasoactive agent, a normal subject will induce an erection with a flow rate of less than 35ml min\(^{-1}\) and a maintenance requirement of less than 5ml min\(^{-1}\) (Wespes and Schulman, 1993). However, there are a number of problems with the use of DICC: there are difficulties with validation as many men with normal erections have been shown to leak, there is no standard
classification for the findings and similarly there is no standardised treatment for venous leak (Ralph and Pryor, 1996).

2.12e Neurological investigation

A patient’s neurological evaluation normally consists of physical examination alone. However, this can be supplemented by neurophysiological testing although it is uncommon to diagnose neurogenic impotence in a patient without either a known neurological disorder or neurological symptoms. The afferent somatic pathway may be initially tested by penile biothesiometry, which measures sensory perception thresholds to vibration, an alternative to which is the cutaneous perception threshold to an electrical current (Ertekin et al, 1985; Newman, 1970). Abnormalities are most often demonstrated in patients with diabetes mellitus or a history of alcohol abuse. If there is evidence of sensory loss, then an assessment of penile nerve conduction velocity and bulbocavernosus reflex (BCR) latency will differentiate between peripheral and sacral spinal pathology. Information on the entire somatic peripheral and central sensory pathway from pelvis to cerebral cortex may be obtained from dorsal nerve somatosensory evoked potentials. These investigations are not part of the routine work up of most patients and all of the above methods are capable of only examining somatosensory penile innervation.

Autonomic dysfunction is suggested by absent NPT with bladder and bowel dysfunction. Corpus cavernosal electromyography (CC EMG) recorded by needle or surface electrodes, is the only method currently available to investigate autonomic motor denervation. It was initially proposed by Wagner as a means of objectively diagnosing autonomic cavernous dysfunction and cavernous smooth muscle degeneration (Wagner et al, 1989). A further development of this technique was the single potential analysis of cavernous activity (SPACE), which has subsequently been deemed an inaccurate term and CC EMG is to be preferred (Stief et al, 1991a; NIH
Consensus Conference, 1993). The results to date suggest that the action of cavernous smooth muscle is very similar to gastrointestinal smooth muscle. The main electrical activity is a slow wave present at all times during flaccidity, but a fast electrical activity which induces muscle contraction has also been described. The injection of drugs with smooth muscle relaxing properties abolishes all fast activity. It is thought that neural elements in the bowel wall smooth muscle merely serve to change the electrical activity and this is in agreement with the findings in neurogenic patients where autonomic denervation is suspected. The continued presence of fast activity during tumescence induced by visual sexual stimulation is thought to indicate either autonomic damage to the cavernous nerves or alternatively a psychogenic aetiology if the cerebral inhibition is disturbed. The relaxation of cavernous smooth muscle cells is thought to occur as a syncytium via gap junctions, and that an abnormal innervation leads to a desynchronisation and degeneration of smooth muscle cells associated with a loss of these junctions (Moreno et al, 1990).

The BCR latency, dorsal nerve conduction velocity, and dorsal nerve somatosensory evoked potentials are not used routinely. CC EMG probably has no utility in the diagnosis of autonomic neuropathy and to what extent it can be used to diagnose pathology in the cavernous smooth muscle remains unanswered. What the true value of CC EMG is in assessing smooth muscle pathology and autonomic innervation dysfunction therefore remains to be seen, at present it does not form part of the routine assessment in most centres but remains a research tool and nevertheless is the only available test of autonomic motor denervation. It is a controversial investigation and its critics convincingly argue that it is only a reflection of normal sympathetic skin activity and it should always be critically evaluated.
2.13 Treatment of erectile dysfunction

With the evolution of a number of effective treatment modalities for erectile dysfunction there has been a change in the approach towards the management of the man which is now much more goal-oriented and less dependent on any great diagnostic precision. While the aetiology remains often indeterminate it should be possible to tailor most patients treatment to their needs.

2.14 Intracavernosal Pharmacotherapy

The serendipitous discovery of the effects of an intracavernosal injection of papaverine (Michal et al, 1977) and the subsequent work of Brindley and Virag has resulted in one of the most important advances to be made in the diagnosis and treatment of ED over the past decade (Brindley, 1983; Virag, 1982b). The original observation was extended by other workers who showed that papaverine and other vasoactive agents could be successfully injected by patients. Men can now learn to self-inject with a vasoactive substance to produce pharmacologically-induced erections, although it should be remembered their ability to do so depends on such factors as adequate manual dexterity and visual acuity (Padma-Nathan et al, 1987a). The technique is perhaps most useful in those with neurogenic and psychogenic impotence, but it is also used with success in a percentage of men with vasculogenic impotence. Intracavernosal self-injection has become the first choice treatment for many clinicians. The institution of such a programme of self-injection should be done under specialist supervision. Patients initially undergo a dose-finding stage when the effects of injection are titrated against dose, while at the same time they are fully instructed in the technique of self-injection. The injection is normally performed with a standard insulin syringe and is relatively painless, but there are also a number of autoinjection devices available. The aim of therapy is to produce an erection which lasts some 30 to 60 minutes. Only one of the agents currently used for this indication
are actually licensed for use in ED and thus particular attention should be exercised by the clinician as regards the education of the patient, and informed written consent should be obtained from all patients.

2.14a Papaverine

The most widely used vasoactive agent has been papaverine, used either by itself or in combination with phentolamine. Its precise mode of action remains incompletely understood, however, it probably has a number of different actions including: a non-specific phosphodiesterase inhibition, alpha-adrenergic blockade and a modulation of intracellular calcium fluxes, all of which will result in smooth muscle relaxation. The development of Peyronie's type penile plaques and cavernosal fibrosis have been reported with papaverine (Juenemann and Alken, 1989; Andersson et al, 1991b). A review of the literature concluded that papaverine may cause prolonged erection in up to 9.5% of patients (Krane et al, 1989; Juenemann and Alken, 1989).

2.14b Prostaglandin E₁

The therapeutic use of PGE₁ was first described by Ishii and co-workers (Ishii et al, 1986; Ishii et al, 1989) and it is becoming increasingly popular as a vasoactive agent used in intracavernosal pharmacotherapy programmes. It produces smooth muscle relaxation by a combination of alpha-adrenergic inhibition, in addition to raising the intracellular concentration of cAMP and via its effect on intracellular calcium (Derouet et al, 1994; Bhargava et al, 1990). The complication of priapism is seen much less frequently with PGE₁ because of the ability of the penile tissues to rapidly metabolise it (Roy et al, 1989), furthermore should any PGE₁ gain access to the systemic circulation it is rapidly inactivated by a single pass through the lungs (Hamberg and Samuelsson, 1971). PGE₁ has not been shown to be produced by
human penile tissues. Despite having a very favourable pharmacological profile of action a significant problem associated with its use is that of a burning/stinging discomfort associated with the injection which lasts throughout the erection and affects up to 40% of patients (Linet and Neff, 1994). A review of the literature concluded that papaverine may cause prolonged erection in up to 9.5% of patients while PGE₁ was associated with a considerably lower rate of 2.4% (Juenemann and Alken, 1989). It is also of interest that PGE₁ is effective in a proportion of papaverine non-responders and has an overall response rate of between 70-80% (Juenemann and Alken, 1989; Stackl et al, 1988). In a more recent review Linet drew similar conclusions from a large number of both short and long term studies (Linet and Neff, 1994).

PGE₁ has been compared with more conventional pharmacological agents in many studies and all have demonstrated its efficacy. It has been shown that compared with papaverine, PGE₁ administration produces a slower onset of erection, a longer duration of erection and fewer side-effects (Chen et al, 1992). The incidence of pain with PGE₁ use is certainly one of the major disadvantages of treatment with intracavernosal PGE₁ and a number of workers have attempted to address this drawback with the addition of local anaesthetics to the injection mixture (Kattan, 1995). Nevertheless, PGE₁ is safer than the other injectable 'erectogens' (Linet and Ogrinc, 1996). Its observed safety is probably due in large part to the capacity of the penis to degrade PGs by the action of PG 15-hydroxydehydrogenase (Roy et al, 1989). Relatively little is known about the pharmacokinetics of PGE₁, but systemic side-effects are rare and this is probably a reflection of the very low doses that are effective, the local degradation of the PG, local stasis in the penis, and because some 90% of circulating PGE₁ will be metabolised by a single passage through the lung (Hamberg and Samuelsson, 1971). PGE₁ would therefore seem to be the drug of choice at present for intracavernosal pharmacotherapy, but one has to bear in mind its considerably greater expense and the high incidence of penile discomfort which may contribute to the high drop out rate from this form of therapy (Althof et al, 1989).
The case for using PGE\textsubscript{1} for intracavernosal pharmacotherapy has been considerably strengthened by it being the only drug that is currently licensed for this indication. The development of penile fibrosis and plaques with PGE\textsubscript{1} use has recently been reported but appears to be of little overall significance (Chen et al, 1996).

2.14c Phenoxybenzamine and phentolamine

Phenoxybenzamine is an alpha-adrenoceptor blocker which has been used in both the diagnosis and treatment of erectile dysfunction (Brindley, 1983). Its effectiveness in the treatment of erectile dysfunction was clearly documented in clinical trials (Keogh et al, 1989). However, it has a number of disadvantages which include pain, priapism and the development of penile fibrosis which make it an unsuitable agent for this application (Juenemann and Alken, 1989).

Phentolamine is another alpha-adrenoceptor, when injected intracavernosally it doesn’t usually produce a satisfactory erectile response (Wespes et al, 1989). However, it is widely used in combination with papaverine when the effects of the two agents appear to be synergistic rather than additive (Zorgniotti and Lefleur, 1985; Juenemann and Alken, 1989).

2.14d Other agents

A large number of other vasoactive agents have been tested as potential candidates for intracavernosal pharmacotherapy. For example, investigators have used well-established drugs such as verapamil (Brindley, 1986), but novel agents such as VIP (Kiely et al, 1989) or CGRP (Djamilian et al, 1993) have also all been used. More recently attempts have been made to use agents which have their effects by virtue of their ability to act as NO donors. The two agents which have been used in
In this respect are sodium nitroprusside and linsidomine chlorhydrate, they do not appear to have any clinical utility at present (Brock et al, 1993; Stief et al, 1992; Porst, 1993; Martinez-Pineiro et al, 1995).

### Complications of intracavernosal pharmacotherapy

The complications of intracavernosal pharmacotherapy can be divided into local and systemic, although it is the local ones that usually predominate. Bruising may occur at the injection site and may spread subcutaneously, however, no specific treatment is usually required as the condition will settle spontaneously. Haematoma formation is rare and its occurrence can be minimised by careful technique (Stackl et al, 1988). Injection into the corpus spongiosum and urethra may result in urethral bleeding and again are the sequelae of a poor injection technique. Fibrosis and smooth muscle hypertrophy have been reported to occur in monkeys which had been injected with papaverine, a response which was seen in some cases after a single injection (Hwang et al, 1991). Such changes may be the result of repeated trauma from injection, but it might also be the result of injecting into the tunica itself, or alternatively it could be a function of the pH of the solution injected or the irritant properties of the substance itself (Seidmon and Samaha, 1989).

Each of the agents that is currently used has its own particular advantages and disadvantages. The main disadvantage of any of these pharmacological agents is the risk of either a painful or prolonged erection, or both. Priapism, defined as a prolonged or painful erection not associated with sexual desire and lasting more than six hours is a particular concern because if inadequately managed it may lead to irreversible cavernosal fibrosis. The development of corporeal fibrosis and nodules, which may ultimately result in penile curvature has been described (Larsen et al, 1987; Corriere et al, 1988; Juenemann and Alken, 1989; Ravnik-Oblak et al, 1990; Schramek and Waldhauser, 1989). As it is thought that injection into the tunica
albuginea may be the cause of localised nodule formation - the possibility of this complication arising should be decreased if patients are instructed never to inject against resistance. In addition, patients should inject alternate sides and at different sites in order to minimise the effects of repeated trauma. Infection as a result of injection has been reported and is also very rare (Montorsi et al, 1993). The systemic side-effects that have been reported are hypotensive episodes and abnormalities in tests of liver function (Andersson et al, 1991b).

2.15 Oral therapies

Because of the unacceptability of intracavernosal self-injection and of the more invasive treatments to some patients, many other agents and treatment modalities have been investigated and these include the use of orally active agents, topically active agents and the relatively new concept of intraurethral preparations.

2.15a Yohimbine

Yohimbine is an indole alkaloid derived from the bark of the Yohimbe tree (*Pausinystalia yohimbine*) which grows in India and Africa. As an oral preparation it has long been used as both an aphrodisiac and a treatment for erectile dysfunction. Pharmacologically, yohimbine has a relatively selective effect on alpha-2 adrenergic receptors (Goldberg and Robertson, 1983). This suggests that it may have a predominantly central effect as it is the alpha-1 subtype that is perhaps of more importance in penile erectile tissues. Central alpha-2 blockade may provide some real benefit in the management of carefully selected cases of erectile dysfunction. Good responses to the administration of yohimbine were reported initially (Morales et al, 1987), but later controlled trials on patients with erectile dysfunction of different aetiologies showed an improved but non-significant result in those patients with an
organic aetiology (Morales et al, 1987). Results which did achieve statistical significance were found in patients with psychogenic impotence with an overall response rate of 57% to the drug (Reid et al, 1987).

The ease of administration of yohimbine coupled with its relatively few side-effects has resulted in some enthusiasm for treatment with this drug, particularly in the USA. Patients should be given a course of the drug and have its effects assessed eight weeks later. If there has been a beneficial effect then this treatment may be continued indefinitely. Yohimbine may be worth a therapeutic trial in many patients before embarking upon other more aggressive therapies.

2.15b Testosterone

The majority of men with ED have serum testosterone levels in the normal range and, as discussed above, only a small proportion will have significant endocrinopathy. Testosterone supplementation should be offered only to those patients with levels below the normal range. Testosterone may be administered either in the form of a course of intramuscular injections (e.g. Sustanon, testosterone propionate), or alternatively as a course of oral therapy (e.g. Restandol) which may prove of benefit to some men. However, the generally accepted opinion is that testosterone acts to increase libido without necessarily having any effect on erectile performance (O'Carroll and Bancroft, 1984). It would seem that the main effect is on central processes and this is supported by the finding of a decreased number of spontaneous erections following the withdrawal of androgen therapy (Kwan et al, 1994).

The problems associated with testosterone therapy are well-recognised, it may cause and exacerbate hypertension, fluid retention, liver disease, decreased spermatogenesis and testicular atrophy. Importantly it should not be used in patients with proven or suspected carcinoma of the prostate. Overall, it would seem that
testosterone therapy has a limited role to play, although it is beneficial in increasing sexual interest, but it is ineffective in the treatment of erectile dysfunction and is associated with potentially serious side-effects.

2.15c Trazadone

A number of largely anecdotal reports of increased libido, sexual function and priapism in patients taking oral trazadone have led to a number of clinicians using it empirically in the treatment of erectile dysfunction (Abber et al, 1987; Lal et al, 1990). Trazadone acts selectively to inhibit the reuptake of serotonin and is used as an antidepressant medication. Recently Lance et al reported the results of a retrospective review of the use of trazadone in patients with impotence in an attempt to put its use on a more scientific footing (Lance et al, 1995). They followed up some 127 patients and reported that overall the medication was very well tolerated; they concluded that trazadone was of most benefit in patients who were younger (less than 60y) and without any known risk factors for their erectile dysfunction (Lance et al, 1995). It would seem reasonable in the light of these findings to continue to empirically use trazadone but perhaps to limit its use to those patients fulfilling the criteria mentioned.

2.15d Apomorphine

The effects of compounds with dopaminergic, serotonergic and adrenergic activity such as L-dopa, apomorphine, bupropion, flenfuramine, yohimbine and trazadone have been studied in animals and humans and have anecdotally been found to improve erectile function (Lal et al, 1984). Apomorphine is a dopamine agonist which when injected in small doses produces an observable erectile response in almost 100% of normal rats (Malmnas, 1976). The specific mechanism of action is incompletely understood but is probably the result of central stimulation (Heaton and
Varrin, 1991). Following these observations apomorphine has been used as a bioassay for the erectile integrity of the rat and abnormalities in the apomorphine test have been reported in recent investigations (Heaton et al, 1991). Following from this there were some encouraging early results from the administration of apomorphine in men with psychogenic erectile dysfunction (Segraves et al, 1991). Unfortunately, these early attempts had to use a parenteral route of administration and the not insignificant side effect of nausea largely limited its clinical application. More recently Heaton et al investigated the utility of a number of different preparations of apomorphine in producing an erectile response in men with predominantly psychogenic erectile dysfunction, they reported that using a tablet the results in a small group were sufficiently encouraging to justify larger clinical studies (Heaton et al, 1995).

2.16 Topical preparations

Intracavernosal pharmacotherapy has disadvantages, as discussed previously, and because of this a number of other agents have been evaluated for use as a topical treatment in erectile dysfunction. Minoxidil and nitroglycerin have been used in this respect and are reviewed below, more recently there have been reports of both papaverine and PGE1 being used in a similar manner (Kim et al, 1995; Kim and McVary, 1995).

2.16a Nitroglycerin

Nitroglycerin is a well known vasodilator and both it and isosorbide dinitrate have been shown to relax isolated strips of human corpus cavernosum in vitro (Moreno et al, 1990). It is presumed that such relaxation is produced by the generation of cGMP via soluble guanylate cyclase. Thus nitroglycerin acting as a
transdermally absorbed smooth muscle relaxant would have obvious advantages over other treatment modalities.

Nitroglycerin was found to be superior to placebo in producing erectile responses to visual erotic stimuli, a result which was later supported by the findings of a double-blind study (Owen et al, 1989). Unfortunately the use of nitroglycerin is associated with systemic side effects, it may produce hypotension and headaches (as a result of cerebral vasodilatation) in both the patient and his partner (Talley and Crawley, 1985). In another study using nitroglycerin plasters an overall response rate of 40% was found in a study population of ten impotent men (Meyhoff et al, 1992). It would be reasonable to conclude that the use of nitroglycerin might be of benefit in a number of men who not only achieve a good erectile response but are also able to tolerate any side-effects, however it is not widely used in the treatment of ED.

2.16b Minoxidil

Minoxidil is an alpha-antagonist originally developed for the treatment of hypertension. A 2% topical solution of minoxidil has been shown to be more effective in increasing the diameter, rigidity and arterial inflow to the penis than either 2% nitroglycerin paste or placebo (Cavallini, 1991), in addition fewer side-effects were reported with minoxidil than with nitroglycerin paste (Cavallini, 1994). Minoxidil was found to be most effective in neurogenic erectile dysfunction and least effective in arteriogenic cases (Cavallini, 1994). Fewer side-effects were reported with minoxidil than with nitroglycerin, however, burning at the application site was reported in a few subjects and it is unclear whether the partner might also absorb the minoxidil solution (Cavallini, 1994). In contrast to this, another study assessed the effects of topically applied 2% minoxidil solution in comparison with intracavernosal pharmacotherapy and external vacuum devices in a study population of men with spinal cord injury resulting in erectile dysfunction (Chancellor et al, 1994). The investigators reported
that in this series the topical application of minoxidil caused little objective or subjective response and furthermore that none of the study subjects wanted a home trial of minoxidil when offered to them (Chancellor et al, 1994). These findings concurred with those of Radomski et al who suggested that 2% topical minoxidil wasn’t effective when applied to the penis as a treatment for erectile dysfunction in a home setting (Radomski et al, 1994). However, another study carried out in 15 spinal cord injured men was rather more optimistic about the use of minoxidil and a small number of patients did express a preference for the continued use of the topical preparation rather than conventional intracavernosal injection (Beretta et al, 1993).

2.16c Intraurethral preparations

A number of investigators have used a novel approach to the delivery of vasoactive agent to the erectile tissues of the penis. The intraurethral application of PGE₂ cream has been tried as a possible alternative treatment to intracavernosal injection and has had encouraging results (Wolfson et al, 1993), in one study a mixed population of men with erectile dysfunction picked at random had a successful response in 60% of cases (Schmidt, 1994). Padma-Nathan et al have reported the successful use of PGE₁ in an intraurethral delivery system and compared the haemodynamic responses to those following conventional PGE₁ injection; the less invasive treatment modality of intraurethral application was found to compare favourably with respect to a number of haemodynamic parameters (Padma-Nathan et al, 1994).
2.17 External Vacuum Devices

Lederer obtained a patent in 1917 for a device which induced an erection-like state by creating a vacuum around the penis; but it was not until 1967 that Osbon designed a device which finally met with FDA approval in 1982 for use in the treatment of ED. A number of different devices are available (e.g. Erecaid, Pos-T-vac, Response and Correctaid) which all depend on the same principle: the device is placed over the flaccid penis and a vacuum is then initiated, the negative pressure results in an accumulation of blood within the penile shaft, sufficient tumescence and rigidity for intercourse are then maintained by constriction bands placed around the base of the penis (Nadig et al, 1986). It is recommended that these bands should not be left in place for more than 30 minutes.

The widespread use of external vacuum devices in the treatment of erectile dysfunction is a relatively recent development, they are suitable for use in virtually any patient provided they have sufficient visual acuity, manual dexterity and strength. Early assessments of the results of such therapy were based on a retrospective review of responses to the manufacturer's own questionnaires and are thus of dubious scientific quality (Witherington, 1989). Prospective studies with a more rigorous methodological approach have been recently undertaken. In a comparison of self-injection therapy with an external vacuum device, it was concluded that both interventions were an effective form of treatment and enhanced sexual functioning, with external vacuum devices having a much lower drop out rate (20%) compared with self-injection (60%). The commonest reasons given in the vacuum therapy group were an insufficient rigidity or duration of rigidity and pivoting erections, a reflection of the fact that rigidity is only produced distal to the constriction band (Turner et al, 1992). Interestingly, a return of spontaneous erectile function was reported in both groups (Turner et al, 1992). Another prospective study specifically studied the partner's evaluation of treatment, it seems that on a number of criteria both self-injection and external vacuum therapies enhanced the sexual lives of both partners; a
similar drop out rate as mentioned above was reported (Althof et al, 1992). The results of both these studies are markedly different from the literature on penile prostheses, in which the postoperative satisfaction of men is much greater than that of woman. Overall vacuum devices work well and their simplicity and non-invasiveness are obvious advantages. However, their cost is a major disadvantage to many patients, the price range being in the region of £200-350. Complications that have been reported include ejaculatory difficulties and initial penile pain, ecchymoses, petechiae and skin necrosis (Witherington, 1989; Kaye and Guay, 1991).

External vacuum devices can be used successfully in the management of ED due to venous leak (Blackard et al, 1993). Those patients who fail to achieve a complete erection-like state are said to have severe caverno-venous leakage via the cavernous drainage at the base of the corpora cavernosa (Blackard et al, 1993). Some investigators have also reported a beneficial effect obtained by a combined use of intracavernosal injections with an external vacuum device (Marmar et al, 1988).

2.18 Penile prostheses

The first attempts to use prostheses in the treatment of erectile dysfunction were in 1936 by Bogaras who used a section of rib cartilage to produce a penile rigidity in a manner analogous to the os penis of walruses, squirrels and other animals (Gee, 1975). These attempts were complicated by a number of problems including infection, extrusion, reabsorption and shape changes. Implantable synthetic penile prostheses were introduced in 1952; the earliest design was a simple specially shaped acrylic splint which was placed outside the tunica albuginea. Over the years the sophistication and variety of designs has increased. The early splints were superseded in turn by polyethylene or silicone prostheses which were placed intracavernosally (Gee, 1975).
Modern penile prosthetic treatment began with the introduction of the Small-Carrion sponge-filled prosthesis and the design of the inflatable prosthesis (Small et al, 1975; Scott et al, 1973). The prosthetic devices currently available may still be grouped into those which are inflatable, and those belonging to the group of semi-rigid prostheses. Small, Carrion and Gordon introduced a pair of silicone implants which filled the entire corpora cavernosa - this prosthesis has become the most widely implanted prosthesis ever developed. Subsequent modifications of this initial design have included the Flexi-Rod (Finney, 1977), the Jonas prosthesis (Jonas and Jacobi, 1980), the AMS 600 (Moul and McLeod, 1986), and most recently the 'Omniphase' and 'Duraphase' prostheses which have a spring-loaded cable allowing the penis to be rendered flaccid on flexion (Krane, 1986).

The inflatable designs were heralded as providing a more physiological erection and a natural appearing flaccid state. These multi-component inflatable devices have become technologically sophisticated pieces of equipment typically consisting of paired silastic corporeal cylinders, a fluid-filled reservoir which is placed beneath the anterior abdominal wall, and the final component which is a pump mechanism implanted in the scrotum. More recently, two-component designs have incorporated the reservoir and pump mechanism into one unit in an attempt to improve both reliability and to facilitate easier operative implantation.

Some 25 000 prostheses are implanted in the USA per annum. The main indication for implantation of a penile prosthesis is erectile dysfunction refractory to other methods of treatment. The choice of an appropriate prosthesis should be carefully deliberated. While the multiple component inflatable penile prostheses are the most physiological, they may not be appropriate in those patients with limited manual dexterity, previous abdominal surgery or cavernosal fibrosis. These issues should be clearly discussed with the patient. In all cases prophylactic antibiotics and careful surgical technique are vital to the successful outcome of the procedure.
The main complications of prosthesis implantation are infection, fibrosis, and displacement with an overall complication rate of 43% (Cumming and Pryor, 1991). The overall revision rate is of the order of 15%, but this figure is much higher in diabetic patients.Leaks have been reported as a problem in some of the high pressure inflatable devices, but a reduced mechanical failure rate can be expected with the continued evolution of prosthesis design. The best results are seen in young men with a high degree of motivation and with careful selection and counselling, preferably of both partners, success rates of 81-90% have been reported (Cumming and Pryor, 1991). The patients who are most dissatisfied are those with a sudden onset of ED and with complications related to the surgery, these are highest in patients with a history of diabetes or priapism. Modifications, improvements and advances in both materials and prosthesis design will continue and will no doubt result in increased functional longevity and reliability. The implantation of penile prostheses will remain a good treatment option in carefully selected patients.

2.19 Vascular procedures

It is in this area of the aetiology and management of erectile dysfunction that perhaps the most controversy has been centred for the last decade. There have been numerous operative descriptions for the correction of abnormal drainage of venous blood from the erect penis, similarly there are a plethora of operative techniques which have promoted as the way to correct for arteriogenic erectile dysfunction. It behoves the surgeon performing this type of surgery to make a careful and critical choice of the procedure that they employ in each case and furthermore to follow up the results of such surgery equally critically.
2.19a Venous leak and the role of venous ligation

The concept of venous ligation was first proposed and undertaken by Wooten in 1902 when he described the procedure of deep dorsal vein ligation (Wooten, 1902). The basic premise underlying this operative procedure is that by ligating the veins draining the corporal bodies, there will be a resultant decrease of excessive venous outflow which occurs in the erect state and is thought to be responsible for the erectile dysfunction. Both the investigation and treatment of veno-occlusive dysfunction addresses pathology outside the tunica albuginea, whereas the underlying pathology is likely to be within the tunica, most likely the smooth muscle of the corpora cavernosa (Claes et al, 1994; Malovrouvas et al, 1994; Wespès et al, 1991; Pickard et al, 1994). Deep dorsal vein ligation (DDVL) was later reintroduced into modern surgical practice (Ebbehoj and Wagner, 1979; Wespès and Schulman, 1985) and since then ever more extensive procedures have been advocated including: ligation of all dorsal vein tributaries (Wespès and Schulman, 1985) arterialisation of the deep dorsal vein (Sarramon et al, 1994), spongiolysis (Gilbert and Stief, 1987), radical excision of DDV, circumflex and cavernosal veins (Williams et al, 1988), as well as corporal plication and corporopexy (Austoni et al, 1992). None of the described surgical techniques is entirely satisfactory - operative access to the cavernosal veins is difficult and more importantly patients may also have significant arteriogenic, myogenic and psychogenic factors contributing to their erectile dysfunction. As in many areas of surgery, a multitude of operations for the same condition usually implies that none of the operations are satisfactory and that perhaps the basic premise underpinning the concept that is being addressed is flawed.

In most of the published studies in the literature the short term results are much better than the long term follow-up. Nevertheless, the reported results of surgery have been very variable: in the short term potency is restored in up to 63% of patients, but this figure generally drops to 30-50% by 1-2 years. The failure of long term success has been attributed by some to the re-opening of collateral channels post-
operatively, although other factors may contribute. Recently the interest of some workers has focused on the role of the suspensory ligament of the penis, it has been postulated that it has a role in the determination of penile suspension and has an extrinsic compression effect on the corpora cavernosa, thus acting to increase penile rigidity. It was postulated that it may thus play a role in the pathogenesis of venous leakage. Procedures which entail the tightening of cavernous bodies and a simultaneous corporopubic suspension have been claimed to have a success rate of up to 77.5% (Austoni et al, 1992). Other approaches that have been tried include the embolisation of the DDV network using balloons or coils although migration is always a potentially dangerous complication with such techniques (Moriel et al, 1993; Fowlis et al, 1994).

The veno-occlusive mechanism is complex and remains incompletely understood, it is clearly simplistic to believe that the problem lies in the extracorporal venous drainage system - that this is the case is borne out by the poor results in published series and indeed by the large number of operations that have been described for this condition. That this should be so is understandable in light of the fact that the failure of the veno-occlusive mechanism appears to be at the level of the corpora cavernosa. Thus, operations that describe ligation of the internal iliac, internal pudendal and periprostatic plexus are completely unwarranted and clearly unphysiological. This accounts for the poor results and failure of these techniques (Freedman et al, 1993; Kerfoot et al, 1994; Fowlis et al, 1994).

Some further understanding of veno-occlusive dysfunction has been made possible from the results of more recent studies where the investigators have concentrated on the abnormalities of the cavernous smooth muscle. Thus, a reduction of the smooth muscle content and it's degeneration and replacement by connective tissue have been reported (Malovrouvas et al, 1994; Wespes et al, 1991). In an elegant study it was demonstrated that in patients with veno-occlusive dysfunction there was evidence of impaired smooth muscle relaxation (Pickard et al, 1994). Thus,
a failure of neurogenic relaxation within the corpora may account for the abnormal findings of cavernosometry which are manifest, and interpreted as isolated venous pathology (Pickard et al, 1994). Furthermore, it should be remembered that both duplex ultrasound and cavernosometry rely on the assumption that complete smooth muscle relaxation is attained. Hatzichristou et al, advocated that smooth muscle relaxation can be achieved by 'redosing' where the patient has repeated intracavernosal injection of a vasoactive erectogen (Hatzichristou et al, 1995). This assumes that the smooth muscle relaxation is dose-dependent, which may not be the case if there is smooth muscle degeneration.

Given the above, improvements in the investigation and selection of patients might serve to improve the outcome of surgery. In the absence of significant arterial disease, corporeal muscle pathology and psychogenic factors, patients with evidence of a pure leak which is of mild or moderate degree will benefit most from DDVL. However, penile surgery for the treatment of venous leakage is not without its complications: penile oedema, altered penile sensation, decreased length and a nodularity secondary to thrombosis have all been reported. Those with a major leak have poor results from simple ligation and these patients might benefit most from the more complex surgical interventions as discussed above. It would seem that the use of less invasive treatment modalities such as an external vacuum device, intracavernosal pharmacotherapy or even a penile prosthesis might be better in the long-term management of these patients. There remain those who argue that such surgery is essentially experimental and should not be part of standard care (Benson, 1992). This is a most powerful argument in the face of the evidence that the tests for establishing the diagnosis remain incompletely validated and in the face of the long-term results of surgery. Fortunately, these factors have tempered the enthusiasm for these procedures which are therefore best done in an investigational setting in specialist centres.

It has been recognised that the contribution to the final rigidity of erection from the ischiocavernosus and bulbocavernosus muscles may be of considerable
import and in this context some workers have reported the beneficial effects of pelvic floor exercises in the treatment of ED due to venous leakage, the results have been encouraging but probably have little to contribute to the management of mild to moderate venous leak although they may have a powerful placebo effect (Claes et al, 1993; Claes and Baert, 1993).

2.19b Penile Revascularisation

Vasculogenic erectile dysfunction is now believed to be the principle cause of organic impotence in 60 to 80% of cases. A large number of technical advances and reports of their varying degrees of success have been published in the field of microvascular penile revascularisation. It would seem that the appropriate selection of patients and their evaluation is one of the most important factors in determining outcome and considerable controversy has been generated in this area and many issues remain unresolved.

There are almost as many descriptions of preoperative assessment as there are available surgical procedures. Duplex ultrasonography, colour-coded duplex scanning, selective pudendal arteriography with intracavernosal papaverine and, most recently, MRI-angiography have all been used in an attempt to define those patients who would most benefit from surgery. However, it needs to be remembered that selective pudendal arteriography demonstrates anatomy and provides a morphological assessment, however, it is an investigation which doesn’t contribute any functional data.

Revascularisation may be undertaken in the presence of demonstrable occlusion of the cavernosal arteries. Michal described an operation (Michal I) in which the inferior epigastric artery was anastomosed directly to the tunica albuginea (Michal et al, 1973), a number of variations of this procedure have been attempted
with success rates of approximately 35% (Metz and Frimedt-Moller, 1983). However, the post operative functional results remained discouraging and a high failure rate was attributed to fibrosis at the anastomosis site and vessel closure (Hawatmew et al, 1982; Zorgniotti et al, 1980). The Michal II procedure was then described in 1980 and comprised an anastomosis of the inferior epigastric artery end to side to the dorsal penile artery and 10 out of 18 patients had improved erections (Michal et al, 1980; Michal et al, 1973). Also in 1980 Virag introduced and reported on the results of anastomosis of the inferior epigastric artery to the deep dorsal vein (Virag, 1982a). The rationale behind this operation was that arterial blood would pass to the subtunical venules and retrogradely fill the cavernous bodies leading to penile erection, Virag later developed a number of modifications of this procedure (Virag, 1982a; Virag et al, 1983). At 18 months of follow up Virag reported that 60% of all cases had good results with a reoperation rate of 20% (Virag et al, 1983).

Somewhat more adventurous was the technique reported by Crespo and colleagues who undertook an anastomosis of the femoral artery to the central artery of the corpus cavernosum or the dorsal penile artery and reported a 77% cure rate (Crespo et al, 1987), unfortunately a number of other centres were unable to duplicate these results. In 1984 Hauri described a technique where the anastomosis was made between the inferior epigastric artery and the deep dorsal vein as well as to one of the dorsal arteries (Hauri, 1984), a success rate of 89% was reported in 44 patients (Hawatmew et al, 1982). In 1986 Goldstein published his results from surgery in young men with vasculogenic impotence secondary to pelvic trauma with a modified Michal II procedure where the inferior epigastric artery was anastomosed end to end to one or both penile arteries, he reported an 80% success rate at 4 years (Goldstein, 1986). Finally, Furlow and Fisher described a modification of Virag’s procedure and reported a success rate of some 61% at 18 months (Furlow and Fisher, 1988).
It is in the area of the outcome and results of revascularisation surgery that the most controversy has been experienced. The problems in this respect were eloquently expressed in an address to the International Society for Impotence Research by Sharlip (Sharlip, 1991). The variation in reported outcome is staggering, success rates varying from 31 to 80% have variously been published. The reasons for this have been critically examined and include the inaccurate selection of patients, inaccurate follow-up, the use of irrational surgical techniques, a powerful placebo effect and author prejudice. For example, those patients with occult venogenic, neurogenic, myogenic and endothelial abnormalities will understandably not benefit from an inappropriate intervention. Importantly, the possible presence of cavernosal fibrosis may limit the overall success rate of an otherwise technically sound procedure. One can only agree with the conclusions of both Sharlip and the NIH Consensus that standardised and controlled tests should form the basis of diagnosis and that reported outcome should be on the basis of objective and ideally independently audited results (Sharlip, 1991; NIH Consensus Conference, 1993).

However, despite these apparently damning critiques a number of authors continue to argue that arterial revascularisation procedures have a specific role in the treatment of erectile dysfunction (Sharaby et al, 1995). Nevertheless, there is general agreement that the best results are seen in young men who have sustained an isolated vascular insult secondary to pelvic trauma (Sharaby et al, 1995). Progress in the understanding of the physiology, pathophysiology and indeed the anatomy of erection might make logical and appropriate interventions commonplace. Whether these improvements will be sufficient in the presence of newer treatment modalities and better penile prosthesis design remains to be seen.
2.20 Future directions and conclusions

Impotence research has veritably exploded over the past decade, this has been instrumental in providing new insight into the underlying physiology and permitting the use of less invasive treatment modalities. We have seen that we now have available a number of effective treatments for the man with erectile dysfunction. There has also been a shift in clinical practice away from an intensive investigation to elucidate the precise cause of the problem in each case and the more recent adoption of what may be described as a goal-oriented approach in which we really try to tailor the treatment to the needs of the patient and to what they may find as being acceptable. There will nevertheless continue to be a need for investigative advances for the purposes of contributing to our understanding of the biology of erection. A number of different experimental paradigms have been utilised in this respect, the experimental model used in the present work is the streptozotocin diabetic rat which is reviewed in a subsequent section.

From the preceding two chapters it should be apparent that there have tremendous new insights into the physiology, pathophysiology and treatment of men with erectile dysfunction. Despite this there remains much that has to be learned and some of the basic questions about the mechanisms that underpin the erectile process are yet to be answered. There have been advances in the physical treatments available, in the surgery that can be offered and the newer approaches which were also discussed. There have been important discoveries in the basic pathways responsible for the relaxation of corporeal smooth muscle relaxation and the critical importance of the balance of forces acting on it to relax and contract it. It seems likely that the molecular biology of erection will continue to be explored and with it comes the possibility of offering patients novel therapeutic interventions as a result of this progress. It is hoped that the work contained in this thesis will contribute to our understanding of normal erectile physiology, alterations in pathological states and perhaps offer some suggestions for appropriate therapeutic intervention.
2.21 Diabetic Rat Model

The streptozotocin (STZ) diabetic rat is the experimental model used in this thesis. It has been the subject of a number of recent reviews (Tomlinson et al., 1994; Sharma and Thomas, 1997), the main points of which have been summarised below.

STZ is a bacterial product initially isolated from *streptomyces achromogens* which was found to have activity against both gram-positive and gram-negative organisms (Lewis and Barbiers, 1959), it was later also reported to have anti-tumour activity (Evans et al., 1965). In 1963 it was reported that an intravenous injection of STZ would induce diabetes mellitus in both rats and dogs (Rakieten et al., 1963). Diabetes has since been successfully induced in a variety of species, however it is of interest to note that the rabbit is resistant to the diabetogenic effects of STZ (Kushner et al., 1969), however, alloxan may be used to render rabbits diabetic. The precise mechanisms of action of either alloxan or STZ remain unclear.

Renold summarised the main hypotheses for both STZ and alloxan toxicity (Renold, 1985). Structurally, STZ is a glucose molecule with a highly reactive side chain. The main site of action of both substances is the pancreatic beta-cell. The binding of STZ is rapid and it is possible that STZ and alloxan are selectively drawn to and taken up by these cells as these insulin producing cells have the ability to both recognise and metabolise glucose. It is postulated that the toxicity may be interfered with by transportable sugars, but this not the case with STZ in contrast to the findings for alloxan. It is known that nicotinamide is able to protect beta-cells if injected as late as 30-20 min after STZ administration. STZ acts in part by inducing islet cell DNA strand breaks with activation of poly (ADP-Ribose) synthetase with a precipitate fall in pancreatic islet cell NAD content; evidence of STZ damage is seen within 30 min of injection.
In terms of histopathological changes, the pancreatic beta-cells show a marked decrease or complete loss of their granules, shrinkage and nuclear pyknosis (Rakieten et al, 1963). The pancreatic alpha-cells remain completely unaffected (Junod et al, 1967). In general, it seems that STZ is both more specific for and active against the beta-cells than alloxan (Rakieten et al, 1963).

With respect to metabolic changes, STZ diabetic rats develop pronounced hyperglycaemia, but have normal levels of blood ketones and plasma free fatty acids. In addition, glycolytic intermediaries, glycogen and citrate in the perfused heart also remain within the normal range (Mansford and Opie, 1968). These observations were confirmed and extended to demonstrate that ketonuria is only a feature of high dose STZ injection (Junod et al, 1969). STZ also duplicates the pancreatic lesions found following beta cell destruction in insulin-dependent DM. The effective dose of STZ varies considerably between and within species, and has been found to depend on the animal's sex, age and nutritional state (Gold et al, 1981). A single intravenous or intraperitoneal injection of STZ induces DM in rats that can be maintained without insulin treatment for one or two years (Powell et al, 1977; Sharma and Thomas, 1974; Zemp et al, 1981).

In the context of neurophysiological abnormalities, it was demonstrated that in alloxan treated rats conduction velocity in the sciatic nerve falls to between 30-50% of the initial velocity within 10 days of treatment (Eliasson, 1964; Eliasson, 1969). This reduction affected both motor and sensory fibres but unmyelinated fibres seemed to be unaffected. This work was subsequently confirmed by a number of other workers and the findings have also been documented in streptozotocin treated rats (Greene et al, 1975; Jakobsen, 1979; Mayer and Tomlinson, 1983). The reductions observed by later workers were not as severe as the earlier findings of Eliasson and it is likely that poor attention to temperature control and the severity of the diabetes are important factors. Many of the more recently carried out studies were performed on animals with longer survival times in order to allow for the assessment of any accompanying
accompanying morphological changes. Insulin may be able to restore the conduction velocity at least in the early stages (Greene et al., 1975; Jakobsen, 1979). The observed reduction of conduction velocity has not yet been fully explained but it is likely to depend on a number of factors. In short term studies it seems that the conduction velocity may be restored by an aldose reductase inhibitor or alternatively by dietary supplementation with myo-inositol (Greene et al., 1975; Mayer and Tomlinson, 1983). The reduction in conduction velocity is correlated with a reduced Na\(^+\)/K\(^+\)-ATPase activity considered to be secondary to the reduced nerve myo-inositol content. This may lower the extrusion of ionised sodium from the axon and lead to an intra-axonal accumulation of ionized sodium that could affect the generation of action potentials.

The observed acute reduction in conduction velocities in severely diabetic animals may be related to the dehydrational shrinkage of the axon as a result of tissue osmolality changes. Thus, the intravenous infusion of hypertonic dextrose in cats led to a progressive fall in nerve conduction velocity (Dyck et al., 1981). A complicating factor of long term observations on conduction velocities in the rat is that as growth occurs over a long time in this species, there is an associated increase in nerve fibre diameter with increased conduction velocity. Untreated diabetes results in growth retardation and thus part of the observed differences in conduction velocity may be due to maturational differences. This effect has been well described in a number of studies (Moore et al., 1981; Sharma and Thomas, 1974). A mild absolute reduction may occur after diabetes of long duration, perhaps a consequence of axonal degeneration although histological confirmation of this is lacking. It is also possible that animals made diabetic after they have reached maturity will have only slight reductions in conduction velocities.

Voltage clamp studies on myelinated fibres from streptozotocin treated rats demonstrated that fibres from diabetic animals were comparable to those from control animals, in relation to both the resting potentials and the action potentials. Alloxan
treated animals however showed substantial voltage-dependent potassium conductance. As this was not seen in fibres of SDR it was considered that this might represent a direct toxic effect of alloxan (Jefferys and Brismar, 1980).

Its use in the laboratory rat provides a permanent DM state which is suitable for longitudinal electrophysiological and morphological studies of peripheral nerves. However, the observations of pathological changes in the autonomic nervous system in the STZ rat have been limited. The type of changes seen in the vagus nerve in human diabetic autonomic neuropathy are not encountered in STZ rats at one year (Sharma and Thomas, 1974). Nevertheless, this model remains one which is used to investigate the effects of substances which are purported to be active against the development of diabetic peripheral and autonomic neuropathy.

Early studies demonstrated that it was indeed possible to correct the reduction in nerve fibre size that was observed in STZ rats in comparison to controls (Jakobsen, 1979). More recently McCallum and his colleagues have demonstrated that 8-week treatment with continuous subcutaneous insulin infusion fully corrects the deficit in weight, skeletal growth and fibre size (McCallum et al, 1986). In addition, a number of workers have now been able to measure the intracavernosal pressure responses to both nerve stimulation as well as performing an ultrastructural analysis in the STZ rat penis (Italiano et al, 1993a; Italiano et al, 1993b); while others have performed the direct intracavernosal injection of vasoactive agents in normal laboratory rats (Chen et al, 1992).
AIMS OF THESIS

The principle aims of this thesis were to study some penile biochemical pathways in depth, and to characterise the abnormalities of cyclic nucleotide metabolism in a rodent model of diabetes mellitus, in an attempt to elucidate what factors in the altered transduction systems may contribute to the aetiology of human diabetic erectile dysfunction. An attempt was also made to compare these results with data from another species to see if the same pathways are present in the NZW rabbit. A variety of different techniques were utilised in this respect:

(i) The anatomical localisation and degree of binding of NOS were determined using low and high resolution autoradiographic techniques. Abnormalities in the STZ diabetic rat were compared to controls. Penile ultrastructure was studied with EM.

(ii) The activity of adenylate and guanylate cyclase were determined by the assessment of cAMP and cGMP production in vitro. Actions of different vasoactive agents were studied in the same system. These experiments were undertaken to elucidate which pathways are present in the experimental models and to observe any alterations in DM.

(iii) An investigation into cAMP and cGMP phosphodiesterase activity by the use of radioactive tritiated precursors was undertaken.

(iv) A study of 5'NT activity was performed to see if this is altered in DM as it forms an important part of vascular defence as well as being a vasodilator.

(v) The effects of vascular risk factors on the cyclic nucleotides in an acute phase in vitro preparation were studied. Furthermore, a clinical study to investigate the role of fibrinogen as a risk factor for ED was also undertaken.
CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The following chemicals and drugs were obtained from:

a) Sigma Chemical Co. (Poole, Dorset, UK):
acetyl choline chloride
adenosine-3'5' cyclic monophosphate
adrenaline bitartrate
Dulbecco's minimum essential medium
isobutylmethylxanthine
guanosine-3'5' cyclic monophosphate
papaverine
prostaglandin E₁
sodium nitroprusside
streptozotocin
vasoactive intestinal polypeptide

b) BDH (Poole, Dorset, UK):
absolute ethanol (Aristar grade)
methanol
polyethylimine cellulose TLC plates
c) Amersham Radiochemicals (Amersham, UK):
Protein binding assay kits for cAMP and cGMP measurement
\[^{3}\text{H}]-\text{L-NOARG}
\[^{3}\text{H}]-\text{Hyperfilm}

d) New England Nuclear (Dreiech, Germany):
\[^{3}\text{H}]-\text{adenosine 5'-monophosphate (120Ci/mmol)}

e) May and Baker Ltd. (Dagenham, UK):
Pentobarbitone (Sagatal)

Polypropylene tubes (2.5ml) for tissue incubation and for radioimmunoassays were purchased from Luckham Ltd. (Middlesex, UK). Micropipettes from Gilson (Anachem Ltd., Luton, Beds., UK), liquid scintillation vials from G & G Chemicals (Berks., UK), liquid scintillation fluid from National Diagnostics (Sussex, UK) and Multistix from Ames Division (Miles Laboratories Ltd., Stoke Poges, Slough, UK). Animals were fed with Mouse No.1 Modified Maintenance Diet (SDS, Litham, Essex, UK).

3.2 Instruments

a) bench centrifuge (Centra 7R), sonicator (Soniprep 150): MSE Instruments (Sussex, UK).
b) shaking water bath: Grant Instruments (Cambridge, UK).
c) vortex mixer, vacuum chamber evaporator, magnetic stirrer: Gallenkamp Ltd. (Middlesex, UK).
d) digital balance: Oertling (Maidstone, Kent, UK).
e) beta particle counter (Rakbeta 1210): LKB Wallace (Turku, Finland).
f) pH meter: Corning Science Products (Middlesex, UK).
3.3 Buffer compositions
(in mmol/l, unless otherwise stated)

a) **Minimum essential medium**: pre-weighed dissolved in 1 litre double distilled water, NaHCO₃ added to 24mmol/l; gassed with 95%O₂ / 5%CO₂.

b) **Phosphate buffer** (for cAMP assay): 8Na₂HPO₄, 5KH₂PO₄, adjusted to pH 7.6 with HCl and/or NaOH.

c) **Tris-gelatin** (RIA): 50 Tris HCl, 1g/l porcine gelatin, adjusted to pH 7.4 with HCl.

3.4 Experimental diabetic rat model

Experiments were carried out using male Sprague Dawley rats with an initial median body weight of 250g. Non-ketonuric, hyperglycaemic diabetes was induced by injecting streptozotocin intravenously via the tail vein at a dose of 65mg.Kg⁻¹ body weight. These rats developed glycosuria but not haematuria or ketonuria. Diabetic rats were fed ad libitum with Mouse No. 1 Modified Maintenance Diet and allowed free access to water. Urine was monitored over the duration of DM for glucose, ketone bodies and proteins with Multistix. Control animals were injected with an equivalent volume of normal saline.

3.5 Preparation of tissues for ex-vivo experiments

After 8 weeks, rats were anaesthetised with pentobarbitone (90 mg.Kg⁻¹ intraperitoneally; Sagatal). Blood was collected by cardiac puncture for measurement of blood glucose and routine clinical chemistry analysis (see appendix 1 and appendix
2). The thoracic aortae and penises were rapidly excised and placed in Dulbecco's Minimum Essential Medium (MEM), pregassed with 95% O₂ / 5% CO₂.

Penises were cut longitudinally into two equal strips and then transversely into segments (to give approximately 16 segments per penis), all excess adventitial tissue was carefully removed. Aortae were cut transversely into rings approximately 2mm wide. Penile and aortic tissues were placed in oxygenated MEM and incubated for 3 hours, at 37°C, to allow the tissues to "settle down" after handling. The carotid artery and penis of New Zealand White rabbits were also used in some of our studies, the preparation of these tissues was identical. Two groups of rats were used in the autoradiographic study, they were rendered diabetic in the same manner but the duration of diabetes was two months in one group and six months in the other.

3.6 Measurement of cAMP

Cyclic AMP concentrations were assayed using a commercially available cAMP [¹²⁵I] assay system (dual range). These kits provide a simple, reliable and precise determination of cAMP in urine, plasma, tissues and other biological samples. This system uses a high specific activity [¹²⁵I] 2'-o-succinyl-cAMP tyrosine methyl ester tracer and a highly specific and sensitive antiserum.

This assay is based on the competition between unlabelled cAMP and a fixed quantity of [¹²⁵I]-labelled cAMP for a limited number of binding sites on a cAMP-specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. The antibody bound cAMP is reacted with a second antibody reagent which contains this second antibody bound to magnetizable polymer particles. Separation of the antibody bound fraction may be achieved by either magnetic separation or centrifugation. Measurement of the
radioactivity in the pellet enables the amount of labelled cAMP in the bound fraction to be calculated.

\[
\begin{array}{c|c}
\text{Free} & \text{Bound} \\
\hline
[^{125}\text{I}] \text{cAMP} &[^{125}\text{I}] \text{cAMP-antibody} \\
\downarrow + \text{antibody} & \downarrow \\
cAMP & \text{cAMP-antibody}
\end{array}
\]

The concentration of unlabelled cAMP in the sample is determined from the use of a standard curve. An increased sensitivity is easily attained by the acetylation of standards and unknown prior to assaying. This enables low cAMP concentrations to be determined and is useful with small tissue quantities. Using a non-acetylation protocol enables cAMP measurement in urine, plasma, tissues and cell cultures in the 15-1600fmol per tube range (Fig. 3.1). Using the acetylation protocol enables a measurement of low levels of cAMP in small quantities of biological materials in the 2-128fmol/tube range (Fig. 3.2).

3.6a cAMP \[^{125}\text{I}\] non-acetylation assay

Reagent preparation

All reagents were allowed to equilibrate at room temperature. Distilled water was used for reagent preparation. Amerlex-M second antibody preparation was supplied ready for use (Amersham, UK). Reconstituted components were stored at 2-8°C.
Fig. 3.1 Standard curve for cyclic AMP (non-acetylated) radioimmunoassay
Fig. 3.2 Standard curve for cyclic AMP (acetylated) radioimmunoassay
**Tracer**

11.0 ml diluted buffer was carefully added. The stopper was replaced and the contents mixed by inversion and swirling.

**Antiserum**

11.0 ml of distilled water was carefully added. The stopper was replaced and the contents gently mixed until a complete solution was obtained.

**Non-acetylation standard**

2.0 ml distilled water was carefully added. The contents of the bottle were mixed until completely dissolved. (NB The final solution contains cAMP at a concentration of 32 pmol/ml in 0.05M acetate buffer.)

**Assay Buffer**

The contents of the bottle were transferred to a 500 ml graduated cylinder by repeated washing with distilled water. The final volume was adjusted to 500 ml with distilled water and mixed thoroughly.

**Preparation of working standards (non-acetylation)**

Seven polypropylene tubes were labelled: 25, 50, 100, 200, 400, 800, and 1600fmol respectively. 500 µl assay buffer was pipetted into each tube. Then 500 µl stock non-acetylation standard (32 pmol/ml) was pipetted into the 1600 fmol tube and mixed thoroughly. 500 µl was then transferred from tube 1600fmol to tube 800fmol and mixed thoroughly. This doubling dilution was repeated successively with the
remaining tubes. 100 µl aliquots from each serial dilution gave rise to 7 standard levels of cAMP ranging from 25-1600fmol.

Measurement of cAMP in tissue in the range 25-1600 fmol/tube:

Assay buffer and standards ranging from 25-1600fmol were prepared as described above. All reagents were equilibrated to room temperature and mixed before use. Tubes were then labelled in duplicate for total counts (TC), zero standard tubes (B0), standards and samples. 100 µl assay buffer was pipetted into the zero standard tube (B0). Then starting with the most dilute, 100 µl of each standard was pipetted into the appropriately labelled tubes. 100 µl of tissue was then pipetted into the appropriately labelled tubes.

NB all tissues were pre-treated as follows:
** 1g (wet weight) tissue was homogenised in 10 ml Hank's balanced salt solution (without calcium and magnesium) containing 5mM EDTA.
** The homogenate was centrifuged for 10 min at 1000G at 4°C.
** 1 ml of supernatant was mixed with 1 ml undiluted acetonitrile. It was vortex mixed for 20 seconds and centrifuged for 10 min at 1500G at 4°C. 1 ml supernatant was then applied to an Amprep SAX 500mg minicolumn.

Once tissues had been pipetted into the appropriately labelled tubes, 100 µl [125I]-cAMP was pipetted into all tubes. This was followed by the addition of 100 µl antiserum into all tubes except the TC. All the tubes were then covered with plastic film and incubated for 3 hours at 2-8°C.

The Amerlex-M second antibody reagent was gently shaken and swirled to ensure a homogenous suspension. 500 µl of reagent was then added to each tube (except the TC). The TC tubes were stoppered and put aside for counting. All tubes
were then vortex mixed thoroughly and incubated for 10 min at room temperature (15-30°C). The antibody fraction was separated by magnetic separation.

Magnetic Separation

A tube rack was attached onto an Amerlex-M Separator base. It was ensured that all tubes were in contact with the base plate and left to separate for 15 min. After separation, the supernatant liquids were poured off and discarded. Keeping the separator inverted, the tubes were then placed on a pad of absorbent tissues and allowed to drain for 5 min. Once any adhering drops of liquid had been removed, the tubes were turned upright again. The radioactivity present in each tube was then determined by counting in a gamma scintillation counter.

3.7 Measurement of cGMP

Cyclic GMP concentrations were assayed using commercially available cGMP \(^{[125]}\text{I}\) assay system. This system uses a highly specific activity \(^{[125]}\text{I}\) 2'-0-succinyl-cGMP tyrosine methyl ester tracer, together with a highly specific and sensitive antiserum. The assay is based on the same principles to that of cAMP as described above.

Cyclic GMP may be measured in the range 2-128fmol per tube using the acetylation protocol (Fig. 3.3) or 50-6400fmol per tube using the non-acetylation protocol. The concentration of cGMP is some 10% that of cAMP, therefore an important aspect of this assay is that it needs to be both sensitive and have little cross-reactivity with cAMP.
Fig. 3.3 Standard curve for cyclic GMP (acetylated) radioimmunoassay
3.7a cGMP $^{125}$I acetylation assay

Reagent preparation

Identical preparation to cAMP$^{125}$I assay system except:

Acetylation standard

A standard curve (range 2-128 fmol/tube) was prepared by carefully adding 10 ml assay buffer to the acetylation standard. The contents were mixed until completely dissolved. The final solution contained cGMP at a concentration of 2.56 pmol/ml.

Preparation of working standards

Seven polypropylene tubes were labelled: 2, 4, 8, 16, 32, 64 and 128fmol respectively. 500 µl was then pipetted into all the tubes. Into the 128fmol standard tube, 500 µl stock standard was pipetted and mixed thoroughly. 500 µl was then transferred from the top standard to the next tube (64fmol) and mixed thoroughly. This doubling dilution was successively repeated with the remaining tubes. 100 µl aliquots from each serial dilution gave rise to 7 standard levels of cGMP ranging from 2-128fmol.

NB For this assay, all tubes had to contain the same volume (i.e. 500 µl). Therefore, 500 µl was removed from the 2fmol standard and discarded.

Measurement of cGMP in tissue in the range 2-128 fmol/tube:

Assay buffer and standards (range 2-128fmol/tube) were prepared as described above. All reagents were equilibrated to room temperature and mixed before use. The
polypropylene tubes for the zero standard and unknowns were labelled. These were the acetylation tubes. Polypropylene tubes were then labelled in duplicate for total counts (TC), zero standard (Bo), each standard dilution and unknowns. These were the assay tubes.

The acetylation reagent was prepared by mixing 1 volume of acetic anhydride with 2 volumes of triethylamine. (Sufficient reagent for 50 acetylations may be attained by mixing 0.5 ml acetic anhydride with 1.0 ml triethylamine). 500 µl assay buffer was then pipetted into the zero standard acetylation tube. 500 µl of each unknown was pipetted into the appropriately labelled acetylation tubes. Tubes containing 500 µl of each working standard were already prepared (see reagent preparation). 25 µl of the acetylation reagent was carefully added to all acetylation tubes containing standards and unknowns. Optimum precision was attained by placing the pipette tip in contact with the test tube wall above the aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid. Each tube was vortexed immediately following addition of the acetylating reagent. Duplicate 100 µl aliquots from all acetylation tubes were then pipetted into the corresponding polypropylene tubes. This was followed by the addition of 100 µl antiserum into all assay tubes except the TC.

All assay tubes were vortex mixed thoroughly and then covered and incubated for one hour at room temperature. 100 µl [125I] cGMP was then pipetted into all the assay tubes. The TC was stoppered and set aside for counting. All assay tubes were vortex mixed again and were then covered and incubated for approximately 18 hours at 2-8°C.

The remainder of the cGMP assay followed an identical procedure to cAMP assay involving Amerlex-M second antibody reagent and magnetic separation, as described previously.
3.8 Calculation of results for cAMP and cGMP assays

(i) The average counts per minute (cpm) for each set of replicate tubes was calculated;
(ii) The percentage $\text{Bq}/\text{TC}$ was calculated using:

$$\% \text{ Bq}/\text{TC} = \frac{\text{Bq cpm} \times 100}{\text{TC cpm}}$$

(iii) The percentage bound for each standard and sample was calculated using:

$$\% \frac{\text{B}}{\text{B}_0} = \frac{(\text{standard/sample cpm}) \times 100}{\text{B}_0 \text{ cpm}}$$

A standard curve is generated by plotting $\% \frac{\text{B}}{\text{B}_0}$ (y axis) against fmol standard/tube (x axis). The fmol/tube value of the samples can be read directly from the plotted graph (for cAMP see Fig. 3.2 and for cGMP see Fig. 3.3).

3.9 Nitric oxide synthase autoradiography

After two months, 10 rats (5 diabetic and 5 control) were placed under terminal anaesthesia using 90mg/kg body weight of pentobarbitone (Sagatal). Their penile tissues were excised and stored immediately at -70°C, in air tight cryotubes. Longitudinal 6μm sections of the rat penises were then cut in a cryostat at approximately -20°C and thaw mounted onto gelatinised microscope slides, which were stored at -70°C in air tight containers until use. Localisation of nitric oxide synthase (NOS) was carried out essentially as described previously (Michel et al,
1991; Kidd et al, 1995), (the autoradiographic methodology used in this study is summarised in an illustrated fashion in Fig. 3.4). Slide-mounted tissue was allowed to equilibrate to room temperature for at least 30 min before incubations were performed. Consecutive sections were incubated (60 min at 22°C) in buffer containing 10nM \(^3\)H-L-N\(^G\)-nitroarginine (\(^3\)H-L-NOARG) (specific activity 55Ci/mmol), the degree of non-specific binding being established by incubating alternate sections in the presence of 10µM unlabelled L-arginine. Slides were washed in buffer (four times for two min) and dried in a stream of cold air. Low resolution autoradiography was carried out by exposing sections to \(^3\)H-Hyperfilm in X-ray cassettes for three months. Photodensitometric analysis was performed on film images using a VIDAS imaging system (Kontron, Thame, UK) and the degree of specific binding determined from curves generated from \(^3\)H-microscales that were co-exposed with slide-mounted tissue. Binding was expressed in terms of radioligand bound per unit area (i.e. d.p.m. per mm\(^2\)). Microscopic localisation (high-resolution autoradiography) of binding was performed by post-fixing tissue in paraformaldehyde vapour (two hours at 80°C) and coating slides in nuclear emulsion (LM-1, Amersham). Slides were then stored in light-proof boxes for 12 weeks at 4°C, after which they were processed in D19 high contrast developer (Kodak, Hemel Hempstead, UK) and fixed (Hypam, Ilford, UK). Underlying tissue was stained with haematoxylin and eosin, high resolution autoradiographs were viewed on an Olympus Vanox microscope and selected sections were photographed where appropriate.

The methodology used for the six month diabetic rats was slightly different. Serial 20 µm longitudinal and transverse sections were cut at -15°C and thaw-mounted onto gelatinised microscope slides which were stored overnight at -20°C. Sections were then incubated using conditions similar to those described above. Briefly, tissues were preincubated in 50mM Tris HCL, pH 7.2 for 30 min at 22°C. Sections were subsequently incubated in tris buffer containing 3mM CaCl\(_2\) and 10nM \(^3\)H-L-NOARG; specific activity 35-70 Ci/mmol - (Amersham) for 60 min at 4°C.
Fig. 3.4 Schematic representation of autoradiograph methodology as used for nitric oxide synthase determination in the rat penis. See text for details.
The degree of non-specific binding was determined by incubating alternate sections in the presence of 10μM L-arginine. After incubation the slides were washed (three times for 10 min) in buffer at 4°C to reduce non-specific binding, dipped in glass distilled water (4°C) and dried in a stream of cold air. Slides were then placed in 24 x 30 cm X-ray cassettes and apposed to ^3H-Hyperfilm for 12 weeks. After exposure the films were processed in undiluted Kodak D19 developer and fixed in Ilford IF-23 fixer (diluted 1:4 with water) washed for 20 min in running tap water and dried. Autoradiographs were examined under a dissecting microscope and photographed on a Nikon macro system. Film images were quantified by densitometric analysis on an IBAS imaging system where the grey levels of the autoradiographs were measured and binding calculated on the basis of curves generated by 125I-microscales that are co-exposed to the film with the tissue section. Binding was expressed in amol/mg protein equivalent. It was also possible to colour code these images using this system.

The results of the experiments were expressed as median and range. The two-tailed Mann-Whitney U test (unpaired values) was used for the statistical analysis of the data.

3.10 Cyclase activities in penile and vascular tissues

Two penile segments and, separately, two aortic rings were placed in 200 μl MEM containing 100 μl IBMX (a non-specific phosphodiesterase inhibitor which prevents hydrolysis of cyclic nucleotides to their respective non-cyclic forms, AMP and GMP). The following agents were added over a wide concentration range:

(a) prostaglandin E₁ (PGE₁), a stimulator of cAMP;
(b) sodium nitroprusside (NaNP), which breaks down to generate NO, a stimulator of cGMP;
(c) acetylcholine chloride (ACh), which is believed to act via stimulation of PGI₂ and possibly adenylate cyclase;
(e) papaverine, a drug commonly used in intracavernosal injection pharmacotherapy, the actions of which include phosphodiesterase inhibition and calcium flux alterations.

Following the addition of one of the above agents, tissues were incubated at 37°C for 20 min, after which time 500 µl of ice-cold absolute ethanol was added and samples were placed in a freezer at -70°C. After thawing, the tissues were then sonicated extensively (six 20 second bursts). This effectively disintegrates plasma membranes and releases cyclic nucleotides (Rosenberg et al, 1982). Validation experiments using various other extraction techniques, for example trichloroacetic acid or perchloric acid, failed to extract any further measurable cAMP or cGMP following extraction with aqueous ethanol and sonication (Fig. 3.5). This method of extraction was concluded to yield virtually all of the nucleotide which was generated in the tissues under study. Tissues were then centrifuged and aliquots of ethanolic extract taken, evaporated under vacuum and reconstituted in assay buffer for measurement of cAMP and cGMP concentrations as described above.

In a further series of experiments we investigated the effects of papaverine and vasoactive intestinal polypeptide on penile and vascular cAMP and cGMP levels. The experimental model used was again the STZ-induced diabetic rat. However, the study also included a series of experiments performed on six non-diabetic NZW rabbits from which the carotid arteries were excised in addition to the penises. The tissues were prepared as described above and VIP or papaverine were then added over a wide concentration range to six duplicate preparations. Penile segments (n=2) or aortic (n=2) or carotid rings (n=2) were placed in 200 µl MEM containing 100µM IBMX. VIP or papaverine were then added over a wide concentration range. The incubations and subsequent measurement of cAMP and cGMP were as previously described.
Fig. 3.5 Validation experiments for cAMP and cGMP extraction methodology. The curves illustrate that with the addition of perchloric acid, no significantly measurable cyclic nucleotide was further extracted from the tissue homogenate (▲, with perchloric acid; ●, without perchloric acid). Note the linearity of the curves.
3.10a Calculation of results and statistical analysis

Basal (i.e. non-stimulated) concentrations of cyclic nucleotides were subtracted from the concentrations after the incubation of tissues with the vasoactive agent under study. Values are expressed as mean concentration ± S.E.M. Significance was determined with the Student's t test. \( P \) values less than 0.01 were taken to be significant.

3.11 Phosphodiesterase activity in penile and vascular tissues

Phosphodiesterase activity was assayed as previously described by Jeremy and co-workers (Jeremy et al, 1988b; Jeremy et al, 1993a). Following routine tissue preparation, penile segments (n=2) or aortic rings (n=2) were placed in polypropylene tubes and [\(^3\)H]-cAMP or [\(^3\)H]-cGMP added (20 \( \mu l \) for aortic rings and 40 \( \mu l \) for penile segments). The tissues were then incubated at 37°C in a water bath. Reactions were stopped by the addition of absolute ethanol (twice the volume of buffer present) at 2.5, 5, 10, 15, 20, 30 and 60 min. Tubes were vortexed and stored in a -70°C freezer. Following centrifugation, the supernatants were applied onto precoated polyethylimine cellulose thin layer chromatography plates (BDH, UK). Unchanged cyclic nucleotides were separated from adenosine or guanosine monophosphate and free nucleotides (adenosine and guanosine) by development of the plates in 50 mM KCl. cAMP and cGMP were detected under ultraviolet light and each band removed from the plate and placed in a liquid scintillation vial. 1 ml of 1M KCl was added and left overnight to extract the nucleotides. Liquid scintillation fluid was added to each vial and the radioactivity associated with each band counted, from which the percentage inhibition of conversion of cAMP to AMP was calculated.
3.12 5' Nucleotidase activity in penile and vascular tissues

The objective of these studies was to investigate the conversion of AMP to adenosine using control and diabetic rat penises and aortae. Following the routine preparation of tissues one aortic ring (6 control and 6 diabetics) or one penile segment (6 controls and 6 diabetics) was placed in each tube (24 tubes in total). 20 µl [³H]-AMP and 300 µL MEM were mixed together and then 20 µl of this mixture was added to each tube. One set of tubes (i.e. control and diabetic) was incubated at 37°C for 1 min. The remaining 5 sets were incubated for 2.5, 5, 10, 20 and 30 min respectively. After these times, 40 µl of absolute ethanol was added to each tube and the tubes were placed in a freezer at -70°C. The tissues were then sonicated extensively (20 second bursts), to disintegrate the plasma membranes and release the nucleotides for assay.

Four polyethylimine (PEI) cellulose plates (two each for aortae and penises) were prepared in grid form. Using separate capillary tubes for each solution (containing an aortic ring or penile segment), lines were drawn across each respective origin line and allowed to dry. All solutions in each respective tube were plated similarly. Approximately 150 ml of 50mM KCl was poured into a chromatography tank. The PEI plates were placed in the tank for approximately one hour - KCl (acting as a solvent) draws nucleotides up the plate and separates them out. Once separation was complete and the plates had dried, they were sub-divided further. These 1cm sections were then cut up and placed into individual vials. To each vial, 1 ml of 1M KCl was added and mixed using a whirlmixer - KCl is used to extract nucleotides from the plate. 5 ml of scintillant was then added and mixed. The vials were left to stand for 5 min and then mixed again. The vials were racked and placed into a Beta-counter to measure the counts per minute (CPM), which were then plotted (Fig. 3.6).
Fig. 3.6 Running positions of $[^3]$H-AMP and $[^3]$H-adenosine separated by TLC on PEI plate. PEI cellulose precoated plastic plates developed in 50mM KCl. Unlabelled AMP and adenosine were also run and visualised under UV light (shown as black rectangles). Following development, plates were dried and cut laterally into 1 cm bands and radioactivity measured. (This figure shows the difference between conversion of AMP to adenosine by aortae from diabetic and control rats)
3.13 Effects of glucose and insulin on cAMP and cGMP synthesis by rat penile tissue, in vitro

The objective of this study was to investigate the effect of varying concentrations of glucose and insulin on cAMP and cGMP by rat corpus cavernosum, in vitro.

Penises were excised from 6 male Sprague Dawley rats and cut into segments as previously described. The penile discs were placed in MEM and incubated for 3 hours at 37°C to allow the tissues to equilibrate prior to stimulation of cyclic nucleotide release. One disc in six duplicate was sequestered into tubes containing buffer (MEM), 250 μM IBMX and varying concentrations of glucose and insulin. IBMX inhibits PDEs therefore allowing cyclic nucleotides to accumulate. The concentrations of glucose and insulin used were approximately in the range of concentrations that are encountered in well- and poorly-controlled diabetes (glucose 6.25-50mM; insulin 0.01-10μ units/ml). One set of tubes was untreated and in the other set, cAMP and cGMP were stimulated with 10μM PGE$_1$ and 10μM sodium nitroprusside (NaNP), respectively. Following incubation for 30 min at 37°C, reactions were then stopped by addition of 300 μl 0.5M perchloric acid. Tissues were then sonicated (two 30 second bursts), centrifuged at 2,500 rpm for 30 min (to extract the nucleotides) and 200 μl aliquots of supernatant taken. The concentrations of cAMP and cGMP in the aliquots were measured by RIA.

3.14 Effects of LDL and oxLDL on cAMP and cGMP synthesis in rat penile tissues in vitro

The objective of this study was to investigate the effect of LDL and oxLDL on cyclic nucleotide synthesis.
Briefly, LDL was obtained from six healthy subjects by high speed centrifugation of plasma. LDL was split into two parts, one half being oxidised by adding metallic copper and incubating with the LDL for 3 hours at 37°C. The degree of oxidation was assessed by measuring T-bars and by HPLC (high-pressure liquid chromatography).

Once provided with LDL and oxLDL, both were dissolved in MEM over a concentration range of 0-10μg/ml. One rat penile disc was incubated in 200 μl MEM containing the LDL preparations for 30 min prior to addition of either 10μM PGE_1 or 10μM NaNP. The tissues were then incubated for a further 30 min at 37°C after which time the reactions were stopped by the addition of 400 μl 0.5M perchloric acid. The tissues were sonicated (to extract the nucleotides), centrifuged at 2,500 rpm for 30 min and aliquots taken and neutralised with 1M K_3PO_4; cAMP and cGMP concentrations were then measured with RIA.

3.15 Ultrastructure of diabetic and control penile tissue

The objective of this study was to examine penile tissues from diabetic rats using the Scanning Electron Microscope (SEM) and the Transmission Electron Microscope (TEM). SEM and TEM specimens were prepared by the team at Kingston University (see appendix 3). Once specimens had been prepared they were examined under the appropriate microscope.

3.15a The Scanning Electron Microscope

The image obtained with the SEM is three-dimensional. The condenser lens of this microscope produces a thin beam of electrons which pass through a magnetic coil
that moves back and forth over the surface of the specimen in a rapid scanning
motion. At each place the scanning beam strikes the specimen, secondary electrons
are emitted from the gold which is coated on the specimen surface. These secondary
electrons are collected by electron detectors and their energy is converted into an
electrical signal, the intensity of which is displayed at the corresponding position on a
television screen. The scanning beam follows the same path as the image-producing
spot on the television screen and travels in synchrony with it so as to build up a
complete image on the screen.

3.15b The Transmission Electron Microscope

The TEM involves the passage of an electron (emitted from an electron gun)
which penetrates the specimen surface. Some electrons are scattered out of the beam
by electron dense parts of the specimen. These electrons are removed from the beam
by the blocking action of a very fine aperture. This aperture allows more contrast in
the image. The electrons that are not scattered are focused by the objective lens to
provide an enlarged image. Once the electrons have passed through the specimen,
they are focused by magnets to form an image on a fluorescent screen or photographic
plate.

3.15c Electron micrographs

Electrons are invisible, so the image formed by the electron beam passing
through the specimen is rendered visible by focusing it on a fluorescent screen, where
the energy of the electrons is converted into light. However, because electrons can
cause silver grains to form in photographic emulsions, photographic film can be used
in place of a fluorescent screen to obtain a photographic record (negative) of what is
known as an electron micrograph.

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Once specimens had been photographed and negatives had been obtained, they were developed as follows:

TEM negatives were focused under a Durst Laborator 1000 enlarger. The darkest area on the image was found using a light sensitive meter. This gave the maximum exposure time required. The image was then set in the grid size being used and Agfa Rapitone Paper (resin-coated) was placed gloss side up under the grid. The timer allowed exposure for the pre-set time; once exposure time had elapsed, the Agfa paper was taken out and put (matt side up) into a Radioprint DP3700 which produced the electron micrographs. An identical procedure was used for the SEM negatives.

3.16 Clinical assessment of fibrinogen and lipids as risk factors for human erectile dysfunction

A clinical study to investigate the prevalence of hyperlipidaemia and hyperfibrinogenaemia among patients with ED was conducted as part of this work. The ED group of patients were recruited from the andrology clinic. Patients with diabetes mellitus or neurogenic impotence were excluded from the study. A total of 57 patients with ED were sampled. An age-matched control population of 18 men with normal erectile function was drawn from patients attending the general urology clinic. A fasting venous blood sample was taken from each patient for estimation of their plasma lipid and fibrinogen levels.
CHAPTER 4

STUDIES ON NITRIC OXIDE SYNTHASE AND ADENYLATE AND GUANYLATE CYCLASE IN CONTROL AND DIABETIC RATS

4.1 Introduction

4.1a Nitric oxide synthase autoradiography

As discussed in chapter one, nitric oxide (NO) is now recognised as being an important mediator of not only vascular smooth muscle relaxation but also of corporeal smooth muscle relaxation and penile erection. NO exerts its effects by increasing the intracellular levels of cGMP via the stimulation of soluble guanylate cyclase (Ignarro et al, 1990). Penile erection depends on both neuronally and endothelially-derived NO (Bush et al, 1992a; Holmquist et al, 1992a; Ignarro et al, 1990; Kim et al, 1991). In this context, it is noteworthy that impaired neurogenic and endothelium-dependent smooth muscle relaxation have been reported in human diabetic tissue as well as in animal models of diabetes mellitus (Saenz de Tejada et al, 1989b; Azadzoi and Saenz de Tejada, 1992; Italiano et al, 1993a).

Nitric oxide synthase (NOS) is the enzyme responsible for the synthesis of NO from L-arginine and has been shown to exist in at least three isoforms: constitutive, inducible and Ec NOS (Lyons et al, 1992; Bredt et al, 1991; Lamas et al, 1992). Immunohistochemical NOS staining has been previously described in the rat and
human penis (Burnett et al, 1992; Burnett et al, 1993). Using an antibody against the constitutive NOS found in rat brain, Burnett et al demonstrated the presence of NOS in the cavernous nerve, its terminal endings in the corpus cavernosum and also in the dorsal penile nerve and its branches of the rat (Burnett et al, 1992). Findings in the human genital tract were similar (Burnett et al, 1993). However, it is of interest to note that NOS was not clearly localised in the endothelium or smooth muscle of the corpus cavernosum by the use of such immunohistochemical techniques. However, Keast, using an NADPH diaphorase staining technique, managed to demonstrate the presence of NOS in the rat penis in both endothelial cells and within the cavernous smooth muscle (Keast, 1992; Dawson et al, 1991).

An impairment of NO synthesis in diabetic vascular beds has been previously reported (Kiff et al, 1991) and given its pivotal role in the mediation of penile erection it is somewhat surprising that it is not known if abnormalities of NOS contribute to the pathogenesis of diabetic and other erectile disorders (Lowenstein et al, 1994). It is reasonable to postulate, however, that a diminution of NOS (and therefore a relative lack of NO) might contribute to the development of ED.

In order to investigate whether diabetes alters NOS activity in the penis, we used $[^3]$H-L-N$^G$-nitroarginine ($[^3]$H-L-NOARG) to label the NOS isoforms. This compound was used since it is the most potent inhibitor of NOS yet described (Moore et al, 1990). It has a rather low affinity for use as a radioligand and has slow inhibition kinetics, both in vivo and in vitro, which suggested its suitability for use in directly labelling NOS (Dwyer et al, 1991). Michel et al reported that $[^3]$H-L-NOARG is suitable for the labelling and localisation of NOS in rat brain cytosol and therefore represented a potentially useful tool for the characterisation of NOS in tissue (Michel et al, 1993). We therefore used this radioligand to label NOS in tissue sections of diabetic (and control) rat penis and used an autoradiographic technique to study the distribution of NOS.
The aim of our present study was to use an autoradiographic methodology not only to localise but also to objectively quantify NOS activity by using \[^{3}\text{H}]-\text{L}-\text{NOARG}. Our principle objectives were to show the distribution of binding and to localise it anatomically by using high resolution emulsions. In addition, we wanted to determine whether there were significant differences between control and diabetic rats and if there were any differences in these profiles between diabetes of two and six months duration.

4.1b Adenylate and guanylate cyclase stimulation experiments

Probably the best known second messengers are the 3'5' cyclic nucleotides cAMP and cGMP. Although they were first discovered some 30 years ago it is only now that we are beginning to understand their complexity and regulation. In the past 10 years a large amount of information has been accumulated about which specific receptors stimulate or inhibit adenylate cyclase and how it is that GTP binding proteins couple cAMP synthesis to receptor occupation (Bentley and Beavo, 1992).

There is also now a clearer understanding of the direct coupling of peptide hormones to cGMP synthesis via membrane bound guanylate cyclase. Moreover, the importance of phosphodiesterases in the control of cyclic nucleotides has been appreciated for a number of years and this aspect of their metabolism is further addressed in the chapter five.

The present studies focus on the ability of penile tissues to generate cAMP and cGMP in response to their incubation with a variety of vasoactive agents in vitro and the comparison of results from diabetic and control animals. A number of different stimulants were used: adrenaline (AD), vasoactive intestinal polypeptide (VIP), papaverine (PAP), prostaglandin E\(_1\) (PGE\(_1\)) acetylcholine (ACh), and sodium nitroprusside (NaNP) over a wide concentration range.
Vasoactive intestinal polypeptide (VIP) is a potent vasodilator consisting of 28 amino acid residues which was first isolated in the gastro-intestinal tract and inhibits contractile activity in many types of smooth muscle (Said, 1981). It interacts with a specific receptor and its mode of action appears to be dependent upon the production of cAMP (Ganz et al, 1986) and/or cGMP (Spessert, 1993), as well as the modulation of NO release (Grider et al, 1992), although data is not available relating to the penis. VIP has been found to be present in high concentrations in the erectile tissues of the human penis, in autonomic nerve fibres which have their endings around cavernous smooth muscle and around penile arteries and arterioles (Polak et al, 1981; Gu et al, 1984; Kirkeby et al, 1992a). It has also been demonstrated in the penis of other species such as rabbits, guinea pigs, and rats (Andersson et al, 1992).

Many workers have independently reported the relaxant effects of VIP upon human erectile tissue. It has a pronounced effect on both the spontaneous activity and on electrically induced contraction in corpus cavernosum strips, but not on NAd contracted preparations (Andersson et al, 1983; Steers et al, 1984; Pickard et al, 1993; Larsen et al, 1981; Adaikan et al, 1986; Hedlund and Andersson, 1985c). The relaxant effect of VIP was found to be unchanged by sympathetic and parasympathetic antagonists, or indeed ganglionic blockade and tetrodotoxin indicating that VIP is acting directly on smooth muscle cells (Lincoln et al, 1991). VIP was also extremely effective in relaxing penile circumflex veins which had been precontracted with NAd (Kirkeby et al, 1992a). The relaxation induced by ACh and VIP together on NAd precontracted human corpus cavernosum was no greater than that seen when ACh or VIP was given separately. It has recently been reported that the use of VIP antiserum and alpha chymotrypsin reduced and/or abolished the relaxant effects of exogenous VIP on human erectile tissue, but had no effect upon the relaxation induced by electrical nerve stimulation (Pickard et al, 1993). On the basis of this indirect evidence it was suggested firstly that VIP is not the neurotransmitter responsible for erection and secondly that it doesn't have its effects via the NO-cGMP pathway (Pickard et al, 1993).
From the results of VIP administration in vitro there is consensus that a relaxant effect is generally produced by the peptide. The results from in vivo experiments are less clear. However, it was reported that the intracavernous injection of VIP and ACh produce a synergistic effect in a canine model of erection, it was therefore suggested that these two agents may play a co-operative role (Takahashi et al, 1992a). However, an intracavernous injection of VIP in monkeys had no effect, and actually caused the detumescence of erections produced by cavernous nerve stimulation (Steers et al, 1984). In more recent experiments using a rabbit model it was found that VIP, peptide histidine methionine and neuropeptide Y were all capable of influencing the smooth muscle tissue involved in the regulation of penile outflow, although interestingly VIP was unable to produce full penile rigidity (Kirkeby et al, 1992b).

Early reports from human studies that tumescence and erection caused by visual sexual stimulation, or by the injection of papaverine or saline, resulted in a marked release of VIP (Virag et al, 1982; Ottesen et al, 1984) were not however supported by subsequent investigations. Thus, Kiely and co-workers measured cavernosal and peripheral VIP concentrations during erections induced by vasoactive compounds in impotent patients, but were unable to detect any rise in intracavernosal VIP concentrations (Kiely et al, 1987).

Following the finding that VIP-containing nerves were depleted in impotent men and that the degree of depletion was related to the severity of erectile dysfunction, it was suggested that VIP was the principal neurotransmitter responsible for penile erection and its depletion was therefore an important factor in ED (Gu et al, 1984). Further support for this idea came from the finding that in impotent diabetic men there was a marked reduction in VIP-like immunoreactivity in the nerves associated with the cavernous smooth muscle (Lincoln et al, 1987). These findings were similar to those previously described in both human diabetes and in the STZ diabetic rat (Crowe et al, 1983).
Other studies in man and canine models have reported that VIP can cause tumescence but not full erection, although a full erection can be achieved when such an intracavernosal injection is supplemented by vibrational or visual sexual stimulation (Adaikan et al, 1986; Kiely et al, 1989; Wagner and Gerstenberg, 1987; Gerstenberg et al, 1992). Better results in impotent men were found using a combination of VIP and phentolamine, all men receiving such an injection thus had erections with sufficient rigidity for penetration (Gerstenberg et al, 1992). In combination with papaverine, VIP produced penile rigidity similar to papaverine and phentolamine, while alone it produced disappointing results (Kiely et al, 1989). These results were interpreted as being consistent with the concept that the main of effect of VIP seemed to be on the mechanism of veno-occlusion (Juenemann et al, 1987). The putative role of VIP as a neurotransmitter is not supported by the results of VIP injection intracavernosally in impotent men in which it fails to produce an erection. Thus, it cannot be the only and indeed is probably not the main NANC mediator of penile smooth muscle relaxation.

Nevertheless, the intracavernosal injection of VIP has been used therapeutically to induce penile erection in the treatment of ED (Kiely et al, 1989; Gerstenberg et al, 1992). Despite VIP having been implicated as an important mediator of penile erection for many years (Andersson et al, 1983; Ottesen et al, 1984) and papaverine being one of the most commonly used pharmacological agents used in the treatment of ED the precise mode of action of these two agents in penile tissues has been unclear.

Papaverine has been extensively used in the intracavernosal pharmacotherapy of ED. It has often been characterised as a non-specific smooth muscle relaxant and has been shown to have the ability to relax all components of the penile erectile system (Kirkeby et al, 1990a). Despite the available evidence from a variety of tissues, its precise intracellular profile of action remains unclear for penile smooth muscle. Papaverine has a non-selective inhibitory effect on the cyclic nucleotide
phosphodiesterases (Poch and Kukovetz, 1972). Thus papaverine possesses the capacity to prevent the hydrolysis of cAMP and cGMP to their constituent nucleotides. However, it is not known if papaverine is able to stimulate the production of either of these cyclic nucleotides de novo. In addition, it has been shown in the guinea pig ureter that papaverine will block voltage operated calcium channels, thereby inhibiting calcium influx (Brading et al, 1983). A further possible action is an inhibition of the release and/or storage of intracellular calcium, as well as increasing calcium efflux (Huddart et al, 1984; Sunagane et al, 1985; Krall et al, 1988). Evidence is also available that papaverine inhibits calcium activated chloride and potassium channels in vascular smooth muscle cells by the depletion of intracellular calcium stores (Wang and Large, 1991). The sum of the available findings is that papaverine acts to lower intracellular calcium levels as well as maintaining/elevating cAMP and cGMP levels: all pro-relaxant factors for smooth muscle cells acting at a number of different levels.

As mentioned previously, recent research interest has focused on the role of corporeal smooth muscle relaxation and its regulatory mechanisms in erection (Saenz de Tejada et al, 1985; Andersson and Holmquist, 1990). Of central importance in this respect are a number of second messenger systems, such as cAMP, cGMP, calcium, potassium and inositol trisphosphate and were recently reviewed by Lerner and colleagues (Lerner et al, 1993). It is important to remember that penile smooth muscle cells are connected by gap junctions (Christ et al, 1991). These facilitate the rapid passage of second messenger molecules between adjoining cells and hence enable the tissue to act as a functional syncytium as in other smooth muscle structures (Christ et al, 1991). Since it is well known that DM is strongly associated with the development of ED in both man and in laboratory animals (McCulloch et al, 1980; Hassan et al, 1993), the aims of the present experiments were firstly to investigate the mode of action of VIP and papaverine on the activity of adenylate and guanylate cyclase in order to further elucidate the mechanisms of penile smooth muscle relaxation.
Secondly, the investigators were keen to assess the suitability of the New Zealand White rabbit as an appropriate model for future experiments into the investigation of the pathophysiology of ED. A number of different experimental models have been used in this respect and while the Sprague Dawley rat is an extremely useful animal it has been noted that it seems to be resistant to the development of atherosclerosis. By performing some initial characterisation of the pathways present in the NZW rabbit it was hoped that interventional experiments as well attempts to monitor intracavernosal pressure changes might be possible in future studies.

4.2 Results

4.2a Nitric oxide synthase autoradiography

In the two month diabetic group, the starting weights (mean [range]) of the control and diabetic animals were similar. (Control rats, n=5: 255g [251-260]; diabetic, n=5: 258g [246-263]). At the end of two months the diabetic rats were significantly lighter than the control animals. (Controls, n=5: 515g [490-527]; diabetic rats, n=5: 228g [219-239]). The serum glucose concentrations were significantly elevated in the diabetic rat group ($P<0.001$) when compared to the control group at two months. (Controls, n=5: 10.1mmol/l [9.8-12.0]; diabetic, n=5: 57.2mmol/l [46.5-59.1], $P<0.01$). Serum chemical pathology profiles were performed on these animals and the reader is referred to Appendix 1 and Appendix 2.
Plate 4.1 Autoradiographic localisation of nitric oxide synthase in diabetic rat corpus cavernosum after two months. Images generated on film (low resolution) from longitudinal sections of rat penis incubated in $[^3]$H-L-NOARG. Top left - autoradiograph from a section of diabetic rat penis after two months (DIAB) incubated in $[^3]$H-L-NOARG alone (total binding, TOT). Top middle - Haematoxylin and eosin stained tissue underlying autoradiograph. Top right - Autoradiograph of non-specific binding to an adjacent section incubated in the presence of 10µM L-arginine (NSB). Bottom - binding and histology from sections of penis from a control animal. Note the dense binding to the corpus cavernosum of the diabetic animal. Binding to this region in the control animal is barely detectable. Scale bar = 1mm.
Plate 4.2 Microscopic localisation of nitric oxide synthase in the control rat corpus cavernosum. High-resolution autoradiographs generated on nuclear emulsion (high-resolution). $[^3H] \text{L-NOARG}$ binding (dark grains) to endothelial cells of corpus cavernosum of the control rat. Tissues stained with haematoxylin and eosin.
Plate 4.3  Microscopic localisation of nitric oxide synthase in diabetic rat corpus cavernosum. High-resolution autoradiographs generated on nuclear emulsion (high-resolution). [3H]-L-NOARG binding (dark grains) to endothelial cells of corpus cavernosum of the diabetic rat of two months duration. Tissues stained with haematoxylin and eosin.
There was dense $[^{3}\text{H}]-\text{L-NOARG}$ binding to tissue sections which was markedly reduced when the tissues were incubated in the presence of L-arginine (Plate 4.1). $[^{3}\text{H}]-\text{L-NOARG}$ was localised to the corpus cavernosum and the urothelial lining of the urethra (Plate 4.2, Plate 4.3). No binding of $[^{3}\text{H}]-\text{L-NOARG}$ to the corpus spongiosum was seen. Examination of the underlying penile tissue under high resolution revealed marked binding of $[^{3}\text{H}]-\text{L-NOARG}$ to the endothelium of the corpus cavernosum, but with no obvious vascular smooth muscle binding.

Densitometric analysis of the film images indicated that binding to the corpus cavernosum was significantly increased in the diabetic rats compared to the controls at two months (Table 4.1). Furthermore, the high resolution autoradiographs showed that this increased binding was primarily to the endothelial cells of the corpus cavernosum (Plate 4.2, Plate 4.3).
Table 4.1 Photodensitometric analysis of \(^{[3]H}\)-L-NOARG binding in cavernosal tissue of normal and diabetic rats at two months

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<th>Median (range)</th>
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<tr>
<td>Control</td>
<td>1.48 (1.32-1.78)</td>
<td></td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.98 (1.76-2.37)</td>
<td></td>
</tr>
</tbody>
</table>

control vs. diabetic $p < 0.0001$
In the six month diabetic group, the starting weights (mean [range]) in both the control and diabetic rat groups were similar (control, n=5: 340g [292-357]; diabetic, n=5: 350g [289-370]). However, at the end of the study the diabetic rats were significantly lighter than the control rats (control, n=5: 750g [670-792]; diabetic, n=5: 215g [187-246], *P* < 0.001). Serum glucose concentrations (mean [range]) were significantly elevated in the diabetic rat group when compared to the control rat group after 6 months (control, n=5: 10.7mmol/l [9.2-11.5]; diabetic, n=5: 75.3mmol/l [54-92], *P* < 0.001).

The six month study group demonstrated specific (displaceable)[^H]-L-NOARG binding sites in the cavernosal regions of control rat penises. These binding sites were markedly increased when the diabetic rat penises were examined (plate 4.4). A densitometric analysis revealed a greater than five fold increase in binding sites when both longitudinal and transverse penile sections from control rats was compared to the same regions of the diabetic rat penis (table 4.2). We also performed a colour coding on these sections to demonstrate the binding differences in an easily appreciable form (plate 4.5).

### 4.2b Adenylate and guanylate cyclase stimulation experiments

The body weights (median [range]) of the experimental and the control group of rats at the time of death were as follows: control, n=5: 475g [449-516]; diabetic, n=5: 239g [220-280]; NZW rabbits, (n=6) were also studied. The glucose concentrations (median [range]) at the time of death were: control, n=5: 8.4mmol/l [6.8-10.3]; diabetic, n=5: 30.3mmol/l [28.2-32.7].
Plate 4.4 Autoradiographic localisation of nitric oxide synthase in diabetic rat corpus cavernosum after six months. Images generated on film (low resolution) from longitudinal sections of rat penis incubated in $[^3H]$-L-NOARG. Top left - autoradiograph from a section of control rat penis (NORM) incubated in $[^3H]$-L-NOARG alone (total binding, TOT). Top middle - Haematoxylin and eosin stained tissue underlying autoradiograph. Top right - Autoradiograph of non-specific binding to an adjacent section incubated in the presence of 10μM L-arginine (NSB). Bottom - binding and histology from sections of penis from a diabetic rat penis of six months duration (DIAB). Note the dense binding to the corpus cavernosum of the diabetic animal. Binding to this region in the control animal is reduced. Scale bar = 1mm.
Table 4.2  Densitometric analysis of specific \[^3\text{H}\]-L-NOARG binding sites in penile tissue from control and diabetic rats 6 months after the initiation of the study. Values are expressed as amol/mg protein equivalent. At least 2 sections were analysed from each penis. Statistical analysis: A vs. B $P<0.001$, C vs. D $P<0.001$ using Mann-Whitney test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Penile section</th>
<th>(n)</th>
<th>amol/mg protein equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control longitudinal</td>
<td>(n=21)</td>
<td>73.4 (70.4-87.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Diabetic longitudinal</td>
<td>(n=16)</td>
<td>416.3 (221.2-883.1)</td>
</tr>
<tr>
<td>C</td>
<td>Control transverse</td>
<td>(n=10)</td>
<td>73.8 (71.1-81.9)</td>
</tr>
<tr>
<td>D</td>
<td>Diabetic transverse</td>
<td>(n=10)</td>
<td>448.7 (377.3-624.8)</td>
</tr>
</tbody>
</table>
Plate 4.5 Colour coded autoradiographic localisation of nitric oxide synthase in diabetic rat corpus cavernosum at six months. Images generated on film (low resolution) from sections of rat penis incubated in $[^3H]$-L-NOARG. Autoradiographs from a section of control rat penis (NORM) and diabetic (DIAB) rat penis in both transverse (TS) and longitudinal section (LS) are shown. The colours have been assigned so that green denotes the areas of highest binding. The binding sites in the diabetic animals of six months duration are clearly increased.
NaNP was found to be a potent stimulator of cGMP synthesis in both penile and aortic tissue (Figs. 4.1, 4.2) but had no effect on cAMP synthesis in either tissue. NaNP-stimulated cGMP synthesis was significantly enhanced \( (P<0.01) \) in the penises of diabetic rats compared with the controls (at low concentrations of NaNP) but the maximal synthesis of cGMP between the two groups was not statistically significant (Fig. 4.1).

ACh stimulated cGMP synthesis in the aorta but to a lesser degree than NaNP (Fig. 4.2). ACh had no effect on cGMP synthesis by penile tissue in either control or diabetic animals (Fig. 4.1). ACh stimulated cGMP synthesis was markedly reduced in the aorta of diabetic rats compared with controls (Fig. 4.2).

PGE\(_1\)-stimulated cAMP synthesis in both aorta and penis was significantly enhanced in diabetic rats compared with controls (Figs. 4.3, 4.4). PGE\(_1\) had no effect on cAMP synthesis over the same concentration range of the eicosanoid. AD-stimulated cAMP synthesis was significantly enhanced in the aorta of diabetic rats compared with controls (Fig. 4.5). AD had no effect on cAMP synthesis in the penis of control or diabetic rats (data not shown). Papaverine had no effect on cAMP or cGMP synthesis in the penis or aorta of control or diabetic animals in this preparation (Table 4.3, Table 4.4).

VIP was a potent stimulator of cAMP synthesis in the penis and aorta of both the rat and rabbit but was without effect on cGMP in both tissues (Figs. 4.6, 4.7, 4.8, Tables 4.5 and 4.6). Papaverine had no effect on either cAMP or cGMP in the penis of the rat and rabbit (Table 4.3). VIP-stimulated cAMP synthesis was significantly enhanced in the penises of diabetic rats compared to controls (Fig. 4.6). Basal cAMP was increased in diabetic rat penile tissue compared to controls (data not shown), and there were significantly higher basal levels of cGMP in both the penis and aorta of the diabetic rat compared to controls (Table 4.6).
Fig. 4.1 Effect of sodium nitroprusside (NaNP) and acetylcholine (ACh) on cGMP synthesis by the penises of diabetic and control rats. ♦, NaNP in diabetic penises. ●, NaNP in control penises. ○, ACh in diabetic penises. O, ACh in control penises. Each point represents the mean ± SEM, n=7. #P<0.01.
Fig. 4.2 Effect of sodium nitroprusside (NaNP) and acetylcholine (ACh) on cGMP synthesis by the aortae of diabetic and control rats. ♦, NaNP in diabetic aortae. ●, NaNP in control aortae. O, ACh in diabetic aortae. ◇, ACh in control aortae. Each point represents mean ± SEM, n=7, # P<0.01.
Fig. 4.3 Effect of prostaglandin E₁ on cAMP synthesis by the aortae of diabetic (♦) and control (●) rats. Each point represents mean ± SEM, n=7.
Fig. 4.4 Effect of prostaglandin E₁ on cAMP synthesis by the penises of diabetic (♦) and control rats (●). Each point represents the mean ± SEM, n=7, # P<0.01
Fig. 4.5 Effect of adrenaline on cAMP synthesis by the aortae of diabetic (♦) and control rats(●). Each point represents the mean ± SEM, n=7. # P < 0.01.
Table 4.3  Effect of various concentrations of papaverine on cAMP (fmol/mg tissue/min) and cGMP (fmol/mg tissue/20min) synthesis by the penis of the rat (n=7) and rabbit (n=6). Each value is the mean ± SEM for six duplicate assays.

<table>
<thead>
<tr>
<th>Papaverine concentration (μmol/l)</th>
<th>Rat penis</th>
<th>Rabbit penis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
</tr>
<tr>
<td>0</td>
<td>5.1 ± 0.4</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>0.1</td>
<td>5.2 ± 0.4</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>1</td>
<td>5.6 ± 0.6</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>10</td>
<td>5.4 ± 0.5</td>
<td>7.2 ± 0.6</td>
</tr>
</tbody>
</table>
Table 4.4 Effect of various concentrations of papaverine on cAMP (fmol/mg tissue/min) and cGMP (fmol/mg tissue/20min) synthesis by the aorta of the rat (n=7) and rabbit (n=6). Each value is the mean ± SEM for six duplicate assays.

<table>
<thead>
<tr>
<th>Papaverine concentration (μmol/l)</th>
<th>Rat aorta</th>
<th>Rabbit aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
</tr>
<tr>
<td>0</td>
<td>4.6 ± 0.4</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>4.5 ± 0.4</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>1</td>
<td>5.2 ± 0.4</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>5.6 ± 0.4</td>
<td>7.4 ± 0.4</td>
</tr>
</tbody>
</table>
Fig. 4.6 Effect of VIP on cAMP synthesis by the penises of diabetic (●, n=10) and control (○, n=7) rats. Each point is the mean ± SEM of six duplicate assays. # P < 0.05.
Fig. 4.7 Effect of VIP on cAMP synthesis by the aortae of diabetic (♦, n=10) and control (○, n=7) rats. Each point is the mean ± SEM of six duplicate assays. # P < 0.05.
Fig. 4.8 Effect of VIP on cAMP synthesis by the penis (●), carotid artery (▲) and aorta (O) of the NZW rabbit (n=6). Each point is the mean ± SEM of six duplicate assays.
Table 4.5 Effect of various concentrations of VIP on cGMP (fmol/mg tissue/20min) synthesis by the penis, aorta and carotid artery of the rabbit (n=6). Each value is the mean ± SEM of six duplicate assays.

<table>
<thead>
<tr>
<th>VIP concentration (µmol/l)</th>
<th>Penis</th>
<th>Aorta</th>
<th>Carotid artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.9 ± 0.4</td>
<td>6.2 ± 0.9</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>0.1</td>
<td>3.8 ± 0.5</td>
<td>7.2 ± 0.9</td>
<td>4.2 ± 0.7</td>
</tr>
<tr>
<td>1</td>
<td>4.1 ± 0.6</td>
<td>6.8 ± 0.6</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>10</td>
<td>4.2 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td>6.2 ± 0.4</td>
</tr>
</tbody>
</table>
Table 4.6  Effect of various concentrations of VIP on cGMP (fmol/mg tissue/20min) synthesis by the penis and aorta of control (n=7) and diabetic (n=10) rats. Each value is the mean ± SEM of six duplicate assays. *P<0.001 when comparing control to diabetic rats at each dose of VIP.

<table>
<thead>
<tr>
<th>VIP concentration (μmol/l)</th>
<th>Penis</th>
<th></th>
<th>Aorta</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>0</td>
<td>6.4 ± 0.6</td>
<td>14.1 ± 1.2*</td>
<td>8.2 ± 2.3</td>
<td>20.8 ± 2.5*</td>
</tr>
<tr>
<td>0.1</td>
<td>7.2 ± 0.7</td>
<td>13.3 ± 1.2*</td>
<td>7.3 ± 1.2</td>
<td>21.9 ± 1.4*</td>
</tr>
<tr>
<td>1</td>
<td>6.6 ± 0.5</td>
<td>14.4 ± 1.3*</td>
<td>7.4 ± 1.2</td>
<td>19.6 ± 1.4*</td>
</tr>
<tr>
<td>10</td>
<td>6.4 ± 0.4</td>
<td>15.2 ± 1.2*</td>
<td>9.3 ± 1.3</td>
<td>17.8 ± 1.8*</td>
</tr>
</tbody>
</table>
4.3 Discussion

This study demonstrated a significant increase in the staining of NOS binding sites on the endothelium of the STZ rat corpus cavernosum at two months duration. This finding should be considered in the light of previous studies which have suggested that a relatively short duration of the diabetic milieu causes alterations in the physiological mechanisms that modulate the relaxation of corpus cavernosum, a crucial step for penile erection. We also demonstrated that the NOS binding sites increased approximately five fold after six months of diabetes in the same model. Whereas in the two month group the observed increase in NOS binding was of the order of 33%. The observation that diabetes increases NOS binding sites in the corpus cavernosum implies an up-regulation of these sites and that they adequately bind L-arginine. We have not however shown that L-arginine is effectively converted to NO. This latter step is of course of the utmost relevance as it is the NO which mediates the relaxation of corporeal smooth musculature.

The observed up-regulation of NOS could be a consequence of a reduction in the bioavailability of the endogenous erectogen NO. Evidence for a reduction in vascular NO production in DM was provided by Kiff and co-workers in the hindquarter vascular bed of diabetic rats with diabetes of one month duration (Kiff et al, 1991). Hyperglycaemia per se, through the production of advanced glycosylation end products, has also been shown to inhibit NO activity (Kiff et al, 1991; Getz, 1995). These advanced glycosylation end products accumulate in human corporeal collagen in diabetes where they quench and inactivate NO (Azadzoi et al, 1990). Hypercholesterolaemia has also been shown to adversely effect endothelial function in the corpus cavernosum (Azadzoi and Saenz de Tejada, 1991). However, cholesterol levels are unlikely to have played a role in the current study since the serum concentrations are not markedly affected in control and two month diabetic animals (Thompson and Mikhailidis, 1992), furthermore the rat seems to be relatively
resistant to atherosclerosis. However, species such as the rabbit are not and would be a fruitful area for further study.

Alternatively, NO generation might be actually increased. Several studies have implicated an increased NO generation in the pathogenesis of vascular dysfunction in early diabetes (Corbett et al, 1992; Tilton et al, 1993). Some further support for this comes from the findings reported in the next section where our studies suggest that the capacity to generate cGMP is not impaired in these vascular beds.

Our findings suggest that any decrease in NO production by the rat corpus cavernosum in experimental DM can not be attributed to a decrease in NOS binding sites. A decrease in NO production may be a consequence of DM neuropathy or a diminished conversion of L-arginine by NOS. Alternatively, the bioavailability of NO may be impaired (e.g. by advanced end glycosylation products) or there may be end-organ resistance. Our findings provide evidence of early and progressive endothelial dysfunction in this tissue in DM. This endothelial dysfunction in the corpus cavernosum of the diabetic rat appears to be progressive and more severe at six months as shown in the studies described above. These findings have obvious implications for the role of NOS in the pathogenesis of diabetic ED.

Several groups have presented convincing evidence that the NO-cGMP axis plays a key role in mediating penile smooth muscle relaxation and erection. In isolated tissue bath preparations it has been demonstrated that relaxation of cavernosal tissue from the rabbit is dependent upon the integrity and presence of the sinusoidal endothelium (Ignarro et al, 1990). In an in vivo dog model, Trigo-Rocha et al, have recently shown that methylene blue, a guanylate cyclase inhibitor, significantly inhibited the erectile response to both NaNP and electrical field stimulation (Trigo-Rocha et al, 1993). Furthermore, they showed that injection of cGMP elicited an erectile response (Trigo-Rocha et al, 1993). In the present experiments, NaNP stimulated cGMP synthesis in the penis in comparable quantities to the aorta.
However, ACh did not elicit the generation of detectable amounts of cGMP in the penis of the rat. This indicates that although NO-stimulated cGMP is present in the penis of the rat the release of NO from the endothelium is not under cholinergic control, at least in this species. Furthermore, cGMP synthesis in response to NaNP was significantly enhanced in the penis of diabetic rats compared to controls. This enhanced capacity to generate cGMP may constitute an adaptive phenomenon aimed at counteracting the reduction of other pro-erectile factors such as VIP, PGI$_2$, and cholinergic receptors (Lincoln et al, 1987; Lincoln et al, 1991).

Apart from the relaxation of erectile tissues, vasodilatation of the pudendal, penile and cavernosal arteries is important in penile erection. As these vessels were too small to study in the rat model, the aorta was used as an indicator of what might be occurring in the vasculature during erection. NaNP-stimulated cGMP synthesis was found to be enhanced in the aortae of diabetic rats although ACh-stimulated cGMP synthesis was significantly reduced. Kamata et al, also demonstrated a reduction of ACh-stimulated cGMP synthesis from the aortae of diabetic rats but the responses of aortic tissue to NaNP (relaxation) were unchanged (Kamata et al, 1989). Taken together, these data indicate that the attenuated responses seen to ACh may be due to either a defect in the ACh receptors linked to NO release or a reduction of NO synthase (NOS) activity. These results suggest that there is a generalised reduction in receptor activity in the vasculature of the diabetic rat which has been ascribed to altered protein kinase C activity (Jeremy et al, 1993b). As such a defect may occur in the vasculature associated with erection, ACh-stimulated NO release from penile vasculature warrants investigation in larger animals. Such work may also reveal important anatomical regional variations in endothelial function.

With regard to adenylate cyclase, the present study demonstrates that PGE$_1$ is a potent stimulator of cAMP synthesis by the penis of the rat although ACh and NaNP had no effect on the synthesis of this nucleotide. Since the intracavernosal injection of PGE$_1$ elicits erection in both man and rat, it can be concluded that PGE$_1$
does so by an activation of adenylate cyclase. The cAMP that is generated probably elicits dilation and/or relaxation of cavernosal tissue via the resequestration of calcium ions (Rasmussen, 1986). Cyclic AMP generated in response to PGE\textsubscript{1} was greater in the penises of diabetic rats than controls. As PGI\textsubscript{2} release was diminished in the penis of diabetic rats (Jeremy et al, 1985b; Jeremy et al, 1986a), this up-regulation of adenylate cyclase activity may constitute an adaptive phenomenon aimed at compensating for the diminished availability of endogenous dilator eicosanoids. Of interest was the fact that ACh failed to elevate cAMP in penises either from controls or diabetic rats.

As ACh stimulates PGI\textsubscript{2} release by cavernosal tissue, a sequence involving ACh-PGI\textsubscript{2}-cAMP probably does not play a part in erection, at least in the rat. In a recent study, Trigo-Rocha et al concluded that cAMP does not play a significant role in erection in the dog because the cAMP inhibitor, N-ethylmaleimide, failed to reduce the response to both electrical field stimulation and NaNP (Trigo-Rocha et al, 1993). It may have been more appropriate to have investigated the effect of N-ethylmaleimide when cAMP synthesis was stimulated with a prostanoid. Certainly the present study indicates that NaNP has no effect on cAMP levels. Similarly, electrical field stimulation is not known to elevate cAMP so it would not be expected that an inhibitor of adenylate cyclase would have an effect on erection elicited by this means. It was also shown that cAMP administration to dogs was much weaker than cGMP in eliciting relaxation (Trigo-Rocha et al, 1993). However, cAMP does not readily enter cells and is rapidly hydrolysed by phosphodiesterases (Ashby, 1990). Therefore to preclude a role for cAMP (and therefore for endogenous prostaglandins) on the basis of this experiment may be spurious since neither field stimulation nor NaNP, as in the present study, elicit cAMP synthesis.

Since adrenaline stimulates PGI\textsubscript{2} release in rat aorta (Jeremy et al, 1985a), it has been suggested that this eicosanoid may elicit relaxation via cAMP elevation (Lincoln et al, 1991; Jeremy et al, 1985a). Hassid and Williams have demonstrated
that agonists that elicit PGI$_2$ release in cultured vascular cells then stimulate cAMP generation (Hassid and Williams, 1982). Furthermore, since adrenaline-stimulated PGI$_2$ is markedly reduced in aortae from diabetic rats (Jeremy et al, 1993b), it would be expected that adrenaline-stimulated cAMP would be reduced. However, this was not the case in the present experiments. Whether there is a direct link between adrenoceptors and adenylate cyclase (perhaps G protein mediated) or whether there is an up-regulation of adenylate cyclase in diabetic vessels will require further experimentation.

In a broader context, the present findings are of relevance to atherogenesis, the incidence of which is increased in diabetes and contributes to impotence (Saenz de Tejada et al, 1989b; Italiano et al, 1993a; Italiano et al, 1993b; Watkins, 1990; Lincoln et al, 1987). Atherogenesis is a complex process which involves proliferation of vascular smooth muscle cells, cholesterol accumulation, platelet adhesion and aggregation and invasion by macrophages (Ross, 1993). Since all these events are counteracted by cGMP and cAMP (and therefore PGI$_2$ and NO), any impairment of adenylate or guanylate cyclase activity may be pro-atherogenic. However, the present study indicates that alterations in the activity of these cyclases is unimpaired and indeed is actually enhanced in penile and vascular tissue from diabetic rats and as such may not contribute to the pathophysiology of diabetic angiopathy. However, defects in receptors (as are known to occur in DM) or NOS activity may indirectly alter the levels of cAMP and cGMP attained in these tissues which in turn may render vascular and/or penile tissue susceptible to atherogenesis. The elucidation of the inter- and intracellular pathways underlying erectile tissue relaxation allows for the identification of targets for novel therapeutic approaches, for example, it may be possible to bypass the receptors to activate the cyclases directly. Although we did not establish whether the diabetic rats used in this study were impotent, there are recent reports that erectile dysfunction occurs in the diabetic rat model (Italiano et al, 1993a; Italiano et al, 1993b; Hassan et al, 1993).
The present experiments also demonstrate that VIP stimulates cAMP synthesis, but not that of cGMP, in both rat and rabbit penile tissue. We have already demonstrated that sodium nitroprusside stimulates cGMP synthesis in the rat penis (Miller et al, 1994a). Thus, although an NO-cGMP axis is in fact present in the penile tissues of these species, a VIP-guanylate cyclase axis appears not to be. Our conclusions concur with the recent results of Pickard et al who demonstrated that VIP-stimulated relaxation of isolated human cavernosal smooth muscle is not mediated by NO and therefore unlikely to be cGMP-dependent (Pickard et al, 1993). These authors also concluded that VIP is not a major relaxant neurotransmitter in the human penis, a view which in the light of evidence that VIP can stimulate the formation of an important second messenger may be somewhat premature. Further support comes from Ganz et al who previously demonstrated that VIP will stimulate cAMP synthesis (and vasodilatation) in the rat aorta and mesenteric vessels (Ganz et al, 1986). We can conclude that VIP elicits erection via the activation of adenylate cyclase and not guanylate cyclase, at least in the rat and rabbit penis. [In a preliminary study using cultured human cavernosal cells we have also demonstrated that VIP stimulates cAMP but not cGMP (Jeremy and Miller, unpublished observations).] The present observations also consolidate the view that penile tissue behaves, physiologically, in a similar fashion to vascular tissue in that VIP stimulates cAMP but not cGMP. The findings also demonstrate that the pathways present in the Sprague Dawley rat are to be found in the NZW rabbit; we were encouraged therefore that we may be able to model other disease states associated with DM in another species. More recent support for our findings comes from the observation that pituitary adenylate cyclase activating peptide (PACAP) is not only found in the human corpus cavernosum but also that it relaxes pre-contracted tissue strips in a similar manner to VIP (Hedlund et al, 1994). Hedlund and colleagues conclude that even if NO is the predominant relaxant neurotransmitter it does not preclude a contributory effect from other transmitters (Hedlund et al, 1994). In this context it is interesting to consider whether the NO-cGMP axis is essential for penile erection. It has been demonstrated by Varrin et al that L-nitroarginine methyl ester administered intravenously failed to abolish
erections in 5 out of 13 Wistar rats, suggesting that NO is not the only mechanism responsible for erection (Varrin et al, 1994). It is becoming apparent that normal penile erection is a multitransmitter event mediated by a complex cascade of second messengers; the NO-cGMP axis is but one aspect of this system.

With regard to diabetes mellitus, several studies have shown that VIPergic nerves are depleted in both impotent and diabetic men (Gu et al, 1984; Lincoln et al, 1987). Similar findings were reported for diabetic rats (Crowe et al, 1983). Our data, however, indicate that there is an increased responsiveness to VIP in the penis of diabetic rats, at least in terms of cAMP generation. We have previously found that cAMP synthesis in response to PGE₁ is also enhanced by the penis of the diabetic rat in a similar fashion to VIP-stimulated cAMP (Miller et al, 1994a). It was postulated that there may be an adaptive increase in adenylate cyclase activity designed to counteract the generalised attenuation of receptor (linked to tumescence) activity and a similar argument may be invoked for the VIP data (Miller et al, 1994a).

The present studies also demonstrate that there is markedly increased basal cGMP (Table 4.6) and cAMP (data not shown) in the diabetic tissues. In this context we have already demonstrated that the NOS content in the diabetic rat penis is elevated. The net effect of these two alterations would be to elevate cGMP (and cAMP) levels and the present data are consistent with these findings. Taken together, our findings may contribute to an explanation of the clinical observation that diabetic men with predominantly neurogenic ED are often markedly sensitive, in terms of their erectile response, to intracavernosal injections (Ravnik-Oblak et al, 1990). This sensitivity may be due to the presence of significantly enhanced transduction mechanisms for smooth muscle relaxation in the presence of a normal blood supply.

The observed lack of effect of papaverine on cAMP and cGMP synthesis by both rat and rabbit penile tissues in the present study is entirely consistent with its previously described action as a phosphodiesterase inhibitor (Poch and Kukovetz,
1972). In the current set of experiments, the activity of the intrinsic phosphodiesterases had already been inhibited by the addition of IBMX. In this context, our observations indicate that papaverine is unable to stimulate cAMP or cGMP formation de novo; it presumably therefore exerts its effects by increasing the bioavailability of both cAMP and cGMP via an inhibition of their normal breakdown by phosphodiesterases. A logical progression from the above findings was to investigate the activity of the intrinsic penile phosphodiesterase systems. This was performed by observing the hydrolysis of cyclic nucleotides using $[^3H]$-radiolabelled ligands and is the subject matter of the following chapter. Clearly it is important to understand the mode of action of agents such as papaverine and PGE$_1$ as future drug development requires a clear understanding of their mode of action as well as the underlying pathophysiology of ED.
CHAPTER 5

STUDIES ON PHOSPHODIESTERASE AND 5'NUCLEOTIDASE ACTIVITIES IN CONTROL AND DIABETIC RATS

5.1 Introduction

5.1a The phosphodiesterases

Apart from receptor activity (a product of both the affinity and number of receptors) and the strength of agonist signals (which were investigated in the previous chapter) as determinants of cAMP and cGMP levels; the intracellular levels of these nucleotides are also controlled by powerful endogenous enzymes known as the phosphodiesterases (PDEs). Thus, the intracellular levels of both cAMP and cGMP are not only dependent on their synthesis, but also on their degradation which is controlled by specific PDEs which hydrolyse the cyclic nucleotides to their respective non-cyclic forms. The PDEs in fact constitute a complex family of isoenzymes and they were recently the subject of an authoritative review (Bentley and Beavo, 1992).

According to the currently available evidence some six different families of PDE isoenzymes are present in most mammalian species. Each of these families is encoded by a different gene or by a series of very closely related genes. It seems inevitable that additional isozymes will be characterised in the future. Given the
previously discussed variety of cyclases it is becoming increasingly clear that the potential number of different combinations of cyclases and phosphodiesterases that can be expressed by a given cell are enormous, this in turn provides the cell with a large number of possible variations for the control of steady state levels of cAMP and cGMP. All mammalian PDEs for which sequence data is available have a highly conserved region of some 270 amino acids, this region is thought to contain the catalytic domain (Charbonneau et al, 1986). Some of the PDEs also have a second domain and studies suggest that this contains a specific cGMP binding site (Stroop et al, 1989; Stroop and Beavo, 1991). In the recent literature much primary sequence data has been reported for several isoenzymes (Novack et al, 1991; Charbonneau et al, 1991; Trong et al, 1990; Bentley et al, 1992; Sonnenburg et al, 1991; Taira et al 1991), this information will allow for more definitive studies to be performed with regard to the structure, function, regulation and localisation of these isoenzymes.

At present our knowledge of the cellular distribution of most of the PDEs is limited. In most cases the major tissues in which a family is predominant have been identified. However, less is known about which cell types contain which particular isoenzyme, a situation which will become clearer as isoenzyme specific probes become available.

The precise regulation of the activity of the PDEs is an area of intense research endeavour. It has been established for some time that calmodulin is important in this respect. More recently other regulatory mechanisms are being described, for example it has been shown that there is a transcriptional regulation of cAMP-specific PDEs in Sertoli cells (Swinnen et al, 1991a; Swinnen et al, 1991b). In another study it was shown that the PDE activity of adipocytes is increased by a phosphorylation mechanism in response to either insulin or cAMP itself (Smith et al, 1991). In both cases the activity increase is produced by the phosphorylation of serine residues. In the case of insulin this implies that one or more serine kinases intervene between the tyrosine kinase activity of the insulin receptor and the activation of the PDE.
Similarly, it has been shown that in cells from the adrenal medulla cGMP is itself a regulator of PDE activity (Whalin et al, 1991).

Alterations of PDE activity have been implicated in some disease states. For example, it has been shown that a genetic defect of photoreceptor PDE in the rd mouse can cause retinal degeneration (Farber et al, 1991; Pittler and Baehr, 1991). In man an excess of cAMP specific PDE activity has been found in one of the forms of diabetes insipidus (Homma et al, 1991). A number of PDE inhibitors such as enoximone and milrinone have been recently used in clinical trials (Lee et al, 1991; O'Connell et al, 1991), whereas aminophylline (a theophylline derivative) has long been established in the clinician's therapeutic armamentarium for the treatment of asthma.

It is in this context that we decided to investigate PDE activity in the penis and aorta of our experimental model. There are few reports on changes of PDE activity in DM, but it is well established that DM results in complex alterations in cyclic nucleotide metabolism (Trovati et al, 1995; Weisbrod et al, 1993). It is therefore possible that the previously observed altered intracellular levels of cyclic nucleotides in response to agonists in the penis of diabetic rats are due, at least in part, to alterations in PDE activity. In order to investigate the possibility that alterations of these key enzymes contribute to ED in DM, we investigated the PDE activity in the penis of control and diabetic rats. PDE activity was assessed by following the hydrolysis of $[^3]H$-cAMP and $[^3]H$-cGMP, and separation of their metabolites by thin layer chromatography. We also studied the aorta to assess any changes which might be present in the extra-penile vasculature subserving erection.
5.1b 5' Nucleotidase

Adenosine and ATP are important substances which have yet to have their roles in the physiology of erection fully appreciated. Firstly, not only do they have important relaxant effects on penile smooth muscle cells, but in common with PGs and NO they also have a protective role in that they prevent the development of atherosclerosis. In this respect we investigated the activity of 5'nucleotidase (5'NT) an enzyme which is central to the metabolism of ATP, AMP and adenosine. Given the above, alterations in the activity of 5'NT in DM may therefore have important consequences with respect to ED and atherosclerosis.

The effects of ATP and adenosine have been investigated in both in vitro and in vivo preparations. Both ATP and adenosine have the ability to produce contraction of the retractor penis muscle of the bull (Klinge and Sjostrand, 1974). However, ATP (and other purines) have been shown to relax rabbit corpus cavernosum strips (Broderick et al, 1991; Tong et al, 1992; Wu et al, 1993). It was found that this relaxation in response to ATP was endothelium-independent (Broderick et al, 1991; Tong et al, 1992). It has been suggested that ATP and other 'purinergic mechanisms' were a component of NANC mediation of penile erection.

This contention seems unlikely in the face of the evidence that none of the purines which have been studied either facilitated or inhibited the response of corporeal smooth muscle to electrical field stimulation (Wu et al, 1993). However, these findings do not preclude a role in neuromodulation. The intracavernosal administration of ATP in a canine model produces both tumescence and erection (Takahashi et al, 1992b). Adenosine produces a full erection when given via the same route (Takahashi et al, 1992c). The physiological role of both ATP and adenosine remain to be fully established.
As alluded to previously, apart from PGs and NO, another important means by which blood vessels are protected against atherogenesis is by the presence of the ectonucleotidases (Fig. 5.1). As their name suggests, the ectonucleotidases are a family of enzymes found on the plasma membrane surface of endothelial and vascular smooth muscle cells. The nucleotides (ATP and ADP) which may be released from platelets and leucocytes are rapidly hydrolysed to AMP. Since both ATP and AMP are known to be pro-atherogenic, their rapid hydrolysis can be considered to be an important protective mechanism for vascular and penile tissues.

Of the family of ectonucleotidases, 5'NT may be considered as being the most important. 5'NT acts to catalyse the conversion of AMP to adenosine. Adenosine is an important physiological entity with respect to vascular tissue, including the penis, as discussed above it is both a vasodilator and relaxant of corporeal smooth muscle as well as a potent inhibitor of VSMC proliferation. There is evidence that adenosine generation may be impaired in atherosclerosis as well as in conditions that predispose to atherosclerosis. Obviously, the study of this enzyme is important as defects in its activity in DM may be responsible for reduced vasodilatation, less relaxation of the corporeal smooth musculature and hence contribute to ED.

5.2 Results

5.2a Phosphodiesterase activity

Body weights (median [range]) of the animal groups at the time of sacrifice were: controls (n=7), 475g (449-516); DM (n=10), 239g (220-280). At sacrifice, the blood glucose concentrations were (median [range]): controls, (n=7): 8.4mmol/l (6.8-10.3); DM, (n=10): 30.3mmol/l (28.2-32.7). In both penile and aortic tissue from diabetic rats the hydrolysis of cAMP and cGMP was significantly reduced compared
ECTONUCLEOTIDASES

PMN

platelet

AMP

ADP

ATP

P42

P21

Platelet

Platelet

P21

5' nucleotidase

endothelium

MEDIA

INHIBITION OF VSMC
PROLIFERATION
VASODILATION

Fig. 5.1 Role of ectonucleotidases in the interaction between leucocytes, platelets and the endothelium. As a result of an endothelial lesion platelets will adhere and be stimulated to release ATP and ADP. These nucleotides are rapidly hydrolysed as shown in the figure above. Ultimately, AMP will be metabolised to adenosine which is both a vasodilator and inhibitor of VSMC proliferation. Thus, as with PGI$_2$ and NO, adenosine plays a dual role in erectile dysfunction: 1) it elicits acute vasodilatation and erection, and 2) it may play a role in preventing atherogenesis (particularly in arteries supplying the penis) and indirectly influence erection.
to age-matched controls (Figs. 5.2, 5.3, 5.4, 5.5). Significance was determined with the student's t test and $P$ values less than 0.05 were taken to be significant.

5.2b 5'nucleotidase activity

Over a time course of 60 min, the conversion of $[^3]$H-AMP to $[^3]$H-adenosine was significantly greater in the penile tissue (Fig. 5.6) and aortic tissue (Fig. 5.7) of diabetic rats when compared to controls.

5.3 Discussion

The present study demonstrates a statistically significant decrease in both cAMP-PDE and cGMP-PDE activity in the penis of the STZ diabetic rat when compared to controls. Similarly, the activity of the PDEs of both cyclic nucleotides was also reduced in the aortae of diabetic rats when compared to controls, but this was not as marked as in the penis. Since a reduction of PDE activity, in situ, would result in an increased intracellular cyclic nucleotide bioavailability (and as such enhanced penile smooth muscle relaxation), these data indicate that a diminished activity of these enzymes is not related aetiologically to the ED associated with DM. Furthermore, this may again constitute an adaptive response designed to counteract the deleterious effect of DM on penile smooth muscle relaxation and vasodilator mechanisms, for example muscarinic, PGs and NO (Abiru et al, 1990; Kamata et al, 1992; Giugliano et al, 1994; Johnstone et al, 1993). In this context, we previously demonstrated that there was increased cAMP and cGMP synthesis in response to PGE$_1$ and NaNP in the penis and aorta of STZ diabetic rats (Miller et al, 1994a). It should be noted, however, that in this latter study, PDE activity was already inhibited by the non-specific PDE inhibitor, IBMX. Thus, the observed elevation of cyclic

179
Fig. 5.2 Hydrolysis of adenosine cyclic monophosphate by the penis of the diabetic rat (♦, n=10) and age-matched controls (●, n=7). Each point = mean ± SEM for six duplicate assays, # p<0.01
Fig. 5.3 Hydrolysis of adenosine cyclic monophosphate by the aorta of the diabetic rat (♦, n=10) and age-matched controls (○, n=7). Each point = mean ± SEM for six duplicate assays, # p<0.01
Fig. 5.4 Hydrolysis of guanosine cyclic monophosphate by the penis of the diabetic rat (●, n=10) and age-matched controls (◆, n=7). Each point = mean ± SEM for six duplicate assays, # p<0.01
Fig. 5.5 Hydrolysis of guanosine cyclic monophosphate by the aorta of the diabetic rat (♦, n=10) and age-matched controls (●, n=7). Each point = mean ± SEM for six duplicate assays, #p<0.01
Fig. 5.6 Percentage conversion of $[^3$H]-AMP to $[^3$H]-adenosine by the penis of the diabetic rat (●, n=10) and age-matched controls (○, n=7) over a time course of 60 min. Each point = mean ± SEM for six duplicate assays, # p<0.001
Fig. 5.7 Percentage conversion of [³H]-AMP to [³H]-adenosine by the aorta of the diabetic rat (●, n=10) and age-matched controls (○, n=7) over a time course of 60 min. Each point = mean ± SEM for six duplicate assays, # p < 0.001
nucleotides was not ascribed to a diminished PDE activity, but rather to an enhanced adenylate and guanylate cyclase activity (Miller et al, 1994a). In a previous study, described in chapter 4, we demonstrated that the autoradiographic binding of NOS was significantly enhanced in the penis of the diabetic rat (Thompson et al, 1994). Taken together, therefore, these data consolidate the concept that an enhanced cyclic nucleotide synthesis via a reduction of PDE activity, increased NOS and increased nucleotide cyclase activity constitutes an adaptive response to counteract the deleterious effects of DM on vasodilator receptors. The raised intracellular levels of cyclic nucleotides may also result in end-organ refractoriness. Thus, smooth muscle relaxation may not occur at intracellular levels of cAMP and/or cGMP which induce this response in healthy tissue.

We are unaware of any reports on alterations of cAMP or cGMP PDE activity in vascular or penile tissues in diabetes. It is also notable that PDEs are calmodulin controlled for full activity (Thompson, 1991). Since several studies have demonstrated that calmodulin activity is diminished in DM, this may explain why we observed a reduced PDE activity. Other intracellular systems disrupted by DM include protein kinases, which in turn are known to control PDE activity. Clearly, further work is required to clarify the significance and mechanisms underlying the present alterations of PDEs.

As mentioned in the introduction it should be remembered that PDEs exist in several forms: both particulate and cytosolic with several subclasses of the enzyme (Thompson, 1991). In the present study, we did not isolate or separate these various forms of PDEs but examined the overall hydrolysis of the cyclic nucleotides by diabetic tissues. It is entirely possible, however, that DM may have differential effects on the different types of PDEs and warrants further investigation by the use of specific PDE subclass inhibitors.
In conclusion, the present study clearly demonstrates that DM in the rat causes a marked overall reduction of both cAMP and cGMP PDE activity in the penis and aorta. The consequences of this, in vivo, would be to elevate the bioavailability of cAMP and cGMP, both of which are associated with smooth muscle relaxation (and therefore erection). Thus, PDEs do not appear to be aetiologically related to ED. On the contrary, the decreased activity of these enzymes may constitute an adaptive response designed to counter the deleterious effect of DM on other vasodilator systems (Miller et al, 1994a). The PDEs constitute a complex homeostatic system and are affected by a number of factors which include calmodulin, cAMP, cGMP as well as insulin. The role of insulin in the metabolism of the cyclic nucleotides forms a central part of the experiments which are described in the next chapter.

In the present study it was found that 5'NT activity (as assessed by adenosine generation from ADP) is markedly enhanced in the penis and aorta of diabetic rats. This was surprising since adenosine is a vasodilator and as such might be expected to be diminished in DM. However, as has been demonstrated previously, adenylate and guanylate cyclase activity are enhanced in the penile tissues of the diabetic rat. Furthermore, it was found that PDE activity was diminished in the penis and aorta of the diabetic rat. In accordance with the previous lines of argument it is possible to speculate that the increased 5'NT activity in the diabetic rat penis constitutes a further adaptive event designed to counter the negative effects of DM on other vasodilator and smooth muscle relaxation systems.

The adaptation of vascular tissue to disease states is not unusual. For example, in hypertension, blood vessels (in particular arteries) adapt to conditions of increased arterial pressure by hypertrophy and hyperplasia and also by alterations of the intracellular biochemical mechanisms that control vasoactivity (Mehta et al, 1997). It has recently been demonstrated that in experimental hepatic portal hypertension in rats the peripheral arterial tissues adapt to the marked alterations in blood pressure through a down-regulation of protein kinase C activity and calcium mobilisation
(Jeremy et al, 1994a). With regards to DM, it has been demonstrated that PG synthesis can either be diminished, enhanced, or remain unaltered in vascular tissues, depending on the type of pathological environment to which these tissues are subjected (Jeremy et al, 1986c; Jeremy et al, 1992). It has also been demonstrated that protein kinase C and calcium mobilisation is enhanced in the arteries of diabetic rats (Jeremy et al, 1993b). Thus, although DM exerts marked deleterious effects on membrane functions (including vasodilator receptor integrity) which cannot be counteracted, the results of the 5'NT study consolidate the proposition that penile tissues adapt to the diabetic condition through modulation of vasodilator systems which control erection.

In a broader context, the increased activity of 5'NT in DM may have implications in the pathoetiiology of atherosclerosis. It was found that adenosine generation was enhanced in the aortae of diabetic rats; DM is associated with an increased incidence of atherosclerosis, one of the important determinants of ED in DM. Adenosine itself is a potent inhibitor of VSMC proliferation (the pathognomic lesion of atherosclerosis), platelet and neutrophil activity as well as the release of some of their mitogenic substances (Mehta et al, 1997). Therefore it would appear that enhanced 5'NT activity may not be associated with diabetic related atherosclerosis but might be a protective phenomenon seen in DM. Indeed, this fact could possibly be exploited in the treatment of atherosclerosis. Whether 5'NT activity is altered in atherosclerosis per se requires further study and the study of hyperlipidaemic Watanabe rabbits may represent a fruitful experimental paradigm in this respect.
CHAPTER 6

RISK FACTORS AND ERECTILE DYSFUNCTION:
THEIR ACUTE EFFECTS ON CYCLIC NUCLEOTIDES;
FIBRINOGEN AND ERECTILE DYSFUNCTION;
DIABETES MELLITUS AND PENILE ULTRASTRUCTURE

6.1 Introduction

6.1a The acute effects of risk factors on cyclic nucleotides

As previously discussed, in contrast to earlier views that ED is predominantly a condition of psychogenic origin, it has become clear that a large proportion of cases are in fact due to organic causes. In particular, it has been established that arterial disease, (viz. atherosclerosis) resulting in arterial insufficiency, is one of the major causes of organic ED (Virag et al, 1985; Feldman et al, 1994). The major risk factors for both atherosclerosis and ED are diabetes mellitus, smoking, hyperlipidaemia and hypertension (Virag et al, 1985; Shabsigh et al, 1991; Feldman et al, 1994).

Diabetes mellitus is a powerful risk factor for the development of ED in both humans and in animal models (McCulloch et al, 1980; Feldman et al, 1994; Hassan et al, 1993). Diabetic men have an increased incidence of atherosclerosis and neuropathy which both contribute to the pathophysiology of ED. The reasons for an increased incidence of atherosclerosis in DM were recently reviewed (Getz, 1995). It is thought
that small vessel disease is the principle mechanism responsible for the development of neuropathy and microscopically the vasa nervorum of these patients are occluded (Dyck et al, 1987). Furthermore, there may also be a contribution from the metabolic sequelae of hyperglycaemia. Thus, it is thought that in DM the peripheral nerves take up glucose from the blood which is then converted to sorbitol by aldose reductase. Sorbitol is converted to fructose by sorbitol dehydrogenase, the fructose which thus accumulates leads to an inhibition of sodium-dependent myoinositol uptake, decreased intraneuronal myoinositol and consequently reduced membrane phospholipid. This results in a depressed $\text{Na}^+/\text{K}^+\text{ATPase}$ activity which in turn leads to altered nerve conduction velocities. Much experimental data supports the key role of aldose reductase in the development of diabetic neuropathy. However, it is only recently that increased levels of this enzyme were associated with the presence of diabetic complications (Nishimura et al, 1994). In man, the administration of aldose reductase inhibitors has led to slight improvements in conduction velocities. Interestingly, in the STZ diabetic rat model there was no benefit of aldose reductase inhibition on either vasoreactivity or prostacyclin release, suggesting that these observed pathological events may be unrelated to an excessive polyol pathway flux (Stevens et al, 1993). Furthermore, the pathophysiological effects of tissue protein glycosylation (advanced glycosylation products) are poorly understood but may contribute to the neuronal malfunction in diabetes mellitus and inhibit NO activity (Getz, 1995; Kiff et al, 1991).

The diabetic neuropathic process affects both the autonomic and peripheral nervous systems, however, it seems to preferentially affect the long parasympathetic fibres first (such as those responsible for the mediation of penile erection). We have previously discussed the changes observed in penile VIPergic innervation in both experimental and human diabetes (Lincoln et al, 1987). Furthermore, dysfunctional cholinergic and adrenergic innervation has also been described in DM (Lincoln et al, 1987; Blanco et al, 1990). In addition, it should be borne in mind that DM may also result in a secondary hyperlipidaemia as well as causing abnormalities of testosterone metabolism (Kissebah, 1992; Sosenko et al, 1980; Barrett-Connor et al, 1982; Murray
et al, 1987). Thus, there are a considerable number of abnormalities which can interplay and contribute to the pathoetiolo gy of diabetic ED which is clearly multifactorial.

There have been a number of studies which have attempted to determine the relative contributions of angiopathy and neuropathy to diabetic ED. The results of these investigations are somewhat at variance with each other. Some workers have stressed the importance and predominance of the neurological factor (Bemelmans et al, 1994; Blanco et al, 1990), while others have stressed the importance of the angiopathic processes (Benvenuti et al, 1993; Wang et al, 1993; Yamaguchi and Kumamoto, 1994). It seems that ED affecting the younger diabetic is usually due to a neuropathy, in contrast to older diabetics who may have a predominant vascular cause (Yamaguchi and Kumamoto, 1994). It is important to remember that there may also be a considerable psychogenic component, not least as a result of the chronic nature of their disease (Veves et al, 1995; Benvenuti et al, 1993). Furthermore, DM also has deleterious effects on endothelial function as well as corporeal smooth muscle relaxation: it has been elegantly demonstrated that DM impairs neurogenic and endothelium-dependent relaxation of rabbit and human penile smooth muscle (Azadzoi and Saenz de Tejada, 1992; Saenz de Tejada et al, 1989b). DM was shown to decrease PGI\textsubscript{2} synthesis in STZ rats (Jeremy et al, 1985b). More recently it has been shown that the density of endothelin binding to rat corpus cavernosum is also significantly increased in STZ-induced DM; clearly this may contribute to ED and is further evidence of the global abnormalities of endothelial dysfunction seen in DM (Bell et al, 1995).

The central role of hyperlipidaemia as well as the altered lipid metabolism seen in DM in the pathogenesis of vascular disease (viz. atherosclerosis) and its deleterious effects on endothelial cell function are well-established for tissues other than the penis. It has been recognised that hyperlipidaemia is a major risk factor for ED (Virag et al, 1985; Shabsigh et al, 1991), as shown in both epidemiological and more recently in a prospective study (Wei et al, 1994). Although it has been previously shown that experimental hypercholesterolaemia impaired endothelium-dependent relaxation of
rabbit corpus cavernosum as well as producing penile smooth cell degeneration (Azadzoi and Saenz de Tejada, 1991; Junemann et al, 1991), it is only relatively recently that a combined structural and functional study has confirmed that hypercholesterolaemia results in penile smooth muscle and endothelial dysfunction in an animal model of penile atheroma (Azadzoi and Saenz de Tejada, 1991). In the context of recent observations of changes in coronary artery reactivity after lipid modification in men with moderately raised lipid levels (Leung et al, 1993), it is interesting to speculate that a similar process may occur in the penis and penile vasculature and furthermore that an altered local synthesis of prostanoids may be involved in this process (Miller and Morgan, 1994).

The chronic effects of atheroma are to produce fixed lesions or plaques which produce a stenosis. Following this, plaque rupture and the subsequent complete occlusion of vessels can present as a variety of clinical manifestations depending on the specific vascular bed that is affected, thus myocardial infarction, cerebrovascular accident, and peripheral vascular disease resulting in the acutely ischaemic limb are all examples of such an event. The clinical presentation of occlusion of the penile vascular bed is usually a less dramatic and not life threatening event. Notwithstanding the effects of established neuropathy and atherosclerotic plaques, of more interest recently has been the concept of vasoreactivity which refers to the ability of healthy arteries to react in terms of dilatation and contraction in response to a suitable stimulus. Clearly, an artery which has been affected by plaque deposition will not be able to cope with the increased demand expected of it in certain situations. Interestingly, it has been shown that altered lipid levels prior to the overt structural changes of plaque formation result in themselves in changes of vessel reactivity, this important observation and it’s potential manipulation by altered lipid levels has important clinical implications (Leung et al, 1993). It is in this context that we investigated the effects of a number of risk factors such as glucose, insulin, LDL and oxLDL in an acute phase situation to see if these might impinge in a deleterious manner on some of the intracellular mechanisms that govern corporeal smooth muscle relaxation.
6.1b Fibrinogen and smoking as risk factors for erectile dysfunction

As discussed above, the role of vascular risk factors such as DM, hyperlipidaemia, hypertension and smoking in the pathogenesis of organic ED is clearly established (Virag et al, 1985; Shabsigh et al, 1991; Wei et al, 1994). Over the last 30 years a number of epidemiological and prospective clinical studies have shown that plasma fibrinogen concentration is another important and independent risk factor for cardiovascular disease and cardiovascular events. The Northwick Park Heart Study first demonstrated that fibrinogen concentration was an independent risk factor for myocardial infarction (Meade et al, 1986), this was followed by further prospective studies which confirmed the relationship between fibrinogen and cardiovascular events (Ernst, 1991). It has therefore been argued that plasma fibrinogen measurement should be part of a cardiovascular risk factor profile (Ernst, 1991).

That fibrinogen is raised after cardiovascular events makes the interpretation of the data a little difficult as it is also an acute phase protein; therefore it will be elevated in the presence of inflammation or tissue necrosis (Crabtree, 1987). However, other studies have shown that fibrinogen levels are also raised before such events occur (Qizilbash et al, 1991; Coull et al, 1991). Some have postulated that it is the presence of inflammation within the atherosclerotic plaque itself that causes the elevation of fibrinogen concentration (Ernst, 1993). Nevertheless, the explanation may also partly lie in the fact that fibrinogen concentration is correlated with nearly all the other cardiovascular risk factors: age, hypertension, hyperlipidaemia, smoking, DM, obesity, stress and the level of physical activity (Ernst, 1990). It should be further remembered that fibrinogen also has chemoattractant and mitogenic properties. We are unaware of any previous investigations to define its existence as a risk factor for ED. In this context a pilot study was undertaken to ascertain whether it may similarly be a risk factor for the development of ED. We present the results of a study in which we compared the serum/plasma levels of lipids and fibrinogen in a population of men with ED and a control population.
6.1c The effects of diabetes mellitus on penile ultrastructure

The final studies performed in this series of experiments were the investigations to document the effects of STZ diabetes mellitus on penile ultrastructure in the rat corpus cavernosum. More specifically, we attempted to identify if damage or other changes were apparent in the cavernosal smooth muscle or the endothelium. Italiano and co-workers have demonstrated that STZ diabetes has both ultrastructural and functional consequences with respect to the neurological integrity of the dorsal nerve of the rat penis (Italiano et al, 1993a; Italiano et al, 1993b). In this respect it has been demonstrated recently that in an animal model of hypercholesterolaemia there is both functional impairment and structural damage to the cavernosal tissue in the rabbit (Kim et al, 1994), we wanted to study the STZ rat in a similar manner.

6.2 Results

6.2a Acute risk factor studies

Varying the glucose concentration was demonstrated to have a biphasic effect on cAMP and cGMP concentrations in non-diabetic rat penile segments. Glucose was found to be maximally stimulating at 12.5 mM and appeared to be inhibitory at concentrations of 25 and 50 mM (Figs. 6.1 and 6.2). In a similar manner, it was shown that glucose also had a biphasic effect on PGE1-stimulated cAMP synthesis and NaNP-stimulated cGMP synthesis (Figs. 6.1 and 6.2).

Varying concentrations of insulin were found to be without effect on the synthesis and subsequent concentration of both cAMP and cGMP from the non-diabetic rat corpus cavernosum when stimulated by 10μM PGE1 and 10μM NaNP respectively (Table 6.1).
Fig. 6.1 Effect of glucose on cAMP synthesis by the penises of non-diabetic rats.

Each point = mean ± SEM, n=6. (●) 10μM PGE₁-stimulated; (▲) without PGE₁.
Fig. 6.2 Effect of glucose on cGMP synthesis by the penises of non-diabetic rats.
Each point = mean ± SEM, n=6. (●) 10μM NaNP-stimulated; (▲) without NaNP.
Table 6.1 Effect of insulin on cAMP and cGMP synthesis by the corpus cavernosum of the rat (non-diabetic), when stimulated by 10μM PGE₁ and 10μM NaNP respectively.

<table>
<thead>
<tr>
<th>Insulin concentration (iU/ml)</th>
<th>cAMP (fmol cyclic nucleotide/mg tissue/min)</th>
<th>cGMP (fmol cyclic nucleotide/mg tissue/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28 ± 2</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>0.0001</td>
<td>21 ± 2</td>
<td>3.8 ± 0.9</td>
</tr>
<tr>
<td>0.001</td>
<td>29 ± 3</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>0.01</td>
<td>33 ± 3</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>0.1</td>
<td>35 ± 3</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>1</td>
<td>27 ± 4</td>
<td>4.6 ± 0.4</td>
</tr>
</tbody>
</table>
Both LDL and oxLDL were demonstrated to inhibit the synthesis and release of cAMP and cGMP from the corpus cavernosum of the non-diabetic rat when stimulated by 10μM PGE₁ and 10μM NaNP respectively (Fig. 6.3). However, oxLDL was found to be a more potent inhibitor than LDL in this preparation.

6.2b Hyperlipidaemia, smoking and fibrinogen in erectile dysfunction

Total cholesterol (TC) in non-smokers with ED (median 6.2mmol/l; range 4.5-8.0mmol/l; n=33) was significantly (p<0.04) increased in comparison to control non-smokers (median 5.1mmol/l; range 3.6-8.1mmol/l; n=18) confirming TC as a risk factor for ED. TC was also significantly (p<0.02) increased in non-smokers with ED compared to smokers with ED (median 5.6mmol/l; range 3.9-6.9mmol/l; n=24). No significant difference in TC between smokers with ED and control non-smokers was found. No significant differences in LDL and triglycerides (TG) were found between all the groups.

High density lipoprotein concentration was significantly (p<0.04) lower in smokers with ED (median 1.0mmol/l; range 0.8-2.2mmol/l) compared with non-smokers with ED (median 1.4mmol/l; range 0.9-2.3mmol/l). The HDL in the ED groups did not differ significantly from that in non-smoking controls (median 1.3mmol/l; range 0.9-2.5mmol/l).

Fibrinogen was significantly higher (p<0.02) in the smokers with ED (median 3.55g/l; range 2.73-5.70g/l) compared to non-smokers with ED (median 3.26g/l; range 2.07-4.33g/l) and (p<0.02) compared to control non-smokers (median 3.05g/l; range 1.20-4.32g/l). No significant difference in fibrinogen was seen in the non-smoking ED and non-smoking control groups (these results are summarised in Table 6.2).
Fig. 6.3 Effect of LDL (●) and oxLDL (▲) on (A) cAMP and (B) cGMP synthesis by the rat corpus cavernosum when stimulated by 10μPGE₁ and 10μM NaNP respectively. Each point = mean ± SEM, for n=6 assays.
Table 6.2 The effects of cigarette smoking on vascular risk factors in men with erectile dysfunction

<table>
<thead>
<tr>
<th>Serum/plasma parameters and age, median (range)</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erectile dysfunction (non-smokers) n=33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erectile dysfunction (smokers) n=24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (non-smokers) n=18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.45</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(0.52 - 4.72)</td>
<td>(0.69 - 3.51)</td>
<td>(0.47 - 6.13)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.2</td>
<td>5.5</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>(4.5 - 8.0)</td>
<td>(3.9 - 6.9)</td>
<td>(3.6 - 8.1)</td>
</tr>
<tr>
<td>High density lipoprotein (mmol/l)</td>
<td>1.4</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>(0.9 - 2.3)</td>
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<td>(2.7 - 5.1)</td>
<td>(2.2 - 5.1)</td>
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<td>3.05</td>
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<td>(2.73 - 5.70)</td>
<td>(1.20 - 4.32)</td>
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<td>(42 - 69)</td>
<td>(42 - 67)</td>
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All results were analysed by Mann Whitney test. Tgs: all non-significant (NS). TC: A vs. B p=0.01; B vs. C=NS; A vs. C p<0.04. HDL: all NS. Age: all NS. Fibrinogen: A vs. B p<0.02; A vs. C=NS; B vs. C p<0.02
6.2c The effects of diabetes mellitus on penile ultrastructure

The transmission electron micrographs of control and diabetic rat corpus cavernosum showed that there was evidence of damage to the endothelium (Plates 6.1, 6.2, 6.3 and 6.4). However, there was no discernible evidence of ultrastructural damage in the scanning electron micrographs in the diabetic sample (Plates 6.5 and 6.6).

6.3 Discussion

The present studies demonstrated that glucose has a biphasic effect on both the basal and stimulated levels of cAMP and cGMP in the non-diabetic rat penis. Normal human glucose concentrations are in the range of 2.0-6.5mmol/l, however, in poorly controlled DM these levels can reach up to 20-30mmol/l (Keen, 1992). Our data indicate that in an acute preparation hyperglycaemia will reduce cyclic nucleotide levels. Given that cAMP and cGMP are important second messengers in the mediation of normal penile erection, the ED in DM may in some part at least be due to an inhibition or decreased activity of these pathways in the presence of transiently elevated glucose levels. In the context of the STZ rat model, the level of hyperglycaemia is comparable to that seen in the untreated human situation (Thompson and Mikhailidis, 1992). Similarly, in the control animals the blood glucose level is close to that in non-diabetic humans.

We have previously demonstrated that in animals with DM of two months duration there is enhanced cyclic nucleotide generation in penile tissues (Miller et al, 1994a). The mechanism implicated was an upregulation of the cyclase enzyme activity perhaps mediated by a G-protein. Furthermore, a decreased PDE activity may contribute to the observed elevations of cAMP and cGMP (Miller et al, 1994b; Miller et al, 1996). It was argued that these changes may be seen as adaptive events to counter the deleterious effects of DM on vasodilatation. Thus, since glucose clearly suppressed
Plate 6.1 Transmission electron micrograph of the control rat corpus cavernosum (x3400). The erectile tissue of the penis consists of broad vascular lacunae or cavernous sinuses supported by trabeculae of fibro-elastic connective tissue containing smooth muscle fibres. The lacunae are lined by vascular endothelium. (CT connective tissue; E endothelium; S smooth muscle).
Plate 6.2 Transmission electron micrograph of the control rat corpus cavernosum (x25000). (C collagen; E endothelium; P pinocytic vesicle).
Plate 6.3 Transmission electron micrograph of the diabetic rat corpus cavernosum (x7100). (E endothelium; S smooth muscle). Some evidence of damage to the endothelium is seen in this specimen (D).
**Plate 6.4** Transmission electron micrograph of the diabetic rat corpus cavernosum (x11000). (E endothelium). Some damage to the endothelium is seen in this view - endothelial cells are dying and sloughing away.
Plate 6.5 Scanning electron micrograph of the control rat corpus cavernosum. Condensed fibro-elastic tissue invests the cavernous bodies, being thickest around the corpora cavernosa and thinner around the corpus spongiosum. (E endothelium; S smooth muscle).
Plate 6.6 Scanning electron micrograph of the diabetic rat corpus cavernosum (Bar = 100 microns). (E endothelium; S smooth muscle). No evidence of ultrastructural damage can be seen.
cAMP and cGMP at concentrations greater than 12.5mM it is possible that the adaptation seen is actually a response to the chronic effects of hyperglycaemia. It would be interesting to compare these results with those of penile tissues of two month diabetic rats which had been treated with insulin and therefore rendered normoglycaemic. In particular, one might expect cyclase activity to be normal if the chronic hyperglycaemia was the mechanism responsible for the observed upregulation.

In contrast, insulin alone showed no effect on either cAMP or cGMP in the penis of the non-diabetic rat. Although human DM is due a relative insulin lack, it is treated by insulin injection and therefore patients will have at least transient periods of hyperinsulinaemia. However, from our previous experimental results it seems unlikely that insulin is a factor in the changes observed in nucleotide synthesis in the DM rat model as these animals are rendered hypoinsulinaemic. Nevertheless, it would be instructive to extend the scope of this particular study.

That there was no observed change in the concentration of either cAMP or cGMP in response to insulin in our acute series of experiments was perhaps a little surprising as it is known that one of the actions of insulin is to lower cAMP concentrations (Butcher et al, 1968). The mechanism in which this is achieved is unknown, but it may be via an activation of a membrane-associated phosphodiesterase (Degerman et al, 1987). If this result was also observed in the tissues of our long-term diabetic rats, then this would perhaps fit in with the general picture of global receptor dysfunction that is observed in DM (Blanco et al, 1990; Lincoln et al, 1987). These results and the underlying mechanisms merit further investigation, especially in the light of our findings with respect to decreased phosphodiesterase activity in untreated experimental DM (Miller et al, 1996), one might conjecture that the oscillations of hyperinsulinaemia in treated human DM could have profound effects on the metabolism of the cyclic nucleotides with acute problems of penile smooth muscle relaxation as a result.
It was found that LDL and oxLDL inhibit cAMP and cGMP synthesis by the penile tissues of the non-diabetic rat in vitro (Fig. 6.3). DM is associated with increased levels of oxLDL (Bowie et al, 1993; Regnstrom et al, 1992), which in turn has been implicated in both atherogenesis and in ED (Ross, 1993; Virag et al, 1985; Steinberg et al, 1989). Once formed by the endothelium, oxLDL may directly injure the endothelial cells and plays an important role in the increased adherence and migration of monocytes and T-lymphocytes into the subendothelial space (Cathcart et al, 1985; Steinbrecher et al, 1990; Rosenfeld et al, 1990). It can induce the formation of adhesive cell-surface glycoproteins, such as VCAM-1 (athero-ELAM) by the endothelium. Once the monocytes and lymphocytes enter the intima of the artery, oxLDL from the endothelium and other substances associated with atherogenesis may participate in the activation and transformation of monocytes into macrophages (Ross, 1993). Uptake of oxLDL by the macrophages will lead to foam cell formation (Ross, 1993).

Notwithstanding the above, our results indicate that LDL and oxLDL may also exert acute deleterious effects on erectile function via the inhibition of the vasodilatory second messengers cAMP and cGMP. The results also indicate that lipid lowering drugs and anti-oxidants may be useful therapeutic approaches to the prevention or treatment of established ED in patients with hyperlipidaemia. It would therefore be interesting to compare the results that might be obtained in an animal model of hyperlipidaemia.

Smoking may act as a risk factor for ED by reducing HDL and increasing fibrinogen levels. Low HDL was a predictor of ED in the Massachusetts Male Ageing and Cooper Clinic (Dallas) studies (Feldman et al, 1994; Wei et al, 1994), furthermore both elevated fibrinogen and reduced HDL concentrations are known predictors of IHD. The findings of our clinical study suggest that ED may share a similar pathogenesis with other forms of vascular disease.

Our results confirm the findings of previous studies which describe hyperlipidaemia as a risk factor for organic ED. In addition, we have shown that
fibrinogen is significantly raised in ED. These findings support the concept that the penis is essentially a modified vascular bed. For reasons already discussed, smoking is an important confounding variable in our present study and we have continued the study to see if fibrinogen elevation is independent of smoking in a larger group of men with ED. As an acute phase reactant it will be elevated in the presence of tissue necrosis and inflammatory processes because of increased hepatic synthesis, therefore we attempted to exclude these conditions from our study groups. In this respect we are continuing this study with an additional measurement of both the white cell count and the erythrocyte sedimentation rate as an index of underlying active disease processes.

Fibrinogen is present in all vertebrate species so far investigated and its structure and function appear similar in all of them (Blomback, 1994). The fibrinogen molecule is similar in structure to immunoglobulin being a dimeric molecule (Blomback, 1994). Thrombin initiates the polymerisation of fibrinogen by the activation of the molecule. The initially formed fibrin polymers create nuclei for the growth of linear polymers in different directions. A network structure containing water is thus formed and is termed a fibrin 'gel'. A relatively small increase in fibrinogen concentration (0.7-1.0 mg/ml) established the association between cardiovascular disease and fibrinogen (Meade, 1992). This may at first be a little puzzling, but the explanation probably lies in that the rate of activation of fibrinogen by thrombin will increase significantly by small increases in fibrinogen - this in turn leads to a dramatic tightness and rigidity of the fibrin gels (Blomback, 1991). The gels formed under these conditions are potentially more thrombogenic and perhaps less prone to thrombolysis.

It has been shown that fibrinogen damages the circulation by a number of mechanisms. It promotes a hypercoagulable state which favours the deposition of thrombus onto atheromatous plaques (Meade et al, 1980; Meade et al, 1986). It is an important determinant of blood rheology (Ernst, 1990), furthermore it links to platelet receptors which is a precondition for their aggregation (Cook and Ubben, 1990). In addition, there are multiple mechanisms for fibrinogen leading to endothelial damage
and dysfunction. Numerous in vitro studies have shown that fibrinogen, fibrin and fibrinogen breakdown products (FDPs) affect a number of biological functions in endothelial cells, vascular smooth muscle cells and macrophages; some of which might occur in vivo and contribute to plaque growth and development. In particular, FDPs stimulate the production of mitogenic substances from endothelial cells in culture (Loppnow and Libby, 1990; Hansson et al, 1989). It is also interesting to note that fibrinogen and FDPs have been demonstrated to be present within atherosclerotic plaques (Smith, 1994). In this respect, if one considers that the penis is a specialised vascular organ, one could postulate that it would therefore be affected by the same disease processes as any other vascular bed and it should be remembered that atherosclerosis is a multisystem disease. Atherosclerosis has been said to be characterised by VSMC proliferation, a further series of experiments using the technique of thymidine incorporation could be undertaken to establish whether this is a dominant process in erectile dysfunction, or whether it is the replacement of smooth muscle with fibrous connective tissue which is the more important factor (Wespes et al, 1991).

In the light of recent work on the effects of lipid modification on vascular reactivity and the availability of specific fibrinogen lowering agents our results have therapeutic implications in the prevention and treatment of ED. Nevertheless, it should be remembered that fibrinogen is also effectively lowered by general measures and a diet rich in fish oil (Shahar et al, 1993). Before making such recommendations for treatment however it is far from certain whether a single plasma measurement is enough. The main concern is that there are a number of sources of variability for any given fibrinogen determination. Firstly, there are problems with assay standardisation in different laboratories. Fibrinogen is normally assayed using the Clauss assay, however, some laboratories now derive a value from other haematological parameters. Secondly, there is a practical difficulty in that the assay has to be performed on fresh plasma. Consequently, fibrinogen concentration is not at present routinely measured and it has been argued that it shouldn't be in future (Machin and Mackie, 1993). In the light of the
above findings it would be interesting to perform some acute phase experiments as above using fibrinogen to determine whether it also has effects upon cyclic nucleotide generation.

The results of the electron microscopy studies into penile ultrastructure unfortunately failed to demonstrate anything conclusive. While, there was some evidence of ultrastructural damage seen in the diabetic TEM specimens, which may possibly be due to diabetes, this was not supported by any evidence from the SEM specimens. At the very best, some tentative conclusions may be made that experimental DM may cause some ultrastructural changes in the vascular component of the diabetic rat penis. Unfortunately, rather more extensive and systematic studies would need to be undertaken to confirm the validity of these preliminary observations.
CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

The principal objectives of this thesis were to explore and characterise the impact of experimental DM on cyclic AMP and cyclic GMP metabolism using a rat model. These cyclic nucleotides are two of the key mediators of penile erection. Furthermore, a number of related issues were also studied: 1) changes in penile ultrastructure in experimental DM, 2) the acute effects of various risk factors in vitro and 3) a clinical pilot study into some of the cardiovascular risk factors for ED. It was hoped that these investigations would provide insights into the effects of DM on the normal biochemical pathways and might also suggest some alternative sites for possible new drug action. A detailed discussion of each topic has been presented at the end of each chapter, although some more recent observations of relevance are mentioned in the present chapter. Therefore, the discussion in this chapter concerns itself mostly with a brief resume of the principal findings, some general remarks and criticisms as well as suggestions for possible further experiments and future directions for the work which has been presented in this thesis.

The studies performed using an autoradiographic localisation of NOS showed that there was an increased binding for this enzyme in diabetic animals at both two and six months when compared with controls. This was a clear and statistically significant result. Nevertheless, one of the major criticisms that can be made of these studies is that we didn’t undertake further studies to confirm the physiological ability of this upregulated NOS to actually generate NO. In other words, does the
experimental observation of increased NOS staining actually reflect an increased biological NOS activity? This conjecture could have been verified by assessing the activity of NOS to synthesise NO using the $[^3]$H-arginine/citrulline assay method (Burnett et al, 1992; Bush et al, 1992c). Unfortunately, such experiments were not undertaken due to the constraint of time. In addition, it is unclear as to what is happening to NOS substrate levels in either the clinical or experimental situations. It may well be that this is a significant factor in the equation and is certainly an aspect that merits closer scrutiny. The precise mechanism of the observed NOS upregulation is not understood but obviously it may be related to substrate levels in the experimental preparation and warrants further investigation.

The series of experiments into nucleotide cyclase activity showed that penile tissues respond to the diabetic environment with an upregulation in their activity. This was argued to be a homeostatic mechanism which helped to maintain the ability of the penile tissues to relax by increasing the bioavailability of cAMP and cGMP. While this is clearly an advantage for a tissue bed which is designed to relax as part of its normal erectile function, such an enhancement of relaxation pathways may not be so advantageous in terms of homeostasis in other parts of the vascular system whose function is to contract and may therefore be deleterious to the organism as a whole. A further series of experiments using compounds such as forskolin, which directly stimulates adenylate cyclase, would have been useful additional confirmatory experiments in the present context. We were also able to show that PGE$_1$ and VIP act via the generation of cAMP and we were able to confirm that papaverine acts as a non-specific PDE inhibitor in penile tissues and is unable to generate nucleotides de novo, these were novel observations in these tissues.

The investigations performed into the alterations of PDE activity showed that rat penile tissues in DM would appear to be changed in a manner which would act to maintain the bioavailability of the cyclic nucleotides cAMP and cGMP, both of which are crucial for corporeal smooth muscle relaxation. Penile tissues from diabetic rats
had a decreased activity of these important enzymes which would contribute to elevated intracellular levels of the cyclic nucleotides. Furthermore, our investigations of 5'NT activity again demonstrated an adaptation of penile tissues to a diabetic milieu in a manner which would act to maintain the levels of adenosine and result in benefits in maintaining relaxation and preventing atherosclerosis. These results are of particular interest as there is now an orally active type 5 specific cGMP PDE inhibitor, sildenafil, which has proved successful in the treatment of human ED in clinical studies where it seems that there was clear efficacy in an unselected group of patients with ED (Boolell et al, 1996). It will be interesting to see if the effect is similar in patients with known organic aetiology. Nevertheless, sildenafil is likely to represent an important and most significant clinical advance. It should be remembered that there are multiple subclasses of the PDEs, whereas we measured overall PDE activity; the exploration of possibly different alterations in subclass activity warrants further investigation.

In another series of experiments we explored the effects of acute exposure to insulin, glucose, LDL or oxLDL on cAMP and cGMP formation in rat penile tissue. These risk factor experiments demonstrated the acute effects of such metabolic upsets on cyclic nucleotide generation. Our observations may help to explain some of the observed clinical effects of these substances. In a more clinical setting we were able to show that fibrinogen may be another important factor in the cardiovascular risk profile for ED. Obviously, a number of errors are possible in such a small study, however the results are sufficiently promising to warrant an extension of this work in a larger series. It would also have been interesting to perform the acute risk factor experiments using fibrinogen to explore its effects on cyclic nucleotide metabolism. A theme that runs through much of this thesis is that the penis may be considered as being a specialised vascular bed. It is nowadays possible to address the primary prevention of ischaemic heart disease, in this context one might begin to speculate about similar preventive strategies for the prevention of human ED. A number of measures may thus be suggested which would prevent the development of
atherosclerosis and contribute to the maintenance of normal corporeal smooth muscle function. Thus, with good control of DM, the correction of lipid abnormalities, cessation of smoking, treatment of hypertension and hyperfibrinogenaemia, it is tempting to suggest that one may be able to maintain an individual's sexual function and perhaps also to confer some overall survival benefit on them (Miller et al, 1994c).

As part of our investigations into the effects of risk factors a series of ultrastructural studies were undertaken to ascertain if discernible smooth muscle or endothelial damage was apparent in the STZ rat model. Disappointingly, these EM studies were probably the least satisfactory of our investigations. A number of other authors have been able to demonstrate the presence of such abnormalities in combined structural and functional studies. Unfortunately, we were unable to be so categorical or emphatic about our findings, nevertheless it would be of considerable interest to pursue our early tentative findings.

The STZ rat model of DM has been used in a large number of studies since it was first described. It is well established as a model of diabetic autonomic neuropathy and perhaps is less well established as a model for peripheral neuropathy (Dyck et al, 1987). Furthermore, it has also been used extensively in cardiovascular research (Tomlinson et al, 1994). However, it seems that it is a model which is relatively resistant to atherosclerosis. As such it is perhaps rather a limited model and may explains the inconclusive findings of our EM studies. Moreover, our diabetic animals were not treated with insulin and therefore represented a model of uncontrolled diabetes, it would be interesting to have compared our results with an insulin treated diabetic model cohort. It becomes clear from our investigations that the STZ diabetic rat model is a rather devastated animal and as such it is probably not a particularly good model of human disease. However, the elucidation of the described alterations in biochemical signalling have contributed to our further understanding of this animal model and its relevance to human disease. Ultimately we would have to conclude that one should turn to other animal models of human disease for further experiments and for interventional studies in particular. In this respect, the use of a rabbit model as
described in some of our experiments is to be preferred. It is an appealing animal to work with because not only is the rabbit susceptible to atherosclerosis but it can also be rendered diabetic with the use of alloxan. The rabbit therefore promises a most fruitful model for further investigations. An interesting aspect that might most usefully be pursued in future experiments is the long term effects of oscillating levels of both glucose and insulin, this is of course what is actually happening in treated human diabetes. It may be that over a period of years that it these factors which are responsible for the changes in endothelial function and basement membrane thickening, in this context it also possible that many of the other effects are due to subtle alterations in the normal regulation by peptide growth factors.

The present work could and perhaps should be replicated and then extended in human penile tissue. Unfortunately, this is not always an easy tissue to obtain especially with respect to the control tissues. This issue is fraught with difficulty and areas which may be explored in this respect are the use of cadaver specimens or perhaps even organ donors. An alternative method of obtaining sufficient tissue which has been used by a number of investigators is the use of cultured human corpus cavernosal cells (Bhargava et al, 1990). In this context it would be interesting to harvest human penile tissue, grow it in culture and then to characterise it biochemically along the lines that have been described for rat and rabbit tissues. Such a series of experiments would enable comparisons to be made between the experimental models and the human tissues, results which would help to establish the validity and usefulness of our animal models. Furthermore, using such cell culture techniques, tissues would be available in reasonable amounts which would allow for the transduction mechanisms to then be studied in greater depth. Ultimately, a variety of both control and disease model cell lines could be cultured and then biochemically characterised in different species. Furthermore, as an adjunct to such biochemical experiments in models of disease, alterations in corporeal smooth muscle relaxation and contraction could be studied in organ baths. In this respect the use of rabbit penile tissue is to again be preferred as it is a much easier preparation to work with.
We have seen in this thesis that penile erection is a multitransmitter event with multiple transduction mechanisms involving a veritable cascade of intracellular molecules, central to the thesis has been the appreciation of the role of the cyclic nucleotides cAMP and cGMP. We have investigated a number of the key mechanisms that control these second messengers and we have shown that they are altered in our diabetic model. The precise manner in which this control may be effected is unknown but is certain to include the G-proteins and protein kinases, it is likely that these too are altered in diabetes mellitus and this should be explored. It has become evident that there is an imbalance in the mechanisms responsible for the control of relaxation and contraction of corporeal smooth muscle in this model of human disease. An increased understanding of the normal pathways and their alterations in disease will lead to the development of novel therapeutic interventions and perhaps a greater understanding of how prevention may be attempted. While we may be encouraged that this will lead to better treatment options for men with ED, we have to balance this with some caution against the very real possibility that there is an enormous danger of this becoming a boom area for potential recreational drug abuse.
Table A1: Chemical pathology profile of diabetic rats

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219
Table A2: Chemical pathology profile of control rats

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<td>103.3</td>
<td>103.2</td>
<td>102.6</td>
<td>105.6</td>
</tr>
<tr>
<td><strong>HCO₃⁻</strong> (mmol/l)</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>28</td>
<td>28</td>
<td>24</td>
<td>26</td>
<td>31</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td><strong>Creatinine</strong> (mmol/l)</td>
<td>64</td>
<td>57</td>
<td>57</td>
<td>52</td>
<td>57</td>
<td>56</td>
<td>55</td>
<td>55</td>
<td>56</td>
<td>60</td>
</tr>
<tr>
<td><strong>Urate</strong> (mmol/l)</td>
<td>0.10</td>
<td>0.07</td>
<td>0.08</td>
<td>0.05</td>
<td>0.11</td>
<td>0.08</td>
<td>0.08</td>
<td>0.04</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>AST</strong> (U/l)</td>
<td>167</td>
<td>114</td>
<td>126</td>
<td>109</td>
<td>220</td>
<td>108</td>
<td>116</td>
<td>103</td>
<td>92</td>
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<tr>
<td><strong>BT</strong> (μmol/l)</td>
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<td>1</td>
<td>1</td>
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<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Calcium</strong> (mmol/l)</td>
<td>2.7</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.6</td>
<td>2.41</td>
<td>2.40</td>
<td>2.51</td>
<td>2.51</td>
<td>2.44</td>
</tr>
<tr>
<td><strong>ALB</strong> (g/l)</td>
<td>31</td>
<td>32</td>
<td>31</td>
<td>30</td>
<td>33</td>
<td>32.2</td>
<td>31.0</td>
<td>32.0</td>
<td>30.9</td>
<td>30.7</td>
</tr>
<tr>
<td><strong>TP</strong> (g/l)</td>
<td>63</td>
<td>60</td>
<td>59</td>
<td>63</td>
<td>65</td>
<td>57.9</td>
<td>57.4</td>
<td>58.9</td>
<td>58.8</td>
<td>57.2</td>
</tr>
<tr>
<td><strong>ALP</strong> (U/l)</td>
<td>99</td>
<td>60</td>
<td>75</td>
<td>88</td>
<td>88</td>
<td>109</td>
<td>97</td>
<td>147</td>
<td>160</td>
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<td><strong>GGT</strong> (U/l)</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td><strong>Blood glucose</strong> (mmol/l)</td>
<td>11.7</td>
<td>11.4</td>
<td>10.4</td>
<td>9.2</td>
<td>9.8</td>
<td>12.07</td>
<td>10.17</td>
<td>10.28</td>
<td>13.29</td>
<td>14.90</td>
</tr>
</tbody>
</table>
APPENDIX 3

Specimen preparation for SEM

The following is the specimen preparation protocol for the SEM and TEM electron micrographs (as carried out by Dr P Daszak at Kingston University):

Penises were cut into blocks approximately 8mm long, rinsed in buffer and post fixed.

(i) Fixation. The primary purpose of the fixative is to cross link proteins, preserving the structure of the cytoplasm without causing damage. Blocks are post-fixed for one hour in Osmium Tetroxide (in 0.1M phosphate buffer). Osmium stains lipids making them electron dense so that membranes and organelles can be detected more easily by the SEM.

(ii) Dehydration.

(iii) Critical Point Dryer. If a liquid is placed in a sealed container, leaving some vapour space around the liquid and the container is heated, vapour pressure and density increase. Conversely, the liquid expands and becomes less dense. There comes a point (known as the critical point (CP)) when vapour density equals liquid density and this known as the Critical Density. The surface tension will equal zero and there will be no distinct liquid and gas phases. By keeping the temperature above the CP and letting pressure return to atmospheric levels, specimens are 'critically point dried':

After repeated changes of arklone, specimens are placed in a container which fits into a critical point dryer apparatus (Polaron). Once sealed, liquid CO₂ is let in. Drying is carried out for 1-2 hours. CO₂ is a useful transitional fluid because its conditions can
be obtained in the laboratory, i.e. the CP of CO$_2$ is 76 atmospheres and the critical temperature is 31°C.

(iv) Mounting. Using a dissecting microscope, adherent tissue on penises (i.e. adventitia) was removed. Mounting involves carefully cutting along dried penis lengths and then placing the lengths on labelled aluminium stubs.

(v) Coating. Biological specimens lack good electrical conductivity and high surface density resulting in poor quality images under the SEM. However, by coating specimens with gold, optimum quality images can be obtained. Using a Sputter Coater (Polaron E5000), specimens are coated with gold. Gold acts as a source of secondary electrons providing a sharper image.

Penis specimens were examined under a Jeol JSM 35 scanning electron microscope.

Specimen preparation for TEM

Specimens for the TEM underwent identical fixative and dehydration procedures as SEM specimens. Specimens are embedded in Araldite after treatment with propylene oxide. Ultra-thin sections (100nm) are cut on a Reichert OMU 4 ultra microtome and mounted onto copper grids. Uranyl acetate and lead citrate are used to stain the sections.

Penis specimens were examined using a Jeol 100S transmission electron microscope.
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Effects of papaverine and vasointestinal polypeptide on penile and vascular cAMP and cGMP in control and diabetic animals: an in vitro study

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The Uro-Andrology Research Group, ¹Department of Urology, ²Department of Chemical Pathology and Human Metabolism, Royal Free Hospital Trust and Medical School, Pond Street, London NW3 2QG, UK, ³Department of Cardiac Surgery, Bristol Royal Infirmary, Bristol BS2 8HW, UK.

Adenosine 3'5'-cyclic monophosphate (cAMP) and guanosine 3'5'-cyclic monophosphate (cGMP) mediate penile erection. We have previously established that adenylate and guanylate cyclase activity is elevated in the diabetic rat penis and aorta. This study investigates the action of papaverine and vasoactive intestinal polypeptide (VIP) on these cyclases. The aortae and penes of Sprague Dawley rats (n=7) were stimulated with VIP and papaverine. Diabetes mellitus (DM) was induced in Sprague Dawley rats (n=7) with streptozotocin and the penile and aortic tissues were treated with VIP. The penes, aortae and carotid arteries of New Zealand White rabbits were similarly processed. cAMP and cGMP generation was measured by radioimmunoassay. In all tissues: VIP stimulated cAMP synthesis; VIP did not increase cGMP levels; papaverine was without effect on either cAMP or cGMP synthesis. VIP-stimulated cAMP was significantly enhanced in the diabetic rat penis and aorta; there was also a significant elevation in the basal levels of cGMP in these tissues. These data: (1) consolidate that cAMP is a mediator of penile erection, (2) indicate that papaverine and VIP elicit erection by different mechanisms, (3) suggest that an enhanced penile capacity to generate cAMP in DM may constitute an adaptive response to counteract the previously reported reduction in VIP content and VIP receptors, and (4) indicate that the penile and vascular tissues of the rabbit respond in a similar manner to VIP and papaverine.

Key words: erectile dysfunction, diabetes mellitus, vasoactive intestinal polypeptide, papaverine, adenylate/guanylate cyclase.

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INTRODUCTION

The intracavernosal injection of substances such as vasoactive intestinal polypeptide (VIP) and papaverine has been used therapeutically to induce penile erection in the treatment of erectile dysfunction (ED). Despite VIP having been implicated as an important mediator of penile erection for many years and papaverine being one of the most commonly used pharmacological agents used in the treatment of ED the precise mode of action of these two agents in penile tissues has been unclear.

VIP is a 28 amino acid residue polypeptide first isolated in the gastrointestinal tract which is a potent inhibitor of smooth muscle contraction. The intracellular mode of action of VIP may be via the generation of cAMP, and/or cGMP, as well as perhaps the modulation of VIP and NO release. Data for penile smooth muscle are lacking, although Pickard et al. provided indirect evidence that VIP does not stimulate the nitric oxide-cGMP pathway in human corpus cavernosal strips. Papaverine is a non-specific smooth muscle relaxant which can relax all components of the penile erectile system. Despite available evidence from a variety of tissues, its precise intracellular profile of action remains unclear for penile smooth muscle. Papaverine has a non-selective inhibitory effect on the cyclic nucleotide phosphodiesterases, however, it is not known if papaverine is able to stimulate the production of cyclic nucleotides de novo. Papaverine also produces smooth muscle relaxation by lowering intracellular calcium concentrations via a number of mechanisms.

Recent research interest has focused on the mechanisms and role of corporeal smooth muscle relaxation in erection, as well as the importance of second messenger systems, such as cAMP, cGMP, calcium and inositol trisphosphate in this process; aspects which were recently reviewed by Lerner and colleagues. It is well known that DM is strongly associated with the development of ED in both man and in laboratory animals. Support for a relaxant neurotransmitter role of VIP came from reports that VIPergic nerves are depleted in impotent men and that the degree of depletion was related to the severity of ED. In addition, it was found that in diabetic men with ED there was a marked reduction in VIP-like reactivity, results which had been previously described in both human ED and streptozotocin-induced diabetic rats. These latter results have not been replicated by other investigators. In a recent study we established that adenylate and guanylate cyclase activity is markedly altered in the penis and aorta of the diabetic rat. Apart from the relaxation of erectile tissues, vasodilation of the pudendal, penile and cavernosal arteries is important in achieving an erection. These vessels are too small to study in the rat and therefore we utilized the aorta as an indicator of what might be occurring in the extra-penile vasculature during erection. In this context it was decided to also study the aorta and carotid arteries of the New Zealand White (NZW) rabbit. Furthermore, it is often argued that the penis may be considered as a specialized vascular organ and it is therefore of interest to observe if the biochemical characteristics of these tissues are similar. The aims of the present study were: (1) to investigate the mode of action of VIP and papaverine on the activity of these cyclases to further elucidate the mechanisms of penile smooth muscle relaxation, and (2) to extend our investigations into another species to assess the suitability of the NZW rabbit as an appropriate model for ED.
INDUCTION OF DIABETES

The experimental model used in this study was the streptozotocin-induced diabetic rat. The experimental group consisted of ten male Sprague Dawley rats with an initial median body weight of 250 g. Non-ketotic, hyperglycaemic diabetes was induced with an intravenous injection of streptozotocin at a dose of 65 mg per kg body weight. These animals developed glycosuria but not ketonuria or haematuria. Diabetic rats were fed ad libitum with Mouse No. 1 Modified Maintenance Diet (SDS, Witham, Essex, UK) and allowed free access to water. The study also included seven age and weight-matched controls which were injected with an equal volume of saline. Urine was monitored over the duration of DM for glucose, ketone bodies and proteins with Multistix (Ames Division, Miles Laboratories Ltd., Stoke Poges, Bucks, UK). After eight weeks, rats were anaesthetized with an intraperitoneal injection of pentobarbitone (90 mg/kg; Sagatal: May and Baker Ltd., Dagenham, UK) and blood was collected by cardiac puncture for the measurement of blood glucose. The thoracic aortae and penes were then rapidly excised and placed in Dulbecco’s minimum essential medium (MEM), pregassed with 95% O₂/5% CO₂. The study also included a series of experiments performed on six non-diabetic NZW rabbits from which the carotid arteries were excised in addition to the aortae and penes.

TISSUE PREPARATION

The penis of each animal was excised at its base, the connective and adventitial tissues were dissected from the penile shaft and the glans penis removed. Penes were cut longitudinally into two equal strips and then transversely into segments (to give approximately 16 segments per penis). Rabbit and rat aortae, and rabbit carotid arteries, were cut transversely into rings approximately 2 mm wide. Penile, carotid and aortic tissue were placed in oxygenated MEM and incubated for 3 h at 37 °C to allow the tissues to achieve steady state prior to the stimulation of cyclic nucleotide formation.

INCUBATION OF TISSUES WITH VIP OR PAPAVERINE

Each incubation was performed with either two penile segments, or two aortic or two carotid rings placed in 200 µl MEM containing 100 µM isobutylmethylxanthine (IBMX). IBMX is a non-specific phosphodiesterase inhibitor which prevents the hydrolysis of cyclic nucleotides to their respective non-cyclic forms (viz cAMP and cGMP). VIP or papaverine were then added over a wide concentration range (see graphs for ranges) to six duplicate preparations. Following the addition of one of the above agents, the tissues were incubated at 37 °C for 20 m after which time 500 µl of ice cold absolute ethanol was added, samples were stored at −70 °C until subsequent assay. After thawing the tissues were extensively sonicated (6×20s bursts). The tissues were centrifuged and aliquots of ethanolic extract taken, evaporated under vacuum and reconstituted in assay buffer for the measurement of cAMP and cGMP concentration. The assays were carried out using commercially available kits.

MATERIALS AND DRUGS

Papaverine, VIP, Dulbecco’s MEM and streptozotocin were all purchased from Sigma Chemical Company (Poole, Dorset, UK). Highly sensitive radioimmunoassay
kits for cAMP and cGMP using $^{125}$I ligands were purchased from Amersham Laboratories (Amersham, UK).

**CALCULATION OF RESULTS AND STATISTICAL ANALYSIS**

Basal (i.e. non-stimulated) concentrations of cyclic nucleotides were subtracted from the concentrations after the incubation of tissues with either papaverine or VIP. Values are expressed as mean concentration ± SEM for each of the six duplicate assays. Significance was determined with the Student’s t-test. P values less than 0.01 were taken to be significant.

**RESULTS**

Body weights (median [range]) of the animal groups at the time of sacrifice were: control rats (n = 10), 475 g (449-516); DM rats (n = 7), 239 g (220-280); NZW rabbits (n = 6), 2.5-3.0 kg. At sacrifice, the blood glucose concentrations of the two rat groups were (mmol/l; median [range]): controls, 8.4 (6.8-10.3); DM, 30.3 (28.2-32.7).

Papaverine had no effect on cAMP or cGMP synthesis by the penis of the rat or rabbit (Table 1), nor on the rat and rabbit aorta (Table 2). VIP was a potent stimulator of cAMP synthesis in the rat penis and aorta (Fig. 1 and Fig. 2), and in the rabbit penis, aorta and carotid artery (Fig. 3). VIP-stimulated cAMP synthesis was significantly enhanced in the penes and aortae of diabetic rats compared to controls.

**TABLE 1.** Effect of various concentrations of papaverine on cAMP (fmol/mg tissue/min) and cGMP (fmol/mg tissue/20 min) synthesis by the penis of the rat (n = 7) and rabbit (n = 6). Each value is the mean ± SEM for six duplicate assays.

<table>
<thead>
<tr>
<th>Papaverine conc. (µmol/l)</th>
<th>Rat penis</th>
<th>Rabbit penis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
</tr>
<tr>
<td>0</td>
<td>5.1±0.4</td>
<td>6.1±0.7</td>
</tr>
<tr>
<td>0.1</td>
<td>5.2±0.5</td>
<td>6.3±0.6</td>
</tr>
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<td>5.6±0.6</td>
<td>6.4±0.5</td>
</tr>
<tr>
<td>10</td>
<td>5.4±0.5</td>
<td>7.2±0.6</td>
</tr>
</tbody>
</table>

**TABLE 2.** Effect of various concentrations of impotence on cAMP (fmol/mg tissue/min) and cGMP (fmol/mg tissue/20 min) synthesis by the aorta of the rat (n = 7) and rabbit (n = 6). Each value is the mean ± SEM for six duplicate assays.

<table>
<thead>
<tr>
<th>Papaverine conc. (µmol/l)</th>
<th>Rat aorta</th>
<th>Rabbit aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
</tr>
<tr>
<td>0</td>
<td>4.6±0.4</td>
<td>6.4±0.4</td>
</tr>
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<td>4.5±0.4</td>
<td>6.4±0.4</td>
</tr>
<tr>
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<td>5.2±0.4</td>
<td>6.9±0.4</td>
</tr>
<tr>
<td>10</td>
<td>5.6±0.4</td>
<td>7.4±0.4</td>
</tr>
</tbody>
</table>
Papaverine and vasointestinal polypeptide: an *in vitro* study

![Graph](image1)

**Figure 1.** Effect of VIP on cyclic AMP synthesis by the penes of diabetic (♦, n=10) and control (●, n=7) rats. Each point is the mean±SEM of six duplicate assays. *# P<0.05.*

![Graph](image2)

**Figure 2.** Effect of VIP on cyclic AMP synthesis by the aortae of diabetic (♦, n=10) and control (●, n=7) rats. Each point is the mean±SEM of six duplicate assays. *# P<0.05.*
The present study demonstrates that VIP stimulates cAMP, but not cGMP, in both rat and rabbit penile tissue. We have previously demonstrated that sodium nitroprusside stimulates cGMP in the rat penis. Thus, although a nitric oxide-cGMP axis is in fact present in the penile tissues of these species, a VIP-guanylate cyclase axis appears not to be. Our conclusions concur with the recent results of

### Discussion

The present study demonstrates that VIP stimulates cAMP, but not cGMP, in both rat and rabbit penile tissue. We have previously demonstrated that sodium nitroprusside stimulates cGMP in the rat penis. Thus, although a nitric oxide-cGMP axis is in fact present in the penile tissues of these species, a VIP-guanylate cyclase axis appears not to be. Our conclusions concur with the recent results of

<table>
<thead>
<tr>
<th>VIP conc. (μmol/L)</th>
<th>Penis (fmol/mg tissue/20 min)</th>
<th>Aorta (fmol/mg tissue/20 min)</th>
<th>Carotid artery (fmol/mg tissue/20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.9±0.4</td>
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<td>5.2±0.8</td>
</tr>
<tr>
<td>0.1</td>
<td>3.8±0.5</td>
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<tr>
<td>1</td>
<td>4.1±0.6</td>
<td>6.8±0.6</td>
<td>5.2±0.8</td>
</tr>
<tr>
<td>10</td>
<td>4.2±0.3</td>
<td>5.9±0.5</td>
<td>6.2±0.4</td>
</tr>
</tbody>
</table>
Papaverine and vasointestinal polypeptide: an in vitro study

Table 4. Effect of various concentrations of VIP on cGMP (fmol/mg tissue/20 min) synthesis by the penis and aorta of control (n=7) and diabetic (n=10) rats. Each value is the mean ± SEM of six duplicate assays. *P < 0.001 when comparing control to diabetic rats at each dose of VIP.

<table>
<thead>
<tr>
<th>VIP conc. (µmol/L)</th>
<th>Control Penis</th>
<th>Diabetic Penis</th>
<th>Control Aorta</th>
<th>Diabetic Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.4±0.6</td>
<td>14.1±1.2*</td>
<td>8.2±2.3</td>
<td>20.8±2.5*</td>
</tr>
<tr>
<td>0.1</td>
<td>7.2±0.7</td>
<td>13.3±1.2*</td>
<td>7.3±1.2</td>
<td>21.9±1.4*</td>
</tr>
<tr>
<td>1.0</td>
<td>6.6±0.5</td>
<td>14.4±1.3*</td>
<td>7.4±1.2</td>
<td>19.6±1.4*</td>
</tr>
<tr>
<td>10</td>
<td>6.4±0.4</td>
<td>15.2±1.2*</td>
<td>9.3±1.3</td>
<td>17.8±1.8*</td>
</tr>
</tbody>
</table>

Pickard et al. who demonstrated that VIP-stimulated relaxation of isolated human cavernosal smooth muscle is not mediated by nitric oxide (NO) and therefore not cGMP. These authors also concluded that VIP is not a major relaxant neurotransmitter in the human penis, a view which in the light of evidence that VIP can stimulate the formation of an important second messenger may be somewhat premature. Further support comes from Ganz et al. who previously demonstrated that VIP will stimulate cAMP (and vasodilatation) in the rat aorta and mesenteric vessels. It is concluded that VIP elicits erection via the activation of adenylate cyclase and not guanylate cyclase at least in the rat and rabbit penis. Using cultured cavernosal cells we have recently demonstrated that VIP stimulates cAMP but not cGMP (Jeremy and Miller, unpublished observations). The present observations also consolidate that penile tissue behaves, physiologically, in a similar fashion to vascular tissue in that VIP stimulates cAMP but not cGMP. The findings also demonstrate that the pathways present in the Sprague Dawley rat are to be found in the NZW rabbit; we are encouraged therefore that we may be able to model other disease states associated with ED in another species. More recent support for our findings comes from the observation that pituitary adenylate cyclase-activating peptide (PACAP) is not only found in the human corpus cavernosum but also that it relaxes pre-contracted tissue strips in a similar manner to VIP. The authors conclude that even if NO is the predominant relaxant neurotransmitter it does not preclude a contributory effect from other transmitters. In this context it is interesting to consider whether the NO-cGMP axis is essential for penile erection. It has been demonstrated by Varrin et al. that L-nitroarginine methyl ester administered intravenously failed to abolish erections in 5 out of 13 Wistar rats, suggesting that NO is not the only mechanism responsible for erection. It is becoming apparent that normal penile erection is a multitransmitter event mediated by a complex cascade of second messengers; the NO-cGMP axis is but one aspect of this system.

With regard to diabetes mellitus, several studies have shown that VIPergic nerves are depleted in both impotent and diabetic men. Similar findings were reported for streptozotocin diabetic rats. Our data, however, indicate that there is an increased responsiveness to VIP in the penis of diabetic rats, at least in terms of cAMP generation. We have previously found that cAMP synthesis in response to PGE1 is also enhanced by the penis of the diabetic rat in a similar fashion to VIP-stimulated cAMP. It was concluded that there may be an adaptive increase
in adenylate cyclase activity designed to counteract the generalized attenuation of receptor (linked to tumescence) activity.

The present study also demonstrates that there is markedly increased basal cGMP (Table 4) and cAMP (data not shown) in the diabetic tissues. In this context we have recently demonstrated that the nitric oxide synthase content (and activity) of the diabetic rat penis is elevated (Thompson et al., unpublished observations). Furthermore in the same experimental model we have shown that the activity of the cGMP (and cAMP) specific phosphodiesterase is decreased (Miller et al., unpublished observations). The net effect of these alterations would be to elevate cGMP (and cAMP) levels and the present data are consistent with these findings. Taken together, our findings may contribute to an explanation of the clinical observation that diabetic men with predominantly neurogenic ED are often markedly sensitive, in terms of their erectile response, to intracavernosal injections.

This sensitivity may be due to the presence of significantly enhanced transduction mechanisms for smooth muscle relaxation in the presence of a normal blood supply. In the present study the observed lack of effect of papaverine on cAMP and cGMP synthesis by both rat and rabbit penile tissues is consistent with its previously described action as a phosphodiesterase inhibitor. In our experiments we have already inhibited the activity of the intrinsic phosphodiesterases with the addition of IBMX. This observation also indicates that papaverine is not able to stimulate cAMP or cGMP formation de novo. It is important to understand the mode of action of such agents as future drug development requires a clear understanding of their mode of action as well as the underlying pathophysiology of ED.

A number of reviews of the streptozotocin-induced diabetic rat as a model for human diabetes are available although these have often concentrated primarily on either the cardiovascular or the neurological aspects of the model. We are unaware of a systematic review of the streptozotocin-induced diabetic rat as a model for human diabetic ED. Nevertheless, many workers continue to use this model as is evidenced by the large number of publications in the literature. We envisage that a biochemical characterization of the model, as in the present study, will contribute not only to the evaluation of the model, but also to the understanding of the pathogenesis of human diabetic ED.

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Papaverine and vasointestinal polypeptide: an in vitro study


Hydrolysis of cyclic guanosine monophosphate and cyclic adenosine monophosphate by the penis and aorta of the diabetic rat

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Objectives To investigate the hydrolysis of adenosine 3'5'-cyclic monophosphate (cAMP) and guanosine 3'5'-cyclic monophosphate (cGMP) by specific phosphodiesterases (PDEs) in the penis and aorta of diabetic rats.

Materials and methods Non-ketonuric diabetes mellitus was induced in 10 Sprague-Dawley rats with streptozotocin. After 2 months, the rats were killed and their penises and aortae excised. The tissues were incubated with [H]-cAMP and [H]-cGMP and the degree of hydrolysis was assessed by separating [H]-cAMP and [H]-cGMP from [H]-AMP and [H]-GMP, respectively, in the incubation supernatants using thin layer chromatography (polyethyleneimine cellulose developed in 50 mmol/L KCl).

Results The hydrolysis of cAMP and cGMP was significantly reduced in penile and aortic tissue from diabetic rats compared to that of seven age-matched controls.

Conclusions Such a reduction of PDE activity would result in increased intracellular cyclic nucleotide levels (and thus corporeal smooth muscle relaxation and erection). Consequently, the altered activity of PDE enzyme systems is not related aetiologically to the pathogenesis of diabetic erectile dysfunction. Furthermore, these data consolidate the concept that enhanced cyclic nucleotide synthesis and decreased degradation constitute an adaptive response to counteract the deleterious effects of diabetes mellitus on ereptogenic mechanisms. The pathophysiology and therapeutic implications of these findings warrant further investigation.

Keywords Erectile dysfunction, diabetes mellitus, phosphodiesterases, cyclic nucleotides

Introduction

Diabetes mellitus is associated with erectile dysfunction in man and in laboratory animals [1,2]. Diabetes is a particularly strong risk factor for the development of erectile dysfunction because diabetic men have an increased incidence of two important complications which contribute to erectile dysfunction; autonomic neuropathy and angiopathy. Notwithstanding the controversy surrounding the nature of the neurotransmitter(s) mediating erection, the role of corporeal smooth muscle relaxation and the transduction mechanisms which control it, are now widely accepted as being of central importance in the mechanism of penile erection. Smooth muscle tone is the result of a balance between relaxant and contractile influences of neuronal and endothelial origin. Penile smooth muscle tone is controlled by several intracellular second messengers, i.e. calcium ions, potassium ions, inositol trisphosphate, cyclic 3'5' adenosine monophosphate (cAMP) and cyclic 3'5' guanosine monophosphate (cGMP) [3].

There is now strong evidence that relaxant neurotransmission, and hence erection, is mediated by nitric oxide (NO) and possibly also VIP [4,5]. NO exerts its effects by activating soluble guanyl cyclase to raise intracellular levels of cGMP [6]. VIP acts via adenylate cyclase to raise cAMP levels [7]. While PGE1 has been shown to act through its effects on calcium ions and cAMP, the overall role of prostaglandins in physiological erection and the pathoetiology of erectile dysfunction remains controversial [8]. The cyclic nucleotides are synthesized by cyclases, the activity of which were investigated in a previous study [9]. In the streptozotocin-induced diabetic rat model, the activity of these cyclases was significantly enhanced when compared with controls [9] and it was proposed that this upregulation in enzyme activity was the tissue's homeostatic response to maintain intracellular cyclic nucleotide levels [9]. This adaptive change may then counter the attenuation of receptor activity linked to these cyclases in diabetes mellitus.

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Apart from receptor activity (affinity and number) and the strength of agonist signals as determinants of cAMP and cGMP levels, the intracellular levels of these nucleotides are also controlled by powerful endogenous enzymes. Thus, the intracellular levels of both cAMP and cGMP are not only dependent on their synthesis; their degradation is controlled by specific phosphodiesterases (PDEs) which hydrolyse the cyclic nucleotides to their respective non-cyclic forms. The PDEs constitute a complex family of isoenzymes [10].

In this context, we investigated the PDE activity in the penis and aorta of the diabetic rat. There are few reports on changes of PDE activity in diabetes mellitus, but it is well established that diabetes results in complex alterations in cyclic nucleotide metabolism [11,12]. It is therefore possible that the altered levels of intracellular cyclic nucleotides in response to agonists in the penis of diabetic rats are due to alterations in PDE activity. To investigate the possibility that alterations of these key enzymes relate to erectile dysfunction in diabetes mellitus, we determined the activity of PDE in the penis of diabetic rats and examined the aorta to assess the changes which might be present in the extra-penile vasculature subserving erection.

Materials and methods

Induction of diabetes

All experiments were carried out using male Sprague Dawley rats (median initial body weight 250 g). Non-ketonuric, hyperglycaemic diabetes was induced by injecting the rats with streptozotocin (Sigma Chemical Co, Poole, UK) intravenously at a dose of 65 mg/kg body weight [13]. These animals developed glycosuria but not ketonuria or haematuria. Diabetic rats had free access to Mouse No.1 Modified Maintenance Diet (SDS, Witham, UK) and water. The study also included seven age-matched control rats. Urine was monitored over the duration of diabetes for glucose, ketone bodies and proteins using Multistix (Ames Division, Miles Laboratories, Stoke Poges, Bucks, UK) and water. The study also included seven age-matched control rats. Urine was monitored over the duration of diabetes for glucose, ketone bodies and proteins using Multistix (Ames Division, Miles Laboratories, Stoke Poges, Bucks, UK).

The percentage conversion of cyclic nucleotide to its monophosphate form was calculated. Results are expressed as the mean (SEM). Significance was determined using Student's t-test and values < 0.05 were taken to indicate significance.

Results

The median (range) body weights of the groups of rats at the time of death were; control rats (n = 7), 475 g (449–516) and diabetic rats (n = 10), 239 g (220–280). At death, the median (range) blood glucose concentrations were: control rats, 30.3 mmol/L (28.2–32.7) and diabetic rats, 30.3 mmol/L (28.2–32.7). In both penile and aortic tissue from diabetic rats the hydrolysis of cAMP and cGMP was significantly reduced compared to that of the age-matched controls (Figs 1–4).

Discussion

There was a decrease in both cAMP-PDE and cGMP-PDE activity in the penis of the diabetic rat; although the activity of the PDEs of both cyclic nucleotides was also
Reduced in the aortae of diabetic rats, this was not as marked as that in the penis. As a reduction of PDE activity in situ would result in increased bioavailability of intracellular cyclic nucleotide (and as such, penile smooth muscle relaxation), these results indicate that diminished activity of these enzymes is not related aetio-
logically to the erectile dysfunction associated with diabetes mellitus. Furthermore, this may constitute an adaptive response designed to counteract the deleterious effect of diabetes on vasodilator mechanisms (e.g. muscarinic, PGE1 and NO) [16–19]. In this context, it was
shown previously that there was increased synthesis of cAMP and cGMP in response to PGE1 and sodium nitroprusside (which generates NO and which in turn stimulates cGMP synthesis), respectively, in the penis and aorta of diabetic rats [9]. However, in that study, PDE activity was inhibited by the PDE inhibitor, isobutylmethylxanthine. Thus, the elevation of cyclic nucleotides was not ascribed to diminished PDE activity, but rather to enhanced adenylate and guanylate cyclase activity [9]. More recently, the level of NO synthase (NOS) was shown to be significantly enhanced in the penis of the diabetic rat [20]. Therefore, taken together these results support the concept that enhanced cyclic nucleotide synthesis via a reduction of PDE activity, increased NOS and increased nucleotide cyclase activity constitutes an adaptive response to counteract the deleterious effects of diabetes mellitus on vasodilator receptors. The raised intracellular levels of cyclic nucleotides may also result in the refractoriness of the end-organ. Thus, smooth muscle relaxation may not occur at intracellular levels of cAMP and/or cGMP which induce this response in healthy tissue.

We are unaware of any reports on alterations of cAMP or cGMP PDE activity in vascular or penile tissues in diabetes. It is also notable that PDEs are controlled by calmodulin for full activity [21]. As several studies have shown that calmodulin activity is diminished in diabetes mellitus, this may explain why the activity of PDE was reduced in the present study. Other intracellular systems disrupted by diabetes include protein kinases, which in turn are known to control PDE activity. Clearly, further work is required to clarify the significance and mechanisms underlying the present alterations of PDEs.

PDEs exist in several forms, both particulate and cytosolic, with several subclasses of the enzyme [21]. In the present study, these various forms of PDE were not isolated or separated, but the overall hydrolysis of the cyclic nucleotides by diabetic tissues was examined. However, it is entirely possible that diabetes mellitus may have different effects on the various types of PDEs.

In conclusion, the present study clearly shows that diabetes mellitus in the rat caused a marked overall reduction of both cAMP and cGMP PDE activity in the penis and aorta. The consequences of this in vivo would be to elevate cAMP and cGMP, both of which are associated with smooth muscle relaxation (and therefore erection). Thus, PDEs do not appear to be aetologically related to erectile dysfunction. On the contrary, the decreased activity of these enzymes may constitute an adaptive response designed to counter the deleterious effect of diabetes mellitus on other vasodilator systems.

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Adenylate and guanylate cyclase activity in the penis and aorta of the diabetic rat: an in vitro study

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Objective To investigate the role of adenylate and guanylate cyclases in the mediation of erection in diabetic rats.

Materials and methods Hyperglycaemic diabetes mellitus was induced in 35 rats using streptozotocin. Two months later the penises and aortae of these rats were excised and cut into rings or segments before being treated with varying concentrations of acetylcholine (Ach), sodium nitroprusside (NaNP), prostaglandin (PGEi) and adrenaline (AD). The levels of adenosine 3'5'-cyclic monophosphate (cAMP) and guanosine 3'5'-cyclic monophosphate (cGMP) so generated were measured by radioimmunoassay and the results compared with those from seven age-matched control rats that had not been given streptozotocin.

Results Ach-stimulated cGMP synthesis was impaired in the aortae in diabetic rats. Ach-stimulated cGMP synthesis was undetectable in the penis. NaNP-stimulated cGMP and PGEi-stimulated cAMP synthesis was enhanced in both the penises and aortae in diabetic rats compared with controls. AD-stimulated cAMP synthesis was enhanced in the aorta in diabetic rats compared with controls, but AD had no effect on cAMP synthesis in the penis.

Conclusion Ach-stimulated nitric oxide (NO) synthesis is impaired in the vasculature of diabetic rats and an Ach-NO axis may not be present in the penis of the rat. The enhanced capacity of the penis and vasculature to generate cAMP and cGMP may constitute an adaptive response to counteract the reduction in receptor-linked NO release. Impaired adenylate or guanylate cyclase activity does not contribute to erectile dysfunction in diabetic rats.

Keywords Impotence, diabetes, adenylate/guanylate cyclases

Introduction

Diabetes mellitus is associated with an increased incidence of erectile dysfunction in men [1] and laboratory animals [2]. As autonomic neuropathy is a common complication of diabetes mellitus [3], damage to the penile autonomic innervation has been suggested as the principal cause of impotence in diabetic men [4]. The presence of vascular disease such as atherosclerosis is also known to contribute to impotence [5] and diabetics have a far greater risk than those without diabetes of developing atherosclerosis [6]. Thus, diabetic men are predisposed to develop complications that contribute to impotence: neuropathy and angiopathy. Recently, interest has been focused on the role of endogenous modulators of smooth muscle relaxation in erection and impotence as well as in the pathophysiology of atherosclerosis. These include endothelium derived relaxing factor (EDRF) which is now thought to be nitric oxide (NO) [7] and prostacyclin (PGI2).

The parasympathetic system, by an hitherto poorly understood combination of cholinergic and non-adrenergic non-cholinergic transmission, stimulates the release of NO from endothelial cells. NO thus generated stimulates relaxation of smooth muscle via activation of guanylate cyclase [8]. Based on studies in several species, including humans, NO released by the penile endothelium appears to play a major role in the initiation and maintenance of tumescence [1, 9–11]. The release of PGI2 from the penis of rats and humans is also stimulated by parasympathomimetics [12]. Although Hedlund and Andersson have shown that PGI2 causes contraction in isolated corpus cavernosa [13], PGI2 release during erection has been suggested as playing a role in increasing blood flow and limiting platelet adhesion via activation of adenylate cyclase [12, 14].

There is evidence that impaired synthesis and release of PGI2 and NO contribute to impotence in diabetic patients. For instance, it has been demonstrated that in impotent diabetic men there is a defect in EDRF-mediated erection [1]. PGI2 synthesis by vascular and penile tissue is reduced in experimentally induced diabetes mellitus [15, 16] and atherosclerosis [17]. Other evidence suggests that as diabetes mellitus is associated with
neuropathy [18], diminished PG\textsubscript{2} and NO release may be a consequence of reduced or impaired cholinergic receptors. Indeed, attenuation of muscarine-receptor linked PG\textsubscript{2} release in the penises of diabetic rats has been documented [19]. In addition, the intracavernosal injection of PGE\textsubscript{1}, an eicosanoid with similar properties to PG\textsubscript{2}, is known to result in erection and is widely used in the treatment of patients with impotence [20]. In a preliminary study intracavernosal injection of the NO donor linsidomine chlorohydrate has been shown to induce erection in men with impotence [21].

PG\textsubscript{2} and NO cause vasodilation mediated by stimulation of adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) synthesis respectively. The present study was undertaken to investigate these cyclic nucleotides in the isolated penis and aorta. The aorta was used because the pudendal artery and associated vessels in rats are too small for analysis. The stimulatory agents investigated were: acetylcholine (Ach), sodium nitroprusside (NaNP), PGE\textsubscript{1}, (which has similar properties to PG\textsubscript{2}), adrenaline (AD) and papaverine.

Materials and methods

The experimental model was the male Sprague-Dawley rat. The initial median body weight of the 35 experimental rats was 250 g. Non-ketonuric, hyperglycaemic diabetes was induced by injecting streptozotocin (Sigma Chemical Co., Poole, UK), intravenously at a dose of 65 mg/kg body weight [15, 16]. The rats developed glycosuria but not ketonuria or haematuria. Diabetic rats were fed ad libitum with Mouse No. 1 Modified Maintenance Diet (SDS, Witham, UK) and allowed free access to water. The study also included seven age-matched controls whose initial median body weight was 250 g. Urine was monitored for glucose, ketone bodies and proteins over the duration of induced diabetes mellitus with Multistix (Ames Division, Miles Laboratories Ltd, Stoke Poges, UK).

After 8 weeks, the rats were anaesthetized with pentobarbitone (Sagatal, May and Baker Ltd, Dagenham, UK) (90 mg/kg body weight) given intraperitoneally. The rats were weighed and blood was collected by cardiac puncture for measurement of blood glucose levels. The thoracic aortae and the penises were excised rapidly and placed in Dulbecco's Minimum Essential Medium (MEM) (Sigma), pre-gassed with 95% oxygen/5% carbon dioxide and the rats were killed by overdose with pentobarbitone.

**Tissue preparation**

Penises were cut longitudinally into two equal strips and then laterally into segments to give approximately 16 segments per penis. Aortae were cut transversely into rings approximately 2 mm wide. Penile and aortic tissues were placed in oxygenated (MEM) and incubated for 3 h, at 37°C, to allow the tissues to stabilize prior to stimulation of cyclic nucleotide release.

**Incubation of tissues with stimulators of cyclic nucleotide synthesis**

Two penile segments and, separately, two aortic rings were placed in 200 µL MEM containing 100 µL isobutyryl-xanthine (a non-specific phosphodiesterase inhibitor which prevents hydrolysis of cyclic nucleotides to their respective non-cyclic forms, AMP and GMP). The following agents were added over a wide concentration range as shown in the graphs: (a) prostaglandin E\textsubscript{1} (PGE\textsubscript{1}) (Sigma), a stimulator of cAMP; (b) NaNP (Sigma), which breaks down to generate NO, a stimulator of cGMP; (c) Ach (acetylcholine chloride, Sigma), which is believed to act via stimulation of EDRF/NO: (d) AD (adrenaline bitartrate, Sigma) which acts via stimulation of PG\textsubscript{2} and possibly adenylate cyclase; and (e) papaverine (Sigma), a drug commonly used in intracavernous injection pharmacotherapy, the actions of which include phosphodiesterase inhibition and calcium flux alterations.

Following the addition of one of the above agents, tissues were incubated at 37°C for 20 min, after which time 500 µL of ice-cold absolute ethanol was added and samples were placed in a freezer at −70°C. The tissues were then sonicated extensively (6 × 20 second bursts). This effectively disintegrates plasma membranes and releases cyclic nucleotides [22]. Validation experiments using various other extraction techniques, for example trichloroacetic acid or perchloric acid, failed to extract any further measurable cAMP or cGMP following extraction with aqueous ethanol and sonication. This method of extraction was concluded to yield virtually all of the nucleotide which was generated in the tissues under study. Tissues were then centrifuged and aliquots of ethanolic extract taken, evaporated under vacuum and reconstituted in assay buffer for measurement of cAMP and cGMP concentrations. Assays were carried out with highly sensitive radioimmunoassay kits using [\textsuperscript{125}I]-ligands (Amersham Laboratories, Amersham, UK).

**Calculation of results and statistical analysis**

Basal (i.e. non-stimulated) concentrations of cyclic nucleotides were subtracted from concentrations measured after stimulators/drugs had been added. Values are expressed as mean concentration ± SEM. Significance was determined by the Student's t-test. P values <0.01 were taken to be significant.
Results

The median body weights of the experimental and the control groups of rats at the time of death were 239 g (range 220-280) and 475 g (range 449-516) respectively. The median glucose concentrations at the time of death were 30.3 mmol/l (range 28.2-32.7) for the experimental group and 8.4 mmol/l (range 6.8-10.3) for the control group.

NaNP was found to be a potent stimulator of cGMP synthesis in both penile and aortic tissue (Figs 1 and 2) but had no effect on cAMP synthesis in either tissue. NaNP-stimulated cGMP synthesis was significantly enhanced (P < 0.01) in the penises of diabetic rats compared with the controls (at low concentrations of NaNP) but the maximal synthesis of cGMP between the two groups was not statistically significant (P < 0.01) (Fig. 1). Ach stimulated cGMP synthesis in the aorta but to a lesser degree than NaNP (Fig. 2). Ach had no effect on cGMP synthesis by penile tissue in either control or diabetic animals (Fig. 1). Ach-stimulated cGMP synthesis was markedly reduced in the aorta of diabetic rats compared with controls (Fig. 2).

PGE1-stimulated cAMP synthesis in both aorta and penis (Figs 3 and 4) was significantly enhanced in diabetic rats compared with controls (Figs 3 and 4). PGE1 had no effect on cGMP synthesis over the same concentration range of the eicosanoid. AD-stimulated cAMP synthesis was significantly enhanced in the aortae of diabetic rats compared with controls (Fig. 5). AD had no effect on cAMP synthesis in the penis in either group of rats (data not shown). Papaverine had no effect on cAMP or cGMP synthesis in the penis or aorta of control or diabetic animals (data not shown).

Discussion

Several research groups have presented convincing evidence that the NO-cGMP axis plays a key role in mediating smooth muscle relaxation and erection of the penis. In isolated tissue bath preparations relaxation of cavernosal tissue from rabbit has been demonstrated to be dependent upon the presence and integrity of the sinusoidal endothelium [11]. In an in vivo dog model, Trigo-Rocha et al. have recently shown that methylene blue, a guanylate cyclase inhibitor, significantly inhibited
Figs. 3-5. Effect of prostaglandin E1, on cAMP synthesis by the aortae of diabetic (●) and control rats (○). Each point = mean ± SEM, n = 7.

The erectile response to both NaNP and electrical field stimulation [9]. Furthermore, they showed that injection of cGMP elicited an erectile response. In the present study, NaNP-stimulated cGMP synthesis in the penis in similar quantities to the aorta. However, Ach did not elicit the generation of detectable amounts of cGMP in the penis of the rat. This indicates that although NO-stimulated cGMP is present in the penis of the rat, the release of NO from the endothelium is not under cholinergic control, at least in this species. Furthermore, cGMP synthesis in response to NaNP was significantly enhanced in the penis of diabetic rats when compared with controls. This enhanced capacity to generate cGMP may constitute an adaptive phenomenon aimed at counteracting the reduction of other pro-erectile factors such as vasoactive intestinal peptide, PGI2 and cholinergic receptors [18].

Apart from the relaxation of erectile tissues, vasodilation of the pudendal, penile and cavernosal arteries is important in achieving an erection. As these vessels were too small to study in the rat model, the aorta was used as an indicator of what might be occurring in the vasculature during erection. NaNP-stimulated cGMP synthesis was found to be enhanced in the aortae of diabetic rats although Ach-stimulated cGMP synthesis was significantly reduced. Kamata et al. also demonstrated a reduction of Ach-stimulated cGMP synthesis from the aortae of diabetic rats but the responses of
aortic tissue to NaNP (relaxation) were unchanged [23]. Taken together, these data indicate that the attenuated responses to Ach may be due to either a defect in Ach receptors linked to NO release or a reduction of NO synthase (NOS) activity. These results suggest that there is a generalized reduction in receptor activity in the vasculature of the diabetic rat which has been ascribed to altered protein kinase C activity [24]. As such a defect may occur in the vasculature associated with erection, Ach-stimulated NO release from penile vasculature warrants investigation in larger animals. Such work might also reveal important regional variations in endothelial function.

With reference to adenylate cyclase, the present study demonstrates that PGE₂ is a potent stimulator of cAMP synthesis by the penis of the rat although Ach and NaNP had no effect on the synthesis of this nucleotide. As intracavernosal injection of PGE₂ elicits erection in both man and rat [27], it can be concluded that PGE₂ does so by activation of adenylate cyclase. The cAMP that is generated probably elicits dilatation and/or relaxation of cavernosal tissue via resequstration of Ca²⁺ [25]. cAMP generated in response to PGE₂ was greater in the penises of diabetic rats than controls. As PGI₂ release was diminished in the penis of diabetic rats [16, 19], this up-regulation of adenylate cyclase activity may constitute an adaptive phenomenon aimed to compensate for the diminished availability of endogenous dilator eicosanoids. Of interest was the fact that Ach failed to elevate cAMP in penises either from controls or diabetic rats.

As Ach stimulates PGI₂ release by cavernosal tissue, a sequence involving Ach-PGI₂-cAMP probably does not play a part in erection, at least in the rat. In a recent study, Trigo Rocha et al. concluded that cAMP does not play a major role in erection in the dog because the cAMP inhibitor, N-ethylmaleimide, failed to reduce the response to both electric field stimulation and NaNP [9]. Investigation of the effect of N-ethylmaleimide when cAMP synthesis was stimulated with a prostanoid may have been more appropriate. Certainly the present study indicates that NaNP has no effect on cAMP levels. Similarly, electrical field stimulation is not known to elevate cAMP so an inhibitor of adenylate cyclase would not have been expected to have an effect on erection elicited by this pathway. Studies have also shown that cAMP administration to dogs was much weaker than cGMP in eliciting relaxation [9]. However, cAMP does not readily enter cells and is rapidly hydrolysed by phosphodiesterases [26]. Therefore to preclude a role for cAMP (and therefore of endogenous prostaglandins) on the basis of this experiment may be spurious as neither field stimulation nor NaNP, as in the present study, elicited cAMP synthesis.

As adrenaline stimulates PGI₂ release in aorta of the rat [27], it has been suggested that this eicosanoid may elicit relaxation via cAMP elevation [18, 27]. Hassid et al. have demonstrated that agonists that elicit PGI₂ release in cultured vascular cells subsequently stimulate cAMP generation [28]. Furthermore, because adrenaline-stimulated PGI₂ is markedly reduced in aortae from diabetic rats [24] adrenaline-stimulated cAMP would be expected to be reduced. However, this was not the case in the present study. Whether there is a direct link between adrenceptors and adenylate cyclase (perhaps G protein mediated) or whether there is an up-regulation of adenylate cyclase in the vessels of diabetic patients requires further experimentation.

In a broader context, the present findings are of relevance to atherogenesis, the incidence of which is increased in diabetics and contributes to impotence [1-4]. Atherogenesis is a complex process which involves proliferation of vascular smooth muscle cells, cholesterol accumulation, platelet adhesion with aggregation and invasion by macrophages [29]. Because all these events are counteracted by cGMP and cAMP (and therefore PGI₂ and NO), any impairment of adenylate or guanylate cyclase may be pro-atherogenic. However, the present study indicates that alterations in the activity of these cyclases is unimpaired and indeed is enhanced in penile and vascular tissue from diabetic rats, and as such may not contribute to the pathophysiology of diabetic angiopathy. Moreover, defects in receptors, as are known to occur in patients with diabetes mellitus, or NOS activity may indirectly alter the levels of cAMP and cGMP seen in these tissues which in turn may render vascular and/or penile tissue susceptible to atherogenesis. The elucidation of the inter- and intra-cellular pathways underlying erectile tissue relaxation allows the identification of targets for novel therapeutic approaches; for example, it may be possible to bypass the receptors to activate cyclases directly. Although the present study did not establish whether the diabetic rats in this study were impotent, there are recent reports that erectile dysfunction occurs in the diabetic rat model [2]. We are currently investigating penile erection in this model, with particular reference to cyclic nucleotide modulators, to further elucidate the relevance of the present findings.

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REVIEW
Eicosanoids, Erections and Erectile Dysfunction

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INTRODUCTION

Although not in itself life-threatening, erectile dysfunction (ED) is a common and distressing condition with far-reaching and obvious implications for the quality of life of those afflicted with it. Over the past 20 years major advances have taken place in our understanding of the basic physiology of normal erection; these advances have resulted in an increasingly sophisticated approach to the investigation of ED, as well as the development of more effective treatments. ED is the term currently used to describe erectile impotence and is defined as 'an inability of the male to achieve an erect penis as part of the overall multifaceted process of male sexual function' (1). It should be remembered that ED is a symptom complex which is often multifactorial in aetiology and does not in itself constitute a diagnosis. More recently, interest has focused on the role of endogenous modulators of vascular tone in erection and detumescence. These include eicosanoids, nitric oxide (NO), vasoactive intestinal polypeptide (VIP), substance P, endothelin etc. An understanding of these systems, in turn, may lead to improved therapeutic interventions.

This review is concerned with the role of eicosanoids in normal erection, their role in the pathophysiology of ED, and the therapeutic uses of prostaglandins (PG) in ED. PGE₁ has now become widely used in the pharmacotherapy of ED and a licensed product is to be shortly available in the UK for this indication.

THE ANATOMY AND PHYSIOLOGY OF ERECTION

Prior to discussing eicosanoids, a resume of the anatomy and physiology of erection is warranted. The erectile tissues of the penis are found within the corpora cavernosa and the corpus spongiosum. The corpus spongiosum is a ventrally situated structure that surrounds the urethra and dilates distally to form the glans penis. The paired corpora cavernosa are dorsally situated structures that are joined by a thin fibrous midline septum. The corpora cavernosa separate proximally to form the crura of the penis which are attached to the inferior aspect of each ipsilateral ischiopubic ramus. The crura and bulb of the penis are covered inferiorly by the ischiocavernosus and bulbocavernosus muscles. Each corpus is ensheathed by a thick, two-layered fibrous sheath, the tunica albuginea. The deep fibres of the tunica albuginea pass circularly around each corpus cavernosum to unite in the midline to form the pectiniform septum of the penis. The septum is incomplete distally and such an arrangement is important in allowing for the communication of blood flow between the paired erectile chambers.

The blood supply to the erectile tissues is derived from the penile arteries, which are branches of the internal pudendal arteries. Each penile artery gives rise to cavernous arteries which pierce the tunica albuginea and give off multiple terminal helicine arteries. The helicine arteries open directly into the sinusoidal spaces. The penis has a rich venous drainage: the peripheral sinusoidal spaces of the corpora cavernosa are drained by small venules that coalesce to form venous plexuses beneath the tunica albuginea. A number of these sub-tunical plexuses unite and drain into the short emissary veins which pass through the tunica albuginea (2).

The corporeal parenchyma consists of a trabecular meshwork formed by smooth muscle fibre bundles, endothelial cells, fibroblasts and a collagenous extracellular matrix. A criss-crossing pattern creates the vascular spaces known as lacunar spaces or sinusoids which are lined by endothelial cells surrounded by smooth muscle cells. This arrangement accounts for the spongy nature of the penile tissues. Histological observations would suggest that human erectile tissue may be considered as being a highly specialised vascular tissue (3, 4). Electron microscopic studies investigating the ultrastructure of the erectile tissues have demonstrated the presence of gap junctions in the human corpus cavernosum (5). It has been postulated that these gap junctions are responsible for the rapid transmission of neural or hormonal stimulation which enables the human corpus cavernosum to behave as a coordinated functional syncytium (6), and electro-physiological investigations...
have demonstrated that human corporeal smooth muscle cells in culture are coupled by gap junctions (7).

Normal penile erection is a complex behavioural and haemodynamic event. The key elements in the process of erection are: (1) an increase, at least initially, of the arterial inflow to the penis; (2) a relaxation of the cavernosal smooth muscle; and (3) an intact veno-occlusive mechanism whereby blood is ‘trapped’ within the penis to maintain erection. It is increasingly recognised that the relaxation of penile trabecular and arteriolar smooth muscle is of central importance in both the initiation and maintenance of erection (8, 9). The extra- and intracellular mechanisms that control this relaxation and modulate smooth muscle tone are the subject of a considerable research effort which has been the subject of recent excellent reviews (10, 11).

The tone of the cavernosal smooth musculature, and its modulation, is influenced by a number of mediators which are principally derived from both neuronal and endothelial elements. Penile smooth muscle tone is a dynamic process and depends on a balance between contractile and relaxant influences. During flaccidity, the cavernosal smooth muscles are contracted (12) and a small quantity of blood flows through the sinusoids (13). However, during erection a relaxation of the smooth muscle of the sinusoidal spaces allows them to fill with blood (14–16) and brings about the mechanical veno-occlusive mechanism (17). Recent dynamic vascular studies have shown that the restriction of penile venous outflow is an essential component of the initiation and maintenance of a rigid erection. These venous events have been studied in humans and in animal models (17–21).

It is now thought that veno-occlusion is a passive, mechanical event which is dependent on an adequate arterial inflow, an intact tunica albuginea and smooth muscle relaxation. During flaccidity, the contracted trabecular smooth muscle allows for unhindered venous drainage via the subtunical venous plexus and emissary veins. During tumescence, there is smooth muscle relaxation and the lacunar spaces fill with blood (14, 15). This instantaneously increased arterial inflow, dilation of the arterial tree and sinusoidal filling results in a compression of the subtunical venous plexus, hence increasing the resistance to blood flow through these vessels and stopping flow in the emissary veins (17). Erection is maintained by this greatly decreased venous outflow and rigidity is imparted by the subsequent rise in intracavernosal pressure which is further aided by the contraction of the bulbocavernous and ischiocavernous muscles (14). The vascular events and smooth muscle tone are controlled by autonomic mechanisms (22). However, it has become apparent that the view that erection is mediated by parasympathetic cholinergic mechanisms, and detumescence and flaccidity by sympathetic adrenergic transmission, represents a gross oversimplification of physiological reality. Thus, as in many other smooth muscle structures relaxation occurs as a result of non-adrenergic non-cholinergic mechanisms (23, 24).

EVIDENCE FOR INVOLVEMENT OF PGS IN ERECTION

A variety of experimental approaches have been used in the attempt to elucidate the role of PGs in normal penile erection. When interpreting the results of these studies, it is as well to remember which species the tissue comes from, which part of the penile anatomy is under study, and whether or not we are witnessing physiological or pharmacological phenomena.

A number of studies have now established that the human penis has the capacity to both produce and to subsequently metabolize PGs (12, 25, 26). It is known that homogenates of human corpora cavernosa can produce PGE, PGF, , PGD, and 6-keto-PGF in vitro (25). Furthermore in the human penis it was shown that the production of PGI was under, at least in part, muscarinic control (26). Other investigators demonstrated that human corpus cavernosal endothelial cells in culture have the ability to produce 6-keto-PGF and PGE, and PGF (12) thus confirming the findings of previous studies. The prostanoids so produced are broken down by PG 15-hydroxydehydrogenase (27). That penile tissues possess the capability to synthesize and degrade PGs is perhaps not surprising since the sinusoidal spaces are lined with endothelium and smooth muscle cells (12).

A role for PGs in erection was first indicated by Klinge and Sjostrand when they reported that PGF contracted the corpus cavernosum and penile artery of bulls (28). Experiments with in vitro tissue strips have elucidated the ability of the various prostanoids to either contract or relax penile tissue in organ bath preparations. Thus, PGF, PGl, PGE and TXA2 analogues were all able to contract corpus cavernosum and arteries (29).

It was suggested that the contraction-mediated prostanoid receptor in the human corpora cavernosa is a TXA2 sensitive receptor (30, 31). Jeremy et al showed that muscarinic receptor stimulation caused PGI production by the human corpora cavernosa (26). A major role for PGI in the erectile response seems unlikely because although it is able to relax segments of penile vessels it was unable to do the same to trabecular tissue which it contracted (29). It may be argued that PGI is important in the initial phase of erection to facilitate an increased blood flow as well as acting to prevent platelet aggregation in a situation of relative stasis (29), but one must remember that this is also probably a function of NO (32).

In these organ bath experiments, PGE, and PGE were found to be effective in relaxing both human trabecular tissue and precontracted segments of cavernous artery
neuronal rather than endothelial sources (42). NO has now evidence that penile NO is largely derived from non-cholinergic transmission in the penis (41); there is no evidence that NO is the final determinant of such non-adrenergic candidates at present would exclude a role for these other substances. It is therefore somewhat surprising that there remains a lack of information of the ability of the human corpora cavernosa to produce PGE₁ (40).

OTHER ENDOGENOUS MEDIATORS OF ERECTION

Many putative relaxant neurotransmitters have been postulated to be responsible for penile erection apart from eicosanoids. Evidence has recently been presented that NO is the final determinant of such non-adrenergic transmission in the penis (41); there is now evidence that penile NO is largely derived from neuronal rather than endothelial sources (42). NO has been shown by many investigators to be of importance in the relaxation of penile smooth muscle, in particular its action has been shown to be mediated via the elevation of intracellular guanosine 3′5′ cyclic monophosphate (cGMP) levels (43). However, many other substances, notably VIP, have also been proposed as being important relaxant neurotransmitters and to preclude a role for these other candidates at present would be premature. It would seem likely that a complex multitransmitter system regulates smooth muscle tone, which is also modulated by other locally acting substances.

It has thus been increasingly recognised that the sinusoidal and vascular endothelium plays a role of pivotal import in the balance and control of the penile vasculature and corporeal smooth muscle tone. The endothelium is able to produce a number of vasoactive substances which are able to either dilate or constrict smooth muscle cells. In the present context it is important to note that the endothelium produces PGs (25, 26), NO (23, 24, 44) and endothelins (45, 46) – factors which all influence smooth muscle tone. Endothelin-1 is synthesized by the corpus cavernosum endothelium and elicits strong sustained contractions of corpus cavernosum smooth muscle (45, 46). It is reasonable, therefore to speculate that endothelin may contribute to the maintenance of penile flaccidity.

A number of intracellular second messengers are responsible for the final control of smooth muscle tone: cAMP, cGMP, calcium ions, potassium ions, and inositol triphosphate. The clinical observation that an erection can be produced by a 20 μg injection of PGE₁ may be explained in the light of the recent demonstration of the existence of gap junctions between penile smooth muscle cells (5, 47). The existence of these connexin-43 type junctions allows for the rapid transmission of second messengers between cells and thus enables the penile smooth musculature to act as a functional syncytium (6). Furthermore, electrophysiological experiments have demonstrated that human corporeal smooth muscle cells in culture are also connected by gap junctions (7). These recent findings have added further complexity to the physiology of human penile erection.

ABNORMALITIES OF PG METABOLISM IN PATHOLOGICAL STATES ASSOCIATED WITH ED

In contrast to previously held views that male ED was predominantly due to psychogenic aetiologies, it has become clear that a considerable number of cases are due to organic causes. In particular, it has been established that arterial disease is one of the major causes of organic ED (48). The major risk factors for both atherosclerosis and ED are diabetes mellitus (DM), smoking, hyperlipidaemia and hypertension (49). The effects of two of these risk factors, namely cigarette smoking and DM, on the production of PGI₂ and other eicosanoids by penile tissues have been studied (50, 51).

DM is a very strong risk factor for the development of ED: 50% of diabetics aged over 50 years will have ED (52). DM has effects on the neural and vascular elements of erection. It causes both a peripheral and an autonomic neuropathy, the latter of which is thought to be of principal importance in the development of ED. However, the vascular substrates are also affected and recent interest has focused on the role of vessel reactivity in addition to the development of atherosclerosis.

The effect of streptozotocin (STZ) induced DM in rats was a marked inhibition of PGI₂ synthesis by the penile tissues and aorta (51). These effects were also related to the duration of DM thus mirroring the human situation.
The diminished PG\textsubscript{12} synthesis was reversible if the animals were treated with insulin (51). These findings have implications for the management and perhaps prevention of DM-related ED especially since they are in agreement with the results of two recently published reports which showed that the incidence of DM complications was decreased by intensive insulin therapy (53, 54). Unfortunately, these latter two studies did not investigate the incidence of erectile dysfunction. The potential beneficial effects for ED may involve delaying the development of both diabetic angiopathy and neuropathy. However, it is salient to remember that PG\textsubscript{12} is not a relaxant of trabecular tissues (29) and any extrapolation of its diminished synthesis to relaxant prostanoids may be an entirely invalid supposition. Thus in DM there is a well-established decrease in PG\textsubscript{12} synthesis, a postulated defect in PGE\textsubscript{1} synthesis, and in addition a recent finding that PGE\textsubscript{1} receptors are decreased in ED (55).

Despite these findings it has recently been shown that the ability of PGE\textsubscript{1} to generate intracellular cAMP is actually enhanced in the STZ rat; it was postulated that this may represent an upregulation of adenylate cyclase activity in response to the reduction in endogenous erectogens (35). In the same study, a similar enhancement of cGMP production (elicited by sodium nitroprusside) was also noted.

It is known that cigarette smoking is an important risk factor for ED (49), which may exert its effects via a number of mechanisms (56). Cigarette smoke extracts inhibit that in vitro synthesis of PG\textsubscript{12} by rat penile tissue (50). It has also been clearly demonstrated that cigarette smoking adversely affects penile arterial blood flow by causing acute vasoconstriction (57) irrespective of any long-term effects on atherosclerosis. These effects may be compounded by a lowered arterial oxygen tension which affects the activity of NO synthase (58) and penile receptor-mediated responses (59).

The role of hyperlipidaemia in the pathogenesis of vascular disease and its effects on endothelial function are well-established in tissues other than the penis. Although it has long been recognised that hyperlipidaemia is a risk factor for ED (48, 49), it is only recently that a combined structural and functional study has demonstrated that hypercholesterolaemia results in penile smooth muscle and endothelial dysfunction in an animal model of penile atheroma (60). In the context of recent observations of changes in vessel reactivity with lipid modification (61) it is interesting to speculate that altered local synthesis of prostanoids may be involved in this process.

Hypertension may have adverse effects again by nature of promoting atherogenesis but also by the changes imparted upon platelet function and in particular the abnormal thromboxane A\textsubscript{2} release (62, 63) which may result in premature detumescence.

It has been suggested that drug effects on local prostanoid synthesis may be a contributing factor to ED. Indeed, many of the drugs that are implicated in the aetiology of ED inhibit prostanoid synthesis in a variety of tissues but data specific for penile tissue is sparse. It is important to remember that the adverse effects of any particular drug are to be mediated by a variety of mechanisms which may act at different sites. For example, beta blockers will not only affect prostanoid synthesis (64, 65), but also act to lower systemic blood pressure which in turn lowers the penile perfusion pressure. In addition these drugs may have anti-erectile actions on the central nervous system (66).

**EVENING PRIMROSE OIL (EPO) AND DM NEUROPATHIES**

Intensive insulin therapy and normoglycaemia may prevent and/or slow the development of the long-term complications of DM (53, 54). However, it is difficult in practice to easily achieve such control and prevent the nerve damage and dysfunction that is seen in DM. In the search for other therapeutic manoeuvres, EPO is an agent that has received much attention in this respect.

Current hypotheses about the aetiology of DM neuropathy centre around the concept that nerve damage occurs as a result of endoneurial hypoxia, it is postulated that this represents the most important contribution to the pathogenesis of DM neuropathy (67–69). It has also been suggested that the effects of hyperglycaemia are to raise intracellular sorbitol levels which then exert neurotoxic effects which are important in the development of DM neuropathy (70, 71).

Ward demonstrated that the decreased PG\textsubscript{12} synthesis by nerves in DM was due to a lack of substrate availability (72); while it had been previously shown that in DM there is a deficient conversion of linolenic acid to gamma-linolenic acid (73). It therefore logically follows that supplementation with gamma-linolenic acid might act to elevate PG\textsubscript{12} production which in turn would enhance the perfusion of neural tissues. It has subsequently been reported that dietary supplementation with EPO, which is a rich source of gamma-linolenic acid, prevents nerve conduction deficits developing in STZ-induced diabetic rats (74, 75). Preliminary reports of clinical trials have recently suggested an improvement in nerve function in human diabetic neuropathy in patients treated with gamma-linolenic acid (76).

The mechanism of this protective action of EPO remains unknown and a number of theories have been proposed to explain these observations. In the present context, it has been suggested that EPO acts by providing a substrate for the production of vascular prostanoids – enhancing PG\textsubscript{1}, PGE\textsubscript{1}, and PGE\textsubscript{2} synthesis by such means would act to improve nerve perfusion (77). In a recent study the detailed effects of EPO on nerve conduction were investigated in STZ-induced diabetic rats (78). The contribution of prostanoids to the beneficial effects was assessed by inhibiting the cyclooxygenase...
pathway with flurbiprofen. Cameron and colleagues demonstrated that the main effects of chronic experimental DM on peripheral nerve function were prevented by EPO. The effects of flurbiprofen were to negate the protective effects of EPO, suggesting that cyclooxygenase mediated metabolism is crucial to its role in ameliorating nerve dysfunction in experimental DM (78). The major metabolites of potential vascular importance of gamma-linolenic acid are PGE\(_1\), PGE\(_2\), and PGI\(_2\). A number of observations have made it unlikely that the action of EPO is mediated by PGE\(_1\) (79, 80) and thus it seems likely that either PGI\(_2\) or PGE\(_1\) are the vital metabolites. A factor of possible benefit in a consideration of ED is that it is known that EPO will decrease TXA\(_2\) synthesis (81) which would increase nerve perfusion and decrease platelet aggregation. EPO may therefore act to restore the normal production of endothelial vasodilators.

These studies have been carried out on the peripheral nerves of the STZ-induced diabetic rat which is an established model for human peripheral neuropathy. The STZ-induced diabetic rat is a less well established model for autonomic neuropathy although it is often used as such, an important point to bear in mind as it is the autonomic neuropathy that is the major contributor to the pathoetiology of ED. Nevertheless, in the light of the findings thus far it is perhaps surprising that no experimental or clinical data relating to ED is available.

**THERAPEUTIC APPLICATIONS OF PGS IN ED**

One of the most important advances to be made in the diagnostic and therapeutic approach to ED was the observation and subsequent development of the fact that an intracavemosal injection of papaverine induced erections (82, 83). This observation was extended by other workers who showed that papaverine and other vasoactive agents could be successfully injected by patients to produce 'pharmacological erections' (83–85). Intracavemosal injection and self-injection therapy with vasoactive agents such as papaverine, phentolamine, or PGE\(_1\) either alone or in combination has become a first choice treatment for many clinicians. Each of the agents that is currently used has its own particular advantages and disadvantages. The main disadvantage of any of these pharmacological agents is the risk of either a painful or prolonged erection, or both. Priapism, defined as a prolonged or painful erection not associated with sexual desire and lasting more than 6 h, is a particular concern because if inadequately managed it may lead to irreversible cavemosal fibrosis. The development of Peyronie's type penile plaques and cavemosal fibrosis has also been reported with papaverine (86). The therapeutic use of PGE\(_1\) was first described by Ishii and co-workers (87, 88). A review of the available literature by Juememann and Alken concluded that papaverine may cause prolonged erection in up to 9.5% of patients, while PGE\(_1\) was associated with a considerably lower rate of 2.4% (86). It is also of interest that PGE\(_1\) is effective in a proportion of papaverine non-responders and overall has a response rate of between 70–80% (86, 89). In a more recent review Linet drew similar conclusions from a large number of short and long-term studies (90).

PGE\(_1\) has been compared with more conventional pharmacological agents in many studies and all have demonstrated its efficacy. Unfortunately PGE\(_1\) injection is associated with a significant incidence of penile pain that affects up to 40% of patients (90). This pain is often described as a burning or stinging sensation and it would appear that this pain is probably a function of the molecule itself. This certainly constitutes one of the major disadvantages of treatment with intracavemosal PGE\(_1\). Nevertheless, PGE\(_1\) is undoubtedly safer than the other injectable 'erectogens' probably because of the capacity of the penis to degrade PGs by PG 15-hydroxydehydrogenase (27). Relatively little is known about the pharmacokinetics of PGE\(_1\), but systemic side-effects are rare and this is probably a reflection of the very low doses that are effective, the local degradation of the PG, local stasis in the penis, and because some 90% of circulating PG will be metabolised by a single passage through the pulmonary bed (91). PGE\(_1\) would therefore seem to be the drug of choice at present for intracavemosal pharmacotherapy, but one has to bear in mind its considerably greater expense and the high incidence of penile discomfort which may contribute to the high drop out rate from this form of therapy (92).

Following the recent elucidation of the role of NO as a final determinant of penile erection, a comparative study between a NO donor (linsidomine) and PGE\(_1\) administered intracavemosally was undertaken (93). The findings led the authors to conclude that the erectile response following linsidomine injection was modest compared to that of PGE\(_1\), and consequently it was unlikely that linsidomine represents a viable alternative to PGE\(_1\) (93).

PGE\(_1\) to TXA\(_2\) ratios have been measured as a potential diagnostic tool to distinguish between arteriogenic and psychogenic impotence (94), not very successfully. In particular it may be important to bear in mind that the time courses may be of vital importance in such a procedure (95). Local artifacts, such as the trauma of puncture, may also come into play as trauma elicits massive PG release from vascular tissues (96).

PGE\(_1\) is now used routinely as an integral part of many of the diagnostic procedures that are used in the evaluation of ED. The administration of PGE\(_1\) intracavemosally in itself constitutes an initial screening test. If the patient manages to obtain an erection then the implication is that the penile arterial blood supply must be sufficient (97). Should an erection not be obtained then a number of further dynamic vascular investigations may be performed. The cavernous arteries and their ability to dilate may be assessed by the use of PGE\(_1\) injection combined with colour-coded duplex scanning
(98–100). Such a study not only provides information about the cavernous arteries and the architecture of the corpora themselves, but it also acts as a screening procedure for venous leakage. If venous leakage is suspected then its site and extent is investigated further with dynamic infusion cavemosometry and cavemosography (101). The use of a smooth muscle relaxant such as papaverine or PGE1 is absolutely essential to make the study as physiological as possible. It is likely that the use of PGE1 in diagnostic applications will increase in the future, especially in the light of the low incidence of priapism associated with its administration intracavernosally.

CONCLUSIONS

Despite the existence of a substantial body of work, the precise role of eicosanoids in the physiology of normal erection remains uncertain. PGs have been shown to both relax and contract penile tissues (29) and it is therefore likely that they play a role in the normal erectile process. In disease models PG metabolism is markedly altered and this may contribute significantly to the pathophysiology of ED.

PG receptors have been demonstrated in penile tissues and are reduced in ED (55). There is abundant evidence that PGs act as neuromodulators of peripheral neurotransmission in a variety of tissues (102), however, it is not known if 'prostanergic' relaxant neurotransmission occurs in the penis or if PGs act solely as neuromodulators in erection. For example, it is known that PGE1 will inhibit adrenergically-mediated contraction and facilitate relaxation and therefore penile erection (33). Intracellulary, PGE1 acts via the elevation of cAMP levels (34, 35) which is in contrast to NO which increases the production of cGMP (43). The underlying physiological reality is likely to involve a number of different neurotransmitters and neuromodulators acting in temporal sequence.

Clinically, PGE1 is certainly a very powerful addition to the therapeutic armamentarium for ED. PGE1 has been demonstrated to be both efficacious and safe. Nevertheless, there remain certain disadvantages associated with its use: (1) penile discomfort of varying degree occurs in up to 40% of patients, (2) intracavernosal injection is not an acceptable mode of administration for all patients, and (3) at present, it is considerably more expensive than conventional pharmacological agents. Thus, in terms of pharmacotherapy, PGE1 is not perfect but it probably has the best profile of action and is the current treatment of choice for intracavernosal pharmacotherapy. Despite the above, it is somewhat surprising that it remains unknown if PGE1 is actually synthesized by the human penis.

Future developments in the treatment of ED with PGE1 may include a different route of administration for those patients who are unable to inject themselves. Another option may come from the results of a recent study which showed that a PGE1 cream prepared from suppositories and mixed with surgical lubricant produced full penile tumescence in 30% of subjects (103). It may also be possible to address the problem of the penile discomfort associated with injection by manipulation of the PGE1 formulation. Lastly, we might see attempts to prevent or delay the onset of diabetic ED with a combination of EPO supplementation, intensive insulin treatment and the correction of abnormal lipid profiles as has been argued elsewhere (104). Such an approach may not only improve erectile function, but it might also confer an overall survival benefit on these patients.

References


CORRIGENDA

page 2, line 14:
monophosphate and 3'5'cyclic adenosine monophosphate respectively. These two important second...

page 6, line 29:
Chapter 4

page 7, line 1:
Chapter 5

page 7, line 11:
Chapter 6

page 7, line 26:
Chapter 7

page 27, line 16:
above, during flaccidity the contracted trabecular smooth muscle allows for unhindered venous...........

page 34, line 16:
stimulation, however it did curtail the erectile response (Andersson et al,.................................

page 51, line 2:
from the above that NO is active via soluble guanylate cyclase. The importance of.......................

page 62, line 14:
inflammatory substances: histamine, serotonin, leukotrienes, interleukins, tumour..........................

page 79, line 2:
has been used to identify the veins which provide abnormal drainage of the corporal......................

page 79, line 24:
erectogen a flow of less then 15ml min''  will be required to maintain the erection...........................

page 83, line 1:
is actually licensed for use in ED and thus particular attention should be exercised........................

page 86, line 18:
Each of the agents that is currently used has its own particular advantages.................................

page 96, line 20:
which have been promoted as the way to correct for arteriogenic erectile dysfunction....................

page 106, line 1:
morphological changes. Insulin may be able to restore the conduction.................................

page 123, line 1:
1993; Kidd et al, 1995), (the autoradiographic methodology used in this study is..........................

page 131, line 9:
nucleotide release. One penile disc per tube was sequestered into each of six duplicate tubes containing

page 152, line 13:
on cGMP synthesis over the some concentration range of the eicosanoid.............................

page 187, line 13:
generation from AMP) is markedly enhanced in the penis and aorta of diabetic rats........................
the rat corpus cavernosum when stimulated by 10µM PGE₁ and 10µM NaNP respectively.

ability of NOS to synthesise NO using the [³H]-arginine/citrulline assay method.

explain the inconclusive findings of our EM studies. Moreover, our diabetic animals.

the structure of the cytoplasm without causing damage. Blocks are fixed for one.
Table 6.2 The effects of cigarette smoking on vascular risk factors in men with erectile dysfunction

<table>
<thead>
<tr>
<th>Serum/plasma parameters and age, median (range)</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erectile dysfunction (non-smokers) n=33</td>
<td>1.45 (0.52 - 4.72)</td>
<td>1.5 (0.69 - 3.51)</td>
<td>1.2 (0.47 - 6.13)</td>
</tr>
<tr>
<td>Erectile dysfunction (smokers) n=24</td>
<td>6.2 (4.5 - 8.0)</td>
<td>5.6 (3.9 - 6.9)</td>
<td>5.1 (3.6 - 8.1)</td>
</tr>
<tr>
<td>Controls (non-smokers) n=18</td>
<td>1.4 (0.9 - 2.3)</td>
<td>1.0 (0.8 - 2.2)</td>
<td>1.3 (0.9 - 2.5)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>Total cholesterol (mmol/l)</td>
<td>High density lipoprotein (mmol/l)</td>
<td>Low density lipoprotein (mmol/l)</td>
</tr>
<tr>
<td>4.1 (2.5 - 5.2)</td>
<td>3.8 (2.7 - 5.1)</td>
<td>3.7 (2.2 - 5.1)</td>
<td>3.26 (2.07 - 4.33)</td>
</tr>
</tbody>
</table>

All results were analysed by Mann Whitney test. Tgs: all non-significant (NS). TC: A vs. B p=0.01; B vs. C=NS; A vs. C p<0.04. HDL: all NS. Age: all NS. Fibrinogen: A vs. B p<0.02; A vs. C=NS; B vs. C p<0.02