CHARACTERIZATION, LOCALIZATION AND SOLUBILIZATION OF P_{2X}-PURINOCEPTORS

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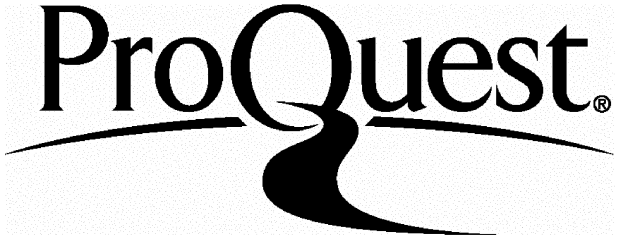

To XB, the poor dreamer

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Abstract

A radioligand binding assay was established to study the biochemical characteristics of P_{2X}-purinoceptors. α,β-Methylene ATP was tritiated, and it was found that the binding of [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) to rat bladder membrane preparations was rapid, fully reversible, and saturable. Competitive displacement showed that the potency order of the unlabelled purinergic ligands in displacing [^{3}H]α,β-MeATP binding was: α,β-methylene ATP > β,γ-methylene ATP > suramin > ATP > 2-methylthio ATP > ADP >> adenosine, which indicates the binding sites are, or are related, to P_{2X}-purinoceptors. Saturation assays revealed high- and low-affinity binding state, with K_d values of approximally 6 and 80 nM respectively. The optimal pH value of the binding was around 7, and the specific binding sites could be completely denatured by heat. The binding was greatly influenced by Na^+, Ca^{2+}, and Mg^{2+} ions in the media. Autoradiographic localization showed that the [^{3}H]α,β-MeATP binding sites were distributed only over the smooth muscle cells of the rat urinary bladder, vas deferens, and the rabbit ear artery, which indicates that the binding is tissue-specific. The basic criteria for the recognition of a ligand binding site as a receptor or a subunit were fulfilled. Further competitive displacement experiments with other nucleotides, base and polyphosphate suggest that the critical structure for the interaction of the ligand with P_{2X}-purinoceptor is the polytriphosphate moiety.

A comparative study carried out on the urinary bladder and urethra of rat, guinea-pig and rabbit showed that rat bladder contained the highest density of [^{3}H]α,β-MeATP binding sites, followed by rabbit and guinea-pig. Semi-quantitation of the autoradiograms revealed a similar order of binding site densities. The urethra of the rat and guinea-pig, a tissue where the P_{2X}-purinoceptor-mediated responses have been shown to be absent, lacked specific labelling.

In human urinary bladder, P_{2X}-purinoceptor-mediated neurogenic contractile responses were reported to be absent or to exist only in some individuals. Both radioligand binding assay and autoradiographic localization showed that specific binding sites of [^{3}H]α,β-MeATP were observed only in about 38 - 42% of the bladder detrusor of old male subjects, and the density was much lower than those in rat, guinea-pig and rabbit bladder.

In many blood vessels extracellular ATP has been reported to elicit contractions of smooth muscle. Radioligand binding assays showed that rabbit ear artery contained a high density of high-affinity [^{3}H]α,β-MeATP binding sites. Autoradiographic localization demonstrated specific binding sites of [^{3}H]α,β-MeATP in many blood vessels from rat, guinea-pig and rabbit. Generally, medium- and small-sized muscular arteries, such as rat saphenous, rat tail, and rabbit ear arteries, contained higher densities of binding sites than large elastic arteries like aorta. In some large arteries such as rabbit carotid, renal and hepatic arteries,

the binding sites were denser over the outer region of the vascular wall than the inner region. Only sparse specific binding was observed in all veins other than the portal vein from all the three species. In the portal veins, the densities over the circular and longitudinal muscles were different.

The high-affinity component of the specific [^{3}H]α,β-MeATP binding sites were successfully solubilized from rat vas deferens with 2% digitonin. The solubilized [^{3}H]α,β-MeATP binding sites still possessed the characteristics of the membrane-bound binding sites, i.e., rapid association and dissociation, reversibility, and saturability. The potency order of the unlabelled ligands in displacing the [^{3}H]α,β-MeATP binding was similar to that obtained from membrane binding experiments. Sucrose density gradient ultracentrifugation showed that the sedimentation coefficient of receptor-detergent complex was 12.1 S. Upon UV irradiation [^{3}H]α,β-MeATP was cross-linked to a molecule of 62,000 daltons in rat vas deferens membrane, which might be the functional receptor or the binding subunit.

Publications

Full papers

Bo, X. & Burnstock, G, (1989), [^{3}H]α,β-Methylene ATP: a radioligand for P_2-purinoceptors, *J. Auton. Nerv. Syst.*, 28:85-88.

Bo, X. & Burnstock, G., (1990), High- and low-affinity binding sites for [^{3}H]α,β-methylene ATP in rat urinary bladder membranes, *Br. J. Pharmacol.*, **101**:291-296.

Bo, X. & Burnstock, G., (1990), The effects of Bay K 8644 and nifedipine on the responses of rat urinary bladder to electrical field stimulation, β,γ-methylene ATP and acetylcholine, *Br. J. Pharmacol.*, **101**:494-498.

Bo, X. & Burnstock, G., (1992). Species differences in characteristics and distribution of [^{3}H]α,β-methylene ATP binding sites in urinary bladder and urethra of rat, guinea-pig and rabbit. *Eur. J. Pharmacol*, 216:59-66.

Bo, X. & Burnstock, G., Heterogeneous distribution of [^{3}H]α,β-methylene ATP binding sites in blood vessels from rat, guinea-pig, and rabbit, (submitted to *J. Vascul. Res.*).

Bo, X. & Burnstock, G., Characterization and autoradiographic localization of [^{3}H]α,β-methylene ATP binding sites in human

urinary bladder (submitted to *J. Urol.*).

Bo, X., Simon, J., Burnstock, G. & Barnard, E.A. (1992). Solubilisation and molecular size determination of P_{2X}-purinoceptor from rat vas deferens. *J. Biol. Chem.*, (in press).

Bo, X. & Burnstock, G., Further characterisation of [^{3}H]α,β-methylene ATP binding sites in rat urinary bladder (ready to be submitted).

Abstracts

Bo, X., Neely, C.F. & Burnstock, G., (1992) Autoradiographic localization of [^{3}H]α,β-methylene ATP binding sites in pulmonary arteries from man and cat. (Abstract for the Annual Meeting of Federation of American Society of Experimental Biology, Anaheim, U.S.A., April, 1992) *FASEB. J.*, 6:A1555.

Bo, X. & Burnstock, G., (1992) Autoradiographic localisation of [^{3}H]α,β-methylene ATP binding sites in human blood vessels. (Abstract for the meeting "Therapeutic Opportunities of Purinergic Transmission", Oxford, U.K., March, 1992)

Bo, X. & Burnstock, G. (1992). Heterogeneous distribution of [^{3}H]α,β-methylene ATP binding sites in blood vessels. (Abstract for the meeting "Purines 92, Pharmacology and Clinical Applications", Milan, Italy, June, 1992), *Int. J. Purine Pyrimidine Res.* 3:65.

Simon, J., Bo, X., Barnard, E.A. & Burnstock, G. (1992). Solubilisation and molecular characterisation of P_{2X}-purinoceptor from rat vas deferens. (Abstract for the meeting "Purines 92, Pharmacology and Clinical Applications", Milan, Italy, June, 1992), *Int. J. Purines Pyrimidine Res.* 3:17.

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Preface

It has been found that extracellular ATP possesses the characteristics of a neurotransmitter, cotransmitter, or neuromodulator in various tissues. The storage, release, postjunctional actions, and the degradation of ATP have been intensively studied and a considerable body of pharmacological and electrophysiological evidence has been accumulated to support the existence of purinergic neurotransmission. The receptors which mediate the responses to extracellular ATP have been classified into P_{2X}- and P_{2Y}-purinoceptors according to the rank order of potencies of agonists and selective antagonism. However, where are these receptors located? What are their biochemical and molecular properties? Such questions have remained unanswered for a long time largely due to the lack of suitable tools for the study of these receptors.

α,β-Methylene ATP is the most potent agonist identified for P_{2X}-purinoceptors so far, which can also induce desensitization of P_{2X}-purinoceptors after prolonged exposure. Such characteristics of α,β-methylene ATP led me to the idea of using its tritiated form as a radioligand for P_{2X}-purinoceptors. In preliminary experiments [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) was shown to have the basic properties of a radioligand for receptor binding assays. Thus, the main aims of this thesis were to establish [^{3}H]α,β-MeATP as a radioligand for P_{2X}-purinoceptors, to use the

radioligand to explore the biochemical properties and tissue distribution of P_{2X}-purinoceptors, and to employ the radioligand for assays in studies of P_{2X}-purinoceptors at the molecular level.

In Chapter 1, the history of purinergic theory and the most recent achievements in the study of purinergic neurotransmission, especially the signal transduction mechanism for P_2-purinoceptors are briefly reviewed. Because [^{3}H]α,β-MeATP has been used as a radioligand for the first time, the optimal conditions for binding assays were carefully examined and the characteristics of the binding sites were systematically studied; the work is described in Chapter 2. A great advantage of [^{3}H]α,β-MeATP over other reported radioligands (probably with the exception of [^{3}H]β,γ-methylene ATP) is that it has no affinity to ectoATPases and or very weak intracellular ATP binding sites. Thus, its specific binding sites could be localized by using the autoradiographic method. This methodology is described in Chapter 3. With a computer-assisted image analysis system, the densities of autoradiographic grains were counted and compared, offering a relative estimate of the P_{2X}-purinoceptor concentration in various tissues. Based on the results from the experiments described in Chapter 2, it could be positively stated that [^{3}H]α,β-MeATP binding sites were, or were linked to, P_{2X}-purinoceptors. Thus, this ligand was used to characterize P_{2X}-purinoceptors with radioligand binding assays and/or autoradiography in different tissues, including urinary bladder, urethra, vas deferens, and various blood vessels , and from different species including rat, guinea-pig, rabbit, and human. The results are described and discussed in Chapters 4, 5,

6, and part of Chapter 7.

In order to understand further the biochemical and molecular properties of P_{2X}-purinoceptors, the membrane-bound receptors have been solubilized. The procedure for the solubilization of the [^{3}H]α,β-MeATP binding sites are described in Chapter 7. The characteristics of the soluble binding sites were studied and compared with those of the membrane-bound sites. The size of the receptor-detergent complex was estimated with sucrose density gradient centrifugation and the molecular weight of the P_{2X}-purinoceptor, or its binding subunit, was determined with photoaffinity labelling. Finally, in Chapter 8 the work in this thesis is reviewed in the context of other studies and future trends for the study of P_2-purinoceptors are discussed.

Chapter 1 Introduction

Oh tell me Lord how it could be,
That though our cells make ATP,
It's not all used for energy,
But sometimes is secreted free,
It puzzles you, it puzzles me,
While Geoffrey Burnstock smiles with glee,
At the many roles of ATP.

— Samuel. C. Silverstein[1]

1 From Preface for Biogical Actions of Extracellular ATP, eds. Dubyak, G.R. & Fedan, J.S., (1990), York Academy of Sciences.

1-1 Brief Review of the History of Purinergic Neurotransmission

Adenosine triphosphate (ATP), the "energy currency of the cells" and the "bricks" of genes, is sometimes secreted from cells. Why are some cells so generous as to give away this precious substance? Surely it is not a meaningless phenomenon. More than 60 years ago, the regulatory effects of purine compounds on cardiovascular system were observed by Drury and Szent-Györgyi (1929). Following this report, pharmacological actions of purine compounds in various tissues, mainly the cardiovascular system, have been studied (Wedd, 1931; Gaddum & Holtz, 1933). The work in this area was summarized by Green and Stoner (1950). The first evidence that ATP might be a neurotransmitter came from the studies on sensory innervation in the 1950's (Holton & Holton, 1953; 1954; Andrews & Holton, 1958; Holton, 1959). In the early 1960's, a nervous component that was neither adrenergic nor cholinergic was recognised in the autonomic nervous system (Burnstock, *et al.*, 1964; Burnstock, 1969). Such non-adrenergic, non-cholinergic nerves have been shown to be present in urinary bladder, gastrointestinal tract, oesophagus, seminal vesicle, uterus, trachea, eye, and parts of the cardiovascular system. Based on the evidence about the synthesis, storage, release, degradation, and postsynaptic effects of purine compounds, in 1972 Burnstock proposed the concept of "purinergic nerves", utilizing ATP or derivatives as the transmitters. Four years later, another proposal was made by Burnstock: ATP might also be a co-transmitter with noradrenaline, acetylcholine and other substances (Burnstock,

1976). This new concept broke the monopoly of the classic adrenergic and cholinergic nerves in the autonomic nervous system, and caused disturbance in the world of neuroscience. Many scientists held a sceptical attitude and did not believe that this ubiquitous substance could have some specific extracellular regulatory functions. However, due to the persistent work of Burnstock and colleagues, and other open-minded scientists, the regulatory roles of extracellular ATP and derivatives have gained better and better understanding. The concept of purinergic neurotransmission is being recognized by more and more scientists.

In 1978, Burnstock proposed the basis to distinguish two main types of purinoceptors, P_1- for adenosine, and P_2- for ATP/ADP (Burnstock, 1978). Seven years later, a further classification of P_2-purinoceptors into P_{2X}-and P_{2Y}-subtypes based on the relative potencies of ATP analogues and selective antagonism were proposed by Burnstock and Kennedy (1985). The classification became two milestones in the progress of the research on purinergic transmission, which pushed the work to a more prosperous period. Nowadays, the physiological effects of extracellular ATP have not only been observed in the autonomic nervous system, but also in the central nervous system (see review by Williams, 1990 and Hoyle & Burnstock, 1991) and the sensory nervous system (see review by Hoyle & Burnstock, 1991). New discoveries about the physiological or pharmacological effects of purine compounds are emerging with increasing speed. Many of the newly observed functions have even passed the traditional boundary of the nervous system, such as the

release of ATP from endothelial cells (see review by Gordon, 1990; Ralevic & Burnstock, 1991), the influence of ATP and analogues on the functions of neutrophils (Ward *et al.*, 1990) and mononuclear phagocytes (Steinberg *et al.*, 1990), and anticancer activities (Rapaport, 1990). The involvement of purinergic transmission in pathological conditions such as ischemia, hypertension, hypoxia, and shock, etc. are under investigation (Burnstock, 1990b; 1991a; Burnstock *et al.*, 1991). The therapeutic potential of ATP and analogues has been shown in patients with ischemia and shock (Chaudry, 1990).

1-2 Purinoceptors

The suggestion that adenine nucleosides and nucleotides may have different pharmacological actions (and therefore different receptors) was first presented by Gillespie (1933), who observed that ATP was more potent than its non-phosphorylated derivatives in causing relaxation of the guinea-pig ileum, and adenosine was more potent than its phosphorylated derivatives in causing vasodilation, or inducing hypotension in cats and rabbits. Purinoceptors were divided into P_1- and P_2-subtypes on the basis of differential rank orders of agonist potency, different antagonism, and different signal transduction mechanisms (Burnstock, 1978).

1-2-1 P_1-purinoceptors

The rank order of potency of purine compounds in activating P_1-

purinoceptors is adenosine ≥ AMP >> ADP ≥ ATP. They are blocked by methylxanthine derivatives. P_1-purinoceptors were further classified into A_1- and A_2-subtypes on the basis of inactivation and activation of adenylate cyclase (Van Calker, *et al.*, 1979). However, in recent years, it has become apparent that at least the A_1 receptor can have effects that are independent of cAMP, including opening K^+ channels (Trussell & Jackson, 1987), closing Ca^{2+} channels (Schubert & Kreutzberg, 1987), and inhibiting (Delahunty *et al.*, 1988) or stimulating (Arend *et al.*, 1989) phosphatidylinositol turnover. Another type, the A_3-adenosine receptor, was described recently (Ribeiro and Sebastiao, 1986; Sebastiao and Ribeiro, 1988; 1989) because this type of receptor is not linked to an adenylate cyclase transduction mechanism, but affects the calcium mobilisation.

Both A_1- and A_2-adenosine receptors have a wide distribution in central and peripheral tissues, including brain, liver, kidney, heart, arteries and veins, gastrointestinal tract, vas deferens, and fallopian tubes. Adenosine arising from released ATP at nerve endings has the character of a neurotransmitter, or a neuromodulator, being involved in the negative feedback regulation of neurotransmitter release. Therefore, high densities of A_1 receptors have been observed in many regions of the brain. However, in other organs, adenosine probably acts as a local hormone rather than a circulating hormone or a neurotransmitter. Unlike classic neurotransmitters, adenosine can be produced by virtually any cell. Adenosine does not appear to be stored in

excytotic vesicles, but rather is produced on demand, in character with prostaglandin and the leukotrienes.

The main physiological role of A_1- and A_2-adenosine receptors seems involved in protecting against the consequences of inadequate oxygenation. A_1 activation almost invariably brings about a decrease in oxygenation. The effects include decrease of heart rate, heart force, lipolysis, renin release, neuronal firing, transmitter release, body temperature and breathing, and contraction of renal afferent arterioles. Most A_2-mediated responses bring about an increase in local oxygen supply, the responses include: vasodilatation, inhibition of platelet aggregation, decreasing neutrophil activation, increasing breathing, dilatation of renal efferent arterioles, increasing renin release, relaxation of gut smooth muscle, and decreasing locomotor activity (see Bruns, 1990 for review).

The distinction of A_1- and A_2-adenosine receptors was not only dependent on their effects on adenylate cyclase, but also defined by their structure-activity relationship. Originally, A_1- and A_2-receptors were distinguished on the basis of the rank order of potency of the agonists N^6-(*R*)-(phenylisopropyl)adenosine (*R*-PIA) and 5'-*N*-ethyl-carboxamidoadenosine (NECA), and the magnitude of the difference in affinity between the *R* and *S* diastereomers of PIA. However, these criteria have problems because the potency order is not consistent in different tissues, particularly between central and peripheral tissues. Recently more selective agonists have been developed. Research on adenosine receptor antagonists

have also proceeded rapidly, many selective antagonists, especially for the A_1-adenosine receptor, have been reported. Due to the availability of these selective agonists and antagonists, and their radiolabelled forms (Schwabe, 1985), the biochemical characterization and distribution of P_1-purinoceptors have been well studied. The A_1-adenosine receptors have been successfully solubilized (Gavish *et al.*, 1982; Nataka & Fujisawa, 1983; Stile, 1985; Klotz *et al.*, 1986; Casadó *et al.*, 1990) and purified (Nataka, 1989; 1990; Olah *et al.*, 1990). Solubilization of A_2-adenosine receptors has also been reported (Ronca-Testoni *et al.*, 1984). Monoclonal antibodies against A_1-adenosine receptors have been raised by an auto-anti-idiotypic approach (Ku *et al.*, 1987). Recently, the cDNAs for canine A_1- and A_2-adenosine receptors have been cloned with homology-screening protocol (Libert *et al.*, 1989; 1991; Maenhaut *et al.*, 1990; reviewed by Linden *et al.*, 1991). cDNAs for A_1-adenosine receptors have also been cloned from rat and bovine brains (Mahan *et al.*, 1991; Reppert *et al.*, 1991; Olah *et al.*, 1992; Tucker *et al.*, 1992). cDNAs for A_2-adenosine receptors have been cloned recently from rat brain (Pollack *et al.*, 1992; Stehle *et al.*, 1992), which encodes a protein of 410 amino acid, while the A_1-adenosine receptor consists of 326 amino acids. Human A_1- and A_{2a}-adenosine receptor cDNAs have also been isolated from ventricle, cerebral cortex, and kidney cDNA libraries (Salvatore *et al.*, 1992). These receptors belong to the G-protein-coupled receptors constituting such a family of proteins encoded by genes with a common ancestor. They share a monomeric structure with seven putative transmembrane domains and the

capacity to modulate the activity of the effector enzymes or ion channels via GTP-binding proteins.

1-2-2 P_2-purinoceptors

The order of potency of purine compounds in activating P_2-purinoceptors is ATP ≥ ADP >> AMP ≥ adenosine. P_2-purinoceptors are not antagonized by methylxanthines. P_2-purinoceptors have been subclassified into P_{2X}- and P_{2Y}-subtypes on the basis of the rank order of potency of ATP analogues and selective antagonism (Burnstock & Kennedy, 1985). Two more subclasses, i.e., P_{2T} and P_{2Z}, were proposed (Gordon, 1986). P_2-purinoceptors also have a wide distribution in various tissues, so far, their functions were mainly observed in peripheral tissues. Activation of P_{2X}-purinoceptors usually leads to contraction of visceral and vascular smooth muscle. P_{2X}-purinoceptor-mediated responses have been reported in the gastrointestinal tract, arteries, veins, vascular beds, urinary bladder, vas deferens, anococcygeus, and nictating membrane. P_{2Y}-purinoceptor-mediated responses are usually inhibitory, such as relaxation of blood vessels. The relaxation of the blood vessels is mainly through the P_{2Y}-purinoceptors on the endothelial cells, the activation of the receptor leads to the release of endothelium-derived relaxation factor (EDRF). P_{2Y}-purinoceptors have also been located on hepatocytes and involved in the control of glycogenolysis (Keppens & De Wulf, 1985; 1986). Insulin secretion from pancreatic β-cells was found to be increased after activation of P_{2Y}-purinoceptors (Bertrand *et al.*, 1987). ATP was also reported to be one of the

most potent secretagogues for surfactant phospholipid secretion, such an effect was mediated by P_{2Y}-purinoceptors (Rice & Singleton, 1987; 1989). P_{2Y}-purinoceptors were also shown to exist on the turkey erythrocyte membranes where they are coupled with G-protein-regulated phospholipase-C (Boyer *et al.*, 1989; Cooper *et al.*, 1989).

The original criteria for the distinction of P_{2X}- and P_{2Y}-purinoceptors are: P_{2X}-: (1) the potency order of ATP analogues: α,β-methylene ATP, β,γ-methylene ATP > ATP > 2-methylthio ATP; (2) antagonism by arylazidoaminopropionyl-ATP ($ANAPP_3$); (3) desensitisation by α,β-methylene ATP; P_{2Y}-: (1) potency order: 2-methylthio ATP >> ATP > α,β-methylene ATP, β,γ-methylene ATP; (2) weak antagonism by $ANAPP_3$ and desensitisation by α,β-methylene ATP (Burnstock & Kennedy, 1985). Some efforts have been made to develop more selective and potent agonists and antagonists for both of the receptor subtypes. L-β,γ-methylene ATP was claimed to be a pure agonist for P_{2X}-purinoceptor, which is also completely resistant to dephosphorylation (Cusack & Hourani, 1984; Hourani, 1984; Hourani *et al.*, 1985). Adenosine 5'-(2-fluorodiphosphate) (ADP-β-F) was reported to be a specific agonist for P_{2Y}-subtype (Hourani *et al.*, 1988). However, in rabbit jugular vein, ADP-β-F may inhibit P_1-purinoceptor mediated responses (Wood *et al.*, 1989). Several new ATP analogues are under test in our laborotary.

Equilibrium competitive antagonists that act specifically and reversibly at the receptor, and that are devoid of agonist or non-

specific activity, have not been reported for either P_{2X}- or P_{2Y}-purinoceptors (Fedan & Lamport, 1990). Several chemicals which either structurally resemble ATP or bear no obvious structure relationship to ATP have been studied. $ANAPP_3$, a photoaffinity analogue of ATP, has been shown to block the P_{2X}-purinoceptor-mediated responses after photoactivation (Hogaboon *et al.*, 1980; Fedan *et al.*, 1982, Westfall *et al.*, 1982). Suramin, a trypanocidal drug, has been reported to be a P_2-purinoceptor antagonist, but it is effective at both P_{2X}- and P_{2Y}-subtypes (Dunn & Blakeley, 1988; Den Hertog, *et al.*, 1989; Hoyle *et al.*, 1990). α,β-MeATP has been used as a selective desensitizer of the P_{2X}-purinoceptors (Kasakov & Burnstock, 1983). Most recently, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid was reported to block the prazosine-resistant component of the neurogenic contraction as well as the α,β-MeATP evoked contraction in rabbit vas deferens (Lambrecht *et al.*, 1992a; 1992b). This compound might become a useful tool for the study of P_2-purinoceptors, although its selectivity needs to be further investigated.

In addition to P_{2X}- and P_{2Y}-purinoceptors, P_{2T}- and P_{2Z}-subtypes were introduced (Gordon, 1986). The P_{2T}-subtype exists on platelets and is activated by ADP, and antagonized by many ATP and AMP analogues. P_{2Z}-subtype is activated by ATP^{4-}, and is involved in the secretion of mast cells (Cockroft & Gomperts, 1979a and b; 1980; Bennett *et al.*, 1981; Tatham *et al.*, 1988). Several other subtypes were also proposed, such as, P_{2S}-, P_3-, (Wiklund & Gustafsson, 1988b; Shinozuka *et al.*, 1988), because they showed different characteristics from the subtypes described above. The

proposal of the new subtypes was mainly based on the finding that the potency order of the agonists was different from that for other subtypes. Further study is required to confirm their existence.

The biochemical characterisation of P_2-purinoceptors has lagged behind mainly due to the lack of selective antagonists. Radioligand binding assays for the P_{2Y}-purinoceptor was first carried out on hepatocytes with adenosine 5'-[α-[^{35}S]thio] triphosphate ([^{35}S]ATPαS) as the radioligand (Keppens & De Wulf, 1986; Keppens *et al.*, 1989). It is suggested that the binding corresponds to the physiological P_{2Y}-purinoceptors involved in the regulation of liver glycogenolysis. Another binding study was carried out on purified rat heart sarcolemma membranes using adenosine 5'-[γ-[^{35}S]thio] triphosphate ([^{35}S]ATPγS) as the radioligand (Zhao & Dhalla, 1990). So far the most thorough study on the biochemical properties of P_{2Y}-purinoceptors was carried out on turkey erythrocyte membranes, which has been summarized by Harden *et al.*, (1990). It was found that activation of the P_{2Y}-purinoceptors leads to the activation of G-protein regulated phospholipase (Harden *et al.*, 1987; 1988; Berrie *et al.*, 1989). P_{2Y}-purinoceptor on the turkey erythrocyte membranes was labelled with adenosine 5'-*O*-2-thio[^{35}S] diphosphate ([^{35}S]ADPβS) (Copper *et al.*, 1989). It was found that the radioligand bound to a homogeneous population of sites with a high affinity, which was modified by guanine nucleotides. The binding sites were successfully solubilized from the membranes and were still coupled

with G-protein (Jeffs *et al.*, 1991).

In transformed 3T6 mouse fibroblasts, P_{2Y}- and P_{2Z}-purinoceptors have been identified by using photoaffinity labelling with [α-^{32}P]3'-O-4-(benzoyl)benzoyl ATP (BzATP) (for both P_{2Y}- and P_{2Z}-purinoceptors) and [α-^{32}P]BzUTP (for P_{2Y}-purinoceptor) (Erb *et al.*, 1992; Weisman *et al.*, 1992). The P_{2Y}-purinoceptor was shown to have a molecular weight of 53 kDa while the P_{2Z}-subtype 300 kDa. It has been claimed that polyclonal antibodies have been raised against the purified 53 kDa and 300 kDa proteins and degenerate oligonucleotide probes corresponding to the N-terminal sequence of these proteins have been synthesized and are being used in an attempt to clone the genes that code for the putative P_{2Y}- and P_{2Z}-purinoceptors in 3T6 cells (Erb *et al.*, 1992).

The first attempt to label the excitatory purinoceptors was carried out on rabbit urinary bladder with [^{3}H]ATP (Levin, *et al.*, 1983). It was concluded that the binding sites were not the substrate binding site on calcium/magnesium ATPase and might be related to the contractile sites on the bladder membrane. However, the existence of possible intracellular binding sites and rapid degradation of [^{3}H]ATP limit its use as a radioligand. More recently the same group used [^{3}H]β,γ-methylene ATP to label P_{2X}-purinoceptors in urinary bladder (Ruggieri *et al.*, 1990). The advantages of [^{3}H]β,γ-methylene ATP are its relative resistance to dephosphorylation and weak affinity to ATPases. Other features observed were that the binding is divalent cation independent and

could be potentially inhibited by EDTA (not reversed by divalent cations). An attempt to probe the molecular properties of P_{2X}-purinoceptors was made by Fedan *et al.* (1985) with a photoaffinity labelling technique by using [^{3}H]$ANAPP_3$ as the photoaffinity radioligand. It was found that upon photolysis [^{3}H]$ANAPP_3$ was incorporated into cellular components of guinea-pig vas deferens membrane with apparent molecular weights of 54 - 66 and 43 - 57 kDa, and the direct antagonism by $ANAPP_3$ of adenine nucleotide-induced responses was a direct result of the covalent interaction at or near the recognition site of cell-surface P_{2X}-purinoceptors.

1-3 Signal transduction mechanisms for P_2-purinoceptors

As the research on the pharmacological responses of various tissues and cell types to extracellular ATP progresses steadily, much information has been obtained on the signal transduction mechanism of purinoceptors. Most of the research indicates that many of the functional responses to extracellular ATP can be ascribed to, or correlated with, alterations in cellular Ca^{2+} homeostasis, usually the increase of cytosolic Ca^{2+} concentration. Such ATP-induced cytosolic Ca^{2+} increase would in turn activate a host of secondary signal transduction processes, including stimulation of diverse Ca^{2+}-dependent regulatory enzymes (e.g., calmodulin-dependent kinases); activation or inhibition of various plasma membrane ion channels (e.g., Ca^{2+}-activated K^+ channels), with consequent changes in membrane potentials and excitability; stimulation of phospholipase A_2 with consequent production of

various eicosanoids. However, comparison of the ATP-induced increase in cytosolic Ca^{2+} concentration observed in diverse cell types has shown that these functional responses appear to involve at least three very different mechanisms of action (reviewed by Dubyak, 1991), and thus strongly support the existence of distinct P_2-purinoceptor subtypes.

In many cell types ATP induces a rapid mobilization of intracellular Ca^{2+} stores, thus, significant biological responses can be triggered even in the medium deficient in extracellular Ca^{2+} (McMillian *et al.*, 1987; Gylfe & Hellman, 1987; Paulmichl & Lang, 1988). Further studies indicate that the increase of the intracellular Ca^{2+} is due to the activation of inositol phospholipid-specific phospholipase C (PI-PLC). Such observations has been reported in aortic endothelial cells (Pi$\hat{r}$otton *et al.*, 1987), adrenal medullary endothelial cells (Forsberg *et al.*, 1987), aortic myocytes (Phaneuf *et al.*, 1987), DDT_1MF-2 smooth muscle cells (Hoiting *et al.*, 1990a; 1990b), cat brain synaptosomes (Huang & Sun, 1988), astrocytes (Pearce *et al.*, 1989), and pituitary cells (Davidson *et al.*, 1990). However, like other members of the superfamily, P_2-purinoceptors appear to indirectly activate PI-PLC effector enzymes via the mediation of GTP-regulatory proteins. It has been shown that in some cell types, the activation of PI-PLC by P_2-purinoceptor agonists requires the presence of GTP and is inhibited by pretreatment of the cells with pertussis toxin (Boyer *et al.*, 1989; Häggblad & Heilbronn, 1988; Nanoff *et al.*, 1990).

As to what subtypes of P_2-purinoceptors are linked to the PI-PLC effector enzymes, more efforts are needed to clarify this matter because only a few reports showed the order of potencies of P_2-purinoceptor agonists in inducing the production of inositol triphosphate. So far, in turkey erythrocyte (Boyer *et al.*, 1989) and rat renal cortex (Nanoff *et al.*, 1990) it has been clearly shown that it is the P_{2Y}-purinoceptor which is coupled to the PI-PLC effector enzymes via G-proteins. In some other cell types, such as aortic endothelial cells (Pirotton *et al.*, 1987), rat hepatocytes (Okajima *et al.*, 1987), astrocytes (Pearce *et al.*, 1989), ATP, ADP, ATPγS, and ADPβS were equipotent to stimulate the production of inositol triphosphate. It is possible that such an effect in these cells is also mediated by P_{2Y}-purinoceptors. In sheep pituitary cells (Davidson *et al.*, 1990) and canine kidney cells (Paulmichl & Lang, 1988), both ATP and UTP are equipotent to elicit the increase of intracellular Ca^{2+}. This receptor may be the tentatively termed P_{2U}-purinoceptor. Some other pathways have also been suggested, such as in rat hepatocyte (Okajima *et al.*, 1987) and in glioma cells (Pianet *et al.*, 1989) one type of P_2-purinoceptors is linked to adenylate cyclase via an inhibitory G-protein. In human promyelocytic cell line HL60 it has been observed that the P_2-purinoceptor-mediated elevation of cytosolic Ca^{2+} is modulated by activation of protein kinase C (Nonotte *et al.*, 1989). ATP-stimulated, G-protein-coupled activation of phospholipase C and D which selectively hydrolyze phosphatidylcholine were also reported in rat liver plasma membrane (Irving & Exton, 1987) and endothelial cells (Martin &

Michaelis, 1989) respectively.

In various smooth muscle preparations such as vas deferens and blood vessels, extracellularly-applied ATP induced depolarization and produced twitches like that elicited by nerve stimulation. Both the ATP-induced depolarization and the nerve-evoked excitatory junction potentials can be inhibited by ATP analogues, such as $ANAPP_3$ or α,β-methylene ATP (Sneddon *et al.*, 1982; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Wakui & Inomata, 1985; Cheung & Fugioka, 1986; Cunnane & Manchanda, 1988). Later on, by using voltage-clamp technique, it has been shown that extracellularly applied ATP can induce an inward current on smooth muscle cells of vas deferens (Nakazawa & Matsuki, 1987; Friel, 1988), ear artery (Benham & Tsien, 1987; Benham *et al.*, 1987; Benham, 1989), and guinea-pig urinary bladder (Marchenko *et al.*, 1987; Inoue & Brading, 1990). The channels which mediate the ATP-induced currents are cation selective, but allow both monovalent and divalent cations to pass across the membrane. Because ATP opens the cation permeable channels with a latency of less than 100 ms and can activate unitary currents in isolated inside-out membrane patches, it is suggested that the purinoceptors are closely coupled to ion channels (Benham & Tsien, 1987). These purinoceptor-gated channels have many similarities to the intensively studied ACh receptor-channel complex that mediates the endplate current at the neuromuscular junction in skeletal muscle. These channels are permeable to a variety of cations such that the reversal potentials are close to zero mV in normal physiological ion gradients. The depolarization of the membrane would trigger

the voltage-dependent Ca^{2+} channels, which elicit contraction of smooth muscles. Such a mechanism explains the sensitivity of purinoceptor-mediated contractile responses to voltage-dependent Ca^{2+} channel antagonists like dihydropyridine derivatives (MacKenzie *et al.*, 1988; Bo & Burnstock, 1990), while ATP-induced depolarization is not affected by Ca^{2+} channel antagonists (MacKenzie *et al.*, 1988).

Apart from smooth muscle cells, ATP-gated ion channels have also been observed in neurones, cardiac myocytes, and cultured developing chicken skeletal muscles. Actually, the first characterization of an ATP-activated conductance under voltage-clamp was reported in snail neurones by Yatani *et al.* (1982). Later on, the properties of the ATP-gated cation channels in mammalian sensory neurones were thoroughly studied by Krishtal and colleagues (Krishtal *et al.*, 1983; 1988a; 1988b; Krishtal & Marchenko, 1984) and also by Bouvier *et al.* (1991). ATP has also been reported to excite a subpopulation of dorsal horn neurones (Jahr & Jessell, 1983; Fyffe & Perl, 1984), raising the possibility that ATP is used as a transmitter released by some primary sensory neurones (Salt & Hill, 1983). Most recently, exciting synaptic potentials and currents from cultured coeliac ganglian neurons were reported to be mimicked by ATP, which indicates that ATP may also be a neurotransmitter in neuro-neuroanl synapses (Evans *et al.*, 1992).

ATP-activated currents have also been recorded in bullfrog

and guinea-pig atrial cells (Friel & Bean, 1988; Matsuusa & Ehara, 1992), and rat ventricular myocytes (Scamps & Vassort, 1990; Zheng *et al.*, 1992; Christie *et al.*, 1992). The best evidence of ATP as a transmitter on controlling heart function has come from the studies on the frog atrium. A positive inotropic effect of nerve stimulation persists even after pharmacological blockade of cholinergic and adrenergic response (Donald, 1985) and this responses can be inhibited by α,β-methylene ATP (Hoyle & Burnstock, 1986). In the ventricles ATP has been reported to increase the mechanical activity (Danziger *et al.*, 1988; Legssyer *et al.*, 1988). As to the sources of the increased cytosolic Ca^{2+} in ventricular myocytes, no consensus of opinion has been reached yet. It may be due to the influx of extracellular Ca^{2+} (Danziger *et al.*, 1988), or the combination of transmembrane influx and release from sarcoplasmic reticulum (De Young & Scarpa, 1989).

ATP-induced conductance has also been reported on cultured developing chicken skeletal muscle (Kolb & Wakelam, 1983; Häggblad *et al.*, 1985; Hume & Thomas, 1988; Thomas & Hume, 1990; Thomas *et al.*, 1991). However, their physiological role is still unclear.

The classification of the purinoceptors which are directly coupled to cation channels is still unsettled matter, because of the existence of discrepancies between pharmacological and electrophysiological evidence, contradictory results from different laboratories, and the lack of a test for various purinoceptor ligands. However, in smooth muscle cells there is enough evidence to indicate that the purinoceptor coupled to

cation channels is the P_{2X}-subtype. In the guinea-pig urinary bladder, ATP, ADP, α,β-methylene ATP, β,γ-methylene ATP can all induce a fast inward transmembrane current (Marchenko *et al.*, 1987), and the ATP-induced conductance can be blocked by α,β-methylene ATP through the desensitization of P_{2X}-purinoceptors (Inoue & Brading, 1990). In rabbit ear artery it has been shown that ATP, ATPγS, and α,β-methylene ATP can all produce large inward current (Benham & Tsien, 1987). In guinea-pig vas deferens, the ATP-induced depolarization can also be inhibited by α,β-methylene ATP (Sneddon & Burnstock, 1984; MacKenzie *et al.*, 1988; Cunnane & Manchanda, 1988), and α,β-methylene ATP itself can also produce a prolonged depolarization of smooth muscle cell membrane of vas deferens (Cunnane & Manchanda, 1988). However, a whole cell voltage-clamp experiment showed that β,γ-methylene ATP did not produce any inward current in vas deferens membrane, which is contradictory to the pharmacological evidence (Friel, 1988).

ATP-induced conductance bears some similarities in frog atrial myocyte to that in smooth muscle cells, which can also be inhibited by α,β-methylene ATP (Friel & Bean, 1988). Although there are many reports which indicate that ATP has inotropic effects on the cardiac ventricles and can increase the cytosolic Ca^{2+} of the ventricular myocytes (Danziger *et al.*, 1988; De Young & Scarpa, 1989; Pucéat *et al.*, 1991; Christie *et al.*, 1992), only one study shows that it is the P_{2Y}-purinoceptor which leads depolarization of the myocyte, which further triggers the voltage-dependent Ca^{2+} channels of the sarcolemma (Björnsson *et al.*,

1989).

The ATP-gated cation conductance as well as the ligand actions in sensory neurones have some similarities to those in smooth muscle cells. β,γ-methylene ATP is an agonist (Krishtal *et al.*, 1988a) and even pyrophosphate has excitatory actions (Salt & Hill, 1983). However, differences in the characteristics of ATP-gated cation channels do exist between these two cell types, as it has been reviewed by Bean & Friel (1990). In guinea-pig coeliac ganglian neurons the purinoceptor which mediates the responses to ATP is probably the P_{2X}-subtype, because both ATP and α,β-MeATP evoked inward currents, which were blocked by suramin. The responeses to ATP could be desensitized by α,β-MeATP (Evans *et al.*, 1992). However, the purinoceptor which mediates the depolarization of guinea-pig intracardiac neurones appeared comparable to the P_{2Y}-subtype (Allen & Burnstock, 1990).

As to the purinoceptors which are coupled to cation channels in developing chicken skeletal muscle, they do not appear to meet the established criteria for belonging to any of the P_2-purinoceptor subtypes (Hume & Honig, 1986; Thomas *et al.*, 1991).

Taken together, the ATP-gated conductance seems to vary widely, the pattern of the ligand selectivity is different from tissue to tissue. Thus, it is possible that a family of closely related P_2-purinoceptors exists which control the ATP-gated cation channels in various tissues. Further biochemical and molecular biological studies will hopefully solve this mystery.

Chapter 2: Radioligand Binding Assay: Determination of the Optimal Binding Conditions for [^{3}H]α,β-Methylene ATP and Characterization of the Binding Sites

Summary

1. The optimal conditions for the binding of [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) to rat urinary bladder membranes were determined and the characteristics of the binding were studied.

2. Membrane fractions of rat urinary bladder were prepared with a differential centrifugation method, and were examined morphologically and biochemically. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) binding assay showed that the membrane preparations were purified about 4.3 times.

3. A filtration method was used to separate the bound and free [^{3}H]α,β-MeATP. Two aliquots of 5 ml buffer were sufficient to wash the filters without obvious loss of the specific binding.

4. It was found that both Tris and HEPES buffers were suitable for [^{3}H]α,β-MeATP binding assay, while the use of phosphate buffer saline (PBS) should be avoided because it inhibited the [^{3}H]α,β-MeATP binding.

5. The optimal pH for [^{3}H]α,β-MeATP binding was near 7. The

specific binding decreased sharply in alkaline media.

6. [^{3}H]α,β-MeATP showed a small amount of false specific binding to glass fibre filters, which could be abolished by presoaking the filters in 20 mM pyrophosphate solutions. Real specific binding was not affected. No binding of [^{3}H]α,β-MeATP to the plastic test tubes was observed.

7. There was a linear relationship between the binding and the membrane protein concentration, but only with a membrane protein concentration less than 75 μg per assay.

8. The [^{3}H]α,β-MeATP binding sites were reduced at a temperature above 40°C, and totally destroyed at 100°C, indicating that the binding sites are bioactive structures.

9. Ethylenediaminetetraacetic acid (EDTA) showed a slight inhibitory effect on [^{3}H]α,β-MeATP binding, while ethyleneglycol-bis(β-aminoethylether) tetraacetic acid (EGTA) and other protease inhibitors tested had no significant influence on the binding.

10. No specific binding of [^{3}H]α,β-MeATP to $Na^+ \cdot K^+ \cdot$ATPase was observed.

11. Mg^{2+}, Ca^{2+}, and Na^+ ions could all inhibit [^{3}H]α,β-MeATP binding to bladder membranes at 30°C, while Ca^{2+} and Na^+ showed a slight potentiating effect at 4°C. All the cations tested potentiated the binding of [^{3}H]α,β-MeATP to the "washed bladder

homogenates".

12. Saturation assays revealed high- and low-affinity states of [^{3}H]α,β-MeATP binding to rat bladder membranes. Mg^{2+} ions in the media could reduce both the high- and low-affinity binding in a concentration dependent manner, while having no significant effect on the affinity of the binding.

13. The binding of [^{3}H]α,β-MeATP to rat bladder membranes was reversible and both the association and dissociation were very rapid at 30°C. The half-life ($t_{\frac{1}{2}}$) of the radioligand-receptor complex was 2.31 min for the high-affinity state and 1.09 min for the low-affinity state.

14. Displacement with unlabelled purinergic ligands confirmed the specificity of [^{3}H]α,β-MeATP binding to the P_{2X}-purinoceptors. In the presence of Mg^{2+} at 30°C, the rank order of potency of the unlabelled ligand was: α,β-methylene ATP > β,γ-methylene ATP > suramin > ATP > ADP > 2-methylthio ATP >> adenosine, which is in agreement with their biological actions on P_{2X}-purinoceptors.

15. In Mg^{2+}-free media at 4°C, the rank order of potencies of the unlabelled ligands in displacing the [^{3}H]α,β-MeATP binding was similar to that in the presence of magnesium ions at 30°C. However, the potencies of all ligands except suramin and adenosine were significantly increased. Such increase may be due to the removal of the influence of Mg^{2+} ions and inhibition of the

enzymic degradation of ATP and ADP.

16. The binding of [^{3}H]α,β-MeATP could also be competitively displaced by other nucleotides such as UTP, CTP, TTP, ITP, and GTP. However, ATP was the most potent displacer. Adenosine, adenine, and xanthine had no significant effect on the [^{3}H]α,β-MeATP binding, while pyrophosphate and tripolyphosphate could effectively displace the binding, indicating that the tripolyphosphate chain in the [^{3}H]α,β-MeATP molecule is the most critical structure in the interaction with P_{2X}-purinoceptors.

2-1 Introduction

More than 100 years ago, Langley (1878) proposed that drugs act by forming a complex with discrete areas of the cell, and in 1905 he used the phrase "receptive substance" to describe the nicotine-sensitive areas of the neurotransmitter junctions (Langley, 1905). In 1907, Ehrlich presented the concept of "chemoreceptor" after his study on drug resistance. After the introduction of the receptor concept, the progress towards understanding receptors was very slow for many decades. However, since the first radioligand receptor binding assay was carried out in late 1960s, using ^{131}I-labelled adrenocorticotropic hormone (ACTH) to demonstrate its specific binding sites (Lefkowitz *et al.*, 1970), the last 20 years have witnessed some dramatic and rapid developments in the studies of receptors. Quantitative radioligand binding assays were developed for receptors for a variety of drugs and transmitters, and ligands radiolabelled with ^{3}H or ^{125}I are now available for the study of many classes of receptors. This widespread availability of suitable ligands has led to a rapid expansion in the use of binding assays with radioligands to characterize receptors and receptor subtypes.

Before the use of radioligand binding assay, the properties of receptors were largely determined form the measurement of biological responses. This approach proved to be productive in the classification of receptors and even led to the identification of subtypes of receptors. However, there exist many problems for the

measurement of the biological responses, for example, the tissue distribution of a drug administered *in vivo* may vary depending on its ability to cross diffusion barriers, such as the blood brain barrier, or on the extent to which the drug binds to plasma proteins. The lipophilic and hydrophilic nature of a compound can determine whether or not it has equal access to all of the receptors in the given tissue. Drugs can also be metabolized before they have an opportunity to interact with a receptor. These metabolic transformations can yield compounds that are either more or less active than the parent drug and thus can markedly alter the observed pharmacological specificity. Drugs not subject to structural alteration are often removed from the extracellular environment by neuronal or extraneuronal uptake mechanisms. Furthermore, *in vivo* the response to a drug is frequently attenuated by compensatory feedback mechanisms. Interpretation of a measured biological response can also be difficult if the drug has multiple sites of action - a phenomenon that can also occur *in vitro* with tissues containing multiple classes of receptor subtypes. In some cases, the receptor subtypes mediate the same physiological response, and they may exert these effects through the same effector system. The observed pharmacological response will then be affected by the degree of the selectivity of the drug and the relative densities of the subtypes present in the tissue. Thus, there are many parameters which need to be taken into account when demonstrating the interaction between a specific drug and a receptor by using biological methods.

Radioligand binding assays can provide specific information

about the initial interaction between a drug and its binding site. For example, kinetic experiments can accurately determine the association and dissociation rate constant of a receptor-radioligand complex. A pharmacological profile of the affinities of a series of unlabelled ligands can be defined by measuring the inhibition of the binding of a radioligand by these unlabelled compounds. The use of radioligands also permits characterization of receptors in the absence of measurable biological responses, which is especially important where the effects of a neurotransmitter are very complicated. The use of binding assays can also result in meaningful estimates of the number or density of receptors in a particular tissue. Consequently, changes in the densities of receptors resulting from pathological and pharmacological interventions can be monitored. Binding assays can also be used to discriminate multiple classes of receptors in a single tissue and to estimate their relative proportions. Moreover, binding assays with radioligands provide the only means by which receptors can be measured during solubilization, purification, and reconstitution - steps necessary for the complete understanding of receptor functions.

Although the radioligand binding assay is a powerful tool for pharmacologists, this technique is full of pitfalls, the major one is studying binding to something other than a receptor. Thus the first step in undertaking a binding study, once binding has been detected, is to establish the nature of the binding sites. If a given binding site is to be recognized to represent a receptor,

some criteria must be fulfilled. (a) The binding of the radioligand should be rapid enough to allow equilibration within the period of the assay; (b) the binding of the radioligand should be fully reversible, except in the specific case of site-directed affinity labels; (c) a component of the radioligand should be inhibited by pharmacologically appropriate concentrations of unlabelled ligands, allowing the definition of a specific and a non-specific component of binding. A set of different ligands should yield the same estimate of non-specific binding; (d) Specific binding should be saturable, so defining a finite concentration of binding sites; (e) there should be a quantitative correlation between the affinities determined by binding assays, and by pharmacological or functional assays on the same receptor type; (f) binding of the same characteristics should be present where receptors for the ligand are known to be present and absent where receptors are known to be absent. There are several other important issues which should be taken into consideration, such as, tissue linearity, temperature dependence, effects of pH and ions. Furthermore, the optimal conditions for a binding assay should be determined in order to obtain the most accurate estimation of the binding sites.

As has been mentioned in Chapter 1, it is imperative to have a radioligand developed for the study of P_{2X}-purinoceptors. Because no suitable specific antagonist for P_{2X}-purinoceptors is available at present, the most potent P_{2X}-purinoceptor agonist, α,β-methylene ATP (α,β-MeATP), was chosen to be tritiated as a radioligand for P_{2X}-purinoceptors. Preliminary experiments have

shown that [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) can specifically bind to rat bladder membrane preparations. In this study, a series of experiments was carried out to determine whether or not the characteristics of [^{3}H]α,β-MeATP binding sites can fulfil the criteria for a receptor. Furthermore, the optimal conditions for [^{3}H]α,β-MeATP binding were also determined. These experiments have formed the basis for the binding assays on other tissues, autoradiographic localization and solubilization of [^{3}H]α,β-MeATP binding sites described in the other chapters. The possible binding mechanism between the P_{2X}-purinoceptor and its ligands are also discussed.

2-2 Materials and methods

2-2-1 Membrane preparation

Male Wistar rats (200 - 250 g) were killed by asphyxiation with CO_2. Urinary bladders were removed immediately and placed in modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, $NaHCO_3$ 16.3, NaH_2PO_4 1.4, glucose 7.7, (pH 7.4). They were trimmed free from surrounding adipose and connective tissues, minced and homogenized in 10 volume of ice-cold 50 mM Tris/HCl buffer (pH 7.4, buffer A) with a motor-driven glass homogenizer at top speed for two 30 s bursts or with a Polytron PT-3000 (Kinematica, Switzerland) twice in 15 s bursts at 30,000 rpm. The homogenate was centrifuged at 170 *g* for 5 min at 4°C. The supernatant was passed through double layers of nylon mesh and centrifuged at 105,000 *g* for 50 min at 4°C in a swingout rotor in a MSE Europa-M 50 ultracentrifuge. The pellets were washed and suspended in buffer A for binding assay. The harvest of the membrane fraction was about 0.3 to 0.4 mg protein per bladder (about 70 to 76 mg wet weight).

2-2-2 Determination of the purity of the membrane preparations

A. Morphological examination with the electron microscope

To ensure the ultracentrifugal pellets contained rich plasma membrane fragments, a morphological examination was performed with

the electron microscopic technique. The ultracentrifugal pellets were fixed for 2 hrs at 4°C with fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4; they were subsequently postfixed in 1% osmium tetroxide fixative, the specimens were dehydrated in a graded ethanol series and embedded in Araldite. Ultrathin (silver colour) sections were stained with uranyl acetate and lead citrate, and subsequently examined using a Philips-300 electron microscope.

B. Estimation of membrane purity with [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding sites as a marker (compared with [^{3}H]α,β-MeATP binding)

Two rat bladders were homogenized, two-thirds of the sample was centrifuged to separate the membrane fractions. The homogenate, ultracentrifugal supernatant and the membrane fraction were used for receptor binding. The tissue samples were incubated with [^{3}H]QNB (final concentration ranging from 0.025 to 1.5 nM) at 30°C for 30 min (final volume 0.5 ml). Non-specific binding was determined in the presence of 10 μM atropine. The reaction was terminated by rapid filtration of the mixture through double layers of Whatman GF/C glass fibre filters under vacuum. The filters were washed with three aliquots of 4 ml ice-cold buffer A, dried under an infrared lamp, and then put into a mini-scintillation vial. To each vial 4 ml OptiPhase 'HiSafe' 3 was added. The vials were put aside for 12 hrs and the radioactivity trapped in the filters was measured in a Beckman LS7500 or SC6100I

scintillation counter with an efficiency of about 56 to 61%.

Parallel experiments were carried out on the binding of [^{3}H]α,β-MeATP to the bladder homogenate, supernatant and the membrane fraction. Samples were incubated with 1.25 to 160 nM [^{3}H]α,β-MeATP in buffer A at 30°C for 15 min (final volume 0.5 ml). At the end of the incubation the assay tubes were put on ice and the reaction mixture was rapidly filtered through double layers of Whatman GF/C filters (24 mm in diameter, presoaked in 20 mM Na_2H_2pyrophosphate solution). The filters were washed with 2 aliquots of 5 ml ice-cold buffer A. Non-specific binding was determined by including 100 μM β,γ-methylene ATP (β,γ-MeATP) in the incubation mixture.

2-2-3 Protein determination

Protein concentration was measured with the method of Lowry *et al.* (1951) and bovine serum albumin was used as a standard. Due to the strong colour reaction of Tris with Folin-reagents, the ultracentrifugal pellets were suspended in double distilled water (ddH_2O) first. After the sample for protein determination was taken, the remaining suspension was diluted with buffer A for further use. In order to determine very low concentrations of protein or for studies in the presence of detergents a modified method of Lowry *et al.* was used (Peterson, 1977; 1979), which will be described in Chapter 7.

2-2-4 Determination of the optimal volume of washing buffer

In the present radioligand binding assay, a filtration method was employed to separate the bound and free radioligand in the sample. The washing process may significantly influence the estimation of final results. In order to minimize the loss of specific binding, while reducing the non-specific binding as much as possible, an optimal volume of buffer used to wash the filters should be determined.

Membrane preparations (protein content was about 25 μg) were incubated with 10 nM [^{3}H]α,β-MeATP in buffer A at 30°C for 15 min (final volume 0.5 ml). At the end of the incubation the reaction mixture was rapidly filtered through double layers of Whatman GF/C filters. The filters were washed with 1 to 7 aliquots of 5 ml ice-cold buffer A. Non-specific binding was determined in the presence of 100 μM β,γ-MeATP.

2-2-5 Choice of biological buffers

In some cases biological buffers can affect the radioligand receptor binding. Therefore, the most suitable buffer should be chosen for the binding assay. Three types of buffers were tested: (i) buffer A, (ii) 20 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid, pH 7.4), and (iii) 50 mM PBS (phosphate buffered saline, pH 7.4). ddH_2O (pH value around 6.56) was also used for comparison. Rat bladder was first homogenized in buffer A and centrifuged at 170 *g*. After ultracentrifugation the pellets

were washed with ice-cold ddH_2O three times, suspended in 2.2 ml ddH_2O, and then 0.5 ml was taken out and diluted with one of the three buffers or ddH_2O. [^{3}H]α,β-MeATP and β,γ-MeATP were prepared in ddH_2O. Incubation was carried out at 30°C for 15 min with 10 nM [^{3}H]α,β-MeATP. The filters were washed with 2 aliquots of 5 ml of the relevant buffer or ddH_2O.

2-2-6 Determination of the optimal pH value

Neurotransmitter receptors operate normally under conditions of physiological pH, thus the optimal pH value for the ligand binding should be near 7.4. Extremes of pH should reduce or destroy the binding. To determine the optimal pH value for [^{3}H]α,β-MeATP binding this radioligand (10 nM) was incubated with membrane preparations in Tris/HCl buffers with different pH values (from 6.0 to 9.0) at 30°C for 15 min. The stock solution of [^{3}H]α,β-MeATP, β,γ-MeATP and the membrane were prepared in the relevant buffers, and the filters were also washed with the corresponding buffers.

2-2-7 Reduction of the binding to glass fibre filters and the examination of the binding to plastic test tubes

Many radioligands carry electrical charges in their molecules which can make the radioligands bind to the non-receptor substances, some of which are even inert materials, such as the binding of insulin to talc (Cuatrecasas and Hollenberg, 1976). This kind of binding can usually be determined as non-specific

binding by including a specific unlabelled ligand in a parallel assay. However, some radioligands can bind to the filters in a manner similar to specific binding. Such false specific binding can be reduced or abolished by including an antiadsorbent in the assay system, or pretreating the filters with antiadsorbent. In preliminary experiments a small amount of false specific binding of [^{3}H]α,β-MeATP to the Whatman GF/B and GF/C glass fibre filters was observed. In order to eliminate such interference, Whatman GF filters were presoaked in buffer A containing 20 mM $Na_2H_2P_2O_7$ for about 1 h. To check whether such treatment of filters can also reduce the real specific binding, incubation mixtures of membranes with 10 nM [^{3}H]α,β-MeATP in the absence or presence of 100 μM β,γ-MeATP were filtered through either the treated or the untreated filters.

In some cases the radioligand tested may bind to the test tubes which decreases the free radioligand concentration and produce incorrect results. In this study [^{3}H]α,β-MeATP in a serial concentration (from 0.625 to 160 nM) was prepared with buffer A in scintillation vials. Half of the solution was taken out and incubated in polyethylene test tubes (final volume 0.5 ml) at 30°C for 15 min. At the end of the incubation the solution was pooled into a scintillation vial, the test tube was washed twice with two aliquots of 0.25 ml buffer A, and the washout was also pooled into the same vial. The radioligand solutions which remained in the vials (unincubated in the test tubes) were also made up to 1 ml with buffer A. The radioactivities in both groups were measured

and compared.

2-2-8 The relationship between the membrane protein concentration and the binding

The membrane preparations from 8 rat bladders were pooled together (about 2.4 mg protein) and the sample was diluted to make serial solutions with different concentrations of membrane proteins. These membrane suspensions were incubated with 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min.

2-2-9 Heat denatur—ation of the binding sites

All neurotransmitter receptors are membrane-bound proteins which should be sensitive to temperature. One of the criteria to distinguish whether the binding sites are biological receptors is that they should be subject to heat denaturization. Membrane preparations were first incubated in a water bath at 30, 40, 56, or 100 °C for 20 min. They were then incubated with 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min. The specific binding in each preparation was determined as described above.

2-2-10 Effect of EDTA, EGTA and other protease inhibitors

In some experiments, such as in the solubilization of the binding sites described in Chapter 7, protease inhibitors were included in the buffer to prevent the degradation of the receptors. However, these protease inhibitors may themselves inhibit the binding.

Thus, the protease inhibitors at commonly used concentrations were tested for their effects on the [^{3}H]α,β-MeATP binding to rat bladder membranes. EDTA and EGTA can chelate divalent ions which may interfere with the binding, they can also inhibit the divalent ion-requiring proteases. Their effects on [^{3}H]α,β-MeATP binding were tested in a range of concentrations. The membrane preparations were incubated with 10 nM [^{3}H]α,β-MeATP in the presence of one of the following chemicals: 0.1 to 10 mM EDTA, 0.1 to 10 mM EGTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01% bacitracin, or 0.002% soybean trypsin inhibitors.

2-2-11 Binding to Na^+•K^+•ATPase

Some potential binding sites other than the purinoceptors for the purine nucleotide radioligand are the ectonucleotidases. In order to check whether [^{3}H]α,β-MeATP can bind to ATPase, a binding assay was carried out on Na^+•K^+•ATPase extracted from porcine cerebral cortex. The reaction mixture contained 117 μg protein (about 0.062 unit of Na^+•K^+•ATPase) and 10 nM [^{3}H]α,β-MeATP. The Tris/HCl buffer used for incubation contained the following protease inhibitors: 1 mM EGTA, 1 mM benzamidine, 0.1 mM PMSF, 0.01% bacitracin, and 0.002% soybean trypsin inhibitors. The incubation was carried out at 4 °C for 2 hr. At the end of incubation the reaction mixture was filtered through a Whatman GF/B filter presoaked in 0.28% polyethyleneimine (M.W. 8000) and 200 mM tetrasodium pyrophosphate, and the filter was washed with

two aliquots of 5 ml buffer A containing 0.2 M NaCl.

2-2-12 Effects of Mg^{2+}, Ca^{2+} and Na^{+} on the binding

Receptor binding should exist in the presence of the physiological concentrations of ions. However, these ions may significantly influence the ligand binding. In this study the effects of Mg^{2+}, Ca^{2+} and Na^{+} on the [^{3}H]α,β-MeATP binding was examined. Two kinds of samples were used, one was the membrane preparations made as described above, the other was the preparation containing all insoluble components of the cells (the "washed homogenate"), which was the resuspended pellet of the bladder homogenate after centrifugation at 105,000 *g* for 50 min. Those two preparations were incubated with 10 nM [^{3}H]α,β-MeATP in buffer A containing different concentrations of Mg^{2+}, Ca^{2+}, (final concentration from 0 to 32 mM) and Na^{+} (final concentration from 0 to 320 mM) either at 30°C for 15 min or at 4°C for 2 h.

The effects of Mg^{2+} at different concentrations on the saturation assay of bladder membrane preparations were also studied to see whether the ion can also influence the affinity of the radioligand to its receptors. Parallel saturation assays were carried out in four kinds of incubation solutions containing either zero, 2 mM, 10 mM, or 25 mM $MgCl_2$. The pooled membrane preparations (in buffer A) from four rat bladders were used for one experiment, with about 25 μg protein per assay. Stock solutions of [^{3}H]α,β-MeATP and β,γ-MeATP were prepared in buffer A. Membrane preparations were incubated with [^{3}H]α,β-MeATP (final

concentration from 1.25 to 160 nM) in the absence or presence of 100 μM β,γ-MeATP at 30°C for 15 min. The reaction was terminated by vacuum filtration and the filters were washed with the buffer containing the corresponding concentration of Mg^{2+}.

2-2-13 Kinetics of the binding

One important criterion for the binding is that it should be reversible and occur with reasonable speed. In this experiment the rate constants of association and dissociation of [^{3}H]α,β-MeATP binding to rat bladder membrane preparations were examined. The membranes were incubated with 10 nM [^{3}H]α,β-MeATP at 30°C. At varying time intervals (from 5 sec to 10 min) the reaction was terminated by rapid filtration of the mixtures under vacuum. For the measurement of dissociation rate constant the membranes were incubated with 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min, and then an aliquot of β,γ-MeATP (final concentration 100 μM) was added. The reaction was terminated at varying times intervals (from 15 sec to 30 min).

2-2-14 Competitive displacement of [^{3}H]α,β-MeATP binding

In order to determine the specificity of the binding sites, several purinergic ligands were used to displace the [^{3}H]α,β-MeATP binding. The displacement experiments were carried out in two groups to compare the effect of Mg^{2+} dependent 5'-nucleotidases: (a) membrane preparations were incubated with 10 nM [^{3}H]α,β-MeATP

in the presence of 25 mM Mg^{2+} and the unlabelled ligand at 30°C for 15 min; (b) assays were performed in Mg^{2+}-free buffer A at 4°C for 2 hr. The concentration ranges of the unlabelled ligands were as follows: α,β-MeATP, β,γ-MeATP, suramin, 2-methylthio ATP (2-MeSATP), 10^{-9} - 10^{-4} M; ATP, ADP, and adenosine, 10^{-9} - 10^{-3} M.

Apart from the commonly used purinergic ligands the following substances were also used for competitive displacement: guanosine triphosphate (GTP), uridine triphosphate (UTP), cytidine triphosphate (CTP), inosine triphosphate (ITP), thymidine triphosphate (TTP), adenine, xanthine, Na_5tripolyphosphate, Na_2H_2pyrophosphate, and $Na_2H_2PO_4$. Incubation was carried out in Mg^{2+}-free buffer at 4°C for 2 hr.

2-2-15 Chemicals

[^{3}H]α,β-MeATP was custom-synthesized by Amersham International plc. (Amersham, U.K.), with a specific activity of 27 (first batch) and 19.2 (second batch) Ci/mmol and chemical purity of 98 - 99%. [^{3}H]QNB with a specific activity of 44 Ci/mmol was purchased from the same company. α,β-MeATP, β,γ-MeATP, ATP, ADP, UTP, GTP, CTP, ITP, TTP, adenosine, adenine, xanthine, tripolyphosphate, pyrophosphate, Tris, HEPES, EDTA, EGTA, benzamidine, PMSF, bacitracin, soybean trypsin inhibitors, Folin reagents were from Sigma Chemical Company Ltd. (Poole, U.K). 2-MeSATP was from Research Biochemical Inc. (Natick, U.S.A.). Atropine sulphate was from Antigen Pharmaceuticals Ltd. (Roscrea, Ireland). Suramin was a gift from ICI Pharmaceuticals, (Alderly Park, U.K.). All the

other chemicals were purchased from BDH (Poole, U.K.).

2-2-16 Data analysis

The results were expressed as mean ± standard error (S.E.M). The experiments were carried out in duplicate or triplicate. Student's *t* test was employed to compare the difference between means. A probability of less than 0.05 was considered to be significant.

The data from radioligand binding assays have to be manipulated many times before they are in a meaningful form. Thus, a general summary of the methods with which the data in this study was calculated is given (Bennett & Yamamura, 1985; Munson, 1984; McGonigle & Molinoff, 1989).

Saturation assay:

The simplest model describing the interaction of a receptor, R, with a radioligand, L, to form a complex, RL, is the bimolecular reaction

$$[L] + [R] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [RL] \qquad \text{Equation 1}$$

where [L] is the "free" or unbound ligand, [R] is the unoccupied receptor and [RL] is the drug-receptor complex (or the amount of drug bound which will be called [B]). k_{+1} and k_{-1} are association and dissociation rate constants respectively.

At apparent equilibrium where the forward and reverse reactions are progressing at the same rate, the law of mass action states that:

$$K_d = \frac{[L]\,[R]}{[B]} \qquad \text{Equation 2}$$

where K_d is the equilibrium dissociation constant, which is equal to k_{-1}/k_{+1}.

Since the total number of receptors, $B_{max} = [R] + [B]$, then,

$$K_d = \frac{[L]\,(B_{max} - [B])}{[B]} \qquad \text{Equation 3}$$

which can be rearranged as

$$[B] = \frac{B_{max}\,[L]}{[L] + K_d} \qquad \text{Equation 4}$$

This equation is identical in form to the Michaelis-Menten equation describing enzymic-substrate kinetics. Equation 4 in essence describes a hyperbolic relationship between the amount of ligand bound to the receptor for a given free ligand concentration. Equation 4 was rearranged by Scatchard (1949):

$$\frac{[B]}{[L]} = \frac{B_{max}}{K_d} - \frac{[B]}{K_d} \qquad \text{Equation 5}$$

A plot of the ratio of bound [B] to free [L] ligand against the concentration of the bound ligand is a straight line that has a slope equal to the negative reciprocal of the dissociation constant, $(-1/K_d)$, and an intercept on the abscissa equal to the

B_{max}.

The interaction of a radioligand with two types of receptors is modelled as the sum of two independent bimolecular reactions. At equilibrium,

$$[B] = \frac{[L]\ B_{max1}}{[L] + K_{d1}} + \frac{[L]\ B_{max2}}{[L] + K_{d2}} \qquad \text{Equation 6}$$

Scatchard transformation of saturation data for the interaction of a selective radioligand with multiple subtypes of receptor will result in a curvilinear plot, which is most effectively used to provide visual confirmation of the existence of a heterogeneous population of the binding sites.

Issues related to cooperativity between sites are often studied using the Hill equation (Hill, 1909)

$$\frac{[B]}{B_{max}} = \frac{[L]^n}{K_d + [L]^n} \qquad \text{Equation 7}$$

where n = number of binding sites per receptor molecule. For convenience in plotting, it is transformed to a logarithmic form,

$$\log \frac{[B]}{B_{max} - [B]} = n \log [L] - \log K_d \qquad \text{Equation 8}$$

The Hill number (n_H) is the slope of the plotted line.

Kinetic analysis

The rate of association of a radioligand with a receptor is determined by measuring the amount of bound ligand [B] as a

function of time. In the simple bimolecular reaction described in Equation 1, the rate of association of the ligand-receptor complex is $k_{+1}[L][R]$, and the rate of dissociation of the complex is $k_{-1}[B]$. Thus, the measured rate of formation of [B] is

$$\frac{d[B]}{dt} = k_{+1}([L_t] - [B_e])(B_{max}-[B_e]) - k_{+1}[B]\,\frac{[L_t]-[B_e](B_{max}-[B_e])}{[B_e]}$$

Equation 10

where $[L_t]$ is the total amount of radioligand added at the start of the reaction; $[B_e]$ is the amount of radioligand bound at the equilibrium. The second order rate equation can be integrated to give

$$\ln\frac{[B_e]([L_t]-[B][B_e])/B_{max}}{[L_t]([B_e] - [B])} = [k_{+1}\ t\ \frac{[L_t](B_{max}-[B_e])}{[B_e]}\ ]$$

Equation 11

The rate constant of association can be determined from the slope of a plot of the expression on the left-hand side of Equation 11 against time.

Usually the total concentration of radioligand $[L_t]$ is much greater than the total concentration of receptor (B_{max}). Under these conditions there is little or no change in the concentration of free ligand [L] as the reaction proceeds to equilibrium. For all practical purposes, [L] can be regarded as a constant, and the reaction can be considered "pseudo-first order" reaction. Thus, Equation 11 can be simplified to (Weiland & Molinoff, 1981)

$$\ln\frac{[B_e]}{[B_e] + [B]} = k_{+1}\ t\ \frac{[L_t]B_{max}}{[B_e]}$$

Equation 12

A plot of the term on the left-hand side of Equation 12 versus

time is called a pseudo-first-order plot. The association rate constant k_{+1} is related to the slope of the pseudo-first-order plot, as follows:

$$k_{+1} = \frac{\text{Slope}}{[L_t]B_{max}/[B_e]} \qquad \text{Equation 13}$$

The slope of the pseudo-first-order plot is called k_{obs}. It can be shown that k_{obs} is related to the ligand concentration [L] as follows:

$$k_{obs} = k_{+1}[L] + k_{-1} \qquad \text{Equation 14}$$

A plot of k_{obs} versus ligand concentration result in a straight line with a slope equal to k_{+1} and intercept on the ordinate equal to k_{-1}. The association rate constants determined by this method can be compared to the rate constants determined at a single concentration of ligand.

The rate of dissociation is determined by stopping the association of the ligand and receptor and measuring the amount of radioligand that remains bound as a function of time. The rate of change of the receptor-ligand complex is defined by

$$d[B]/dt = -k_{-1}[B] \qquad \text{Equation 15}$$

Integration of Equation 15 yields

$$\ln[B]/[B_o] = -k_{-1}\ t \qquad \text{Equation 16}$$

where $[B_o]$ is the concentration of receptor-ligand complex just

prior to the addition of a competing ligand. The half-life of the complex can be calculated by

$$t_{\frac{1}{2}} = 0.693/k_{-1} \qquad \text{Equation 17}$$

and the dissociation constant (K_d) can also be calculated by

$$K_d = k_{-1}/k_{+1} \qquad \text{Equation 18}$$

which can be compared with the K_d values obtained from saturation assay.

Competitive displacement

In a typical competitive experiment, the binding of a fixed concentration of radioligand is inhibited by increasing concentrations of an unlabelled ligand. The amount of radioligand that is bound to the receptor is

$$[B] = [B_o] - \frac{[B_o] - [I]}{[I] + IC_{50}} \qquad \text{Equation 19}$$

where [I] is the concentration of competing inhibitor. Equation 19 can be rearranged to a simpler form:

$$[B] = \frac{[B_o]}{1 + [I]/IC_{50}} \qquad \text{Equation 20}$$

The equilibrium dissociation constant k_i of an unlabelled competing ligand is related to the IC_{50} value, as described by Cheng & Prusoff (1973)

$$k_i = \frac{IC_{50}}{1 + [L]/K_d} \qquad \text{Equation 21}$$

A Hill plot can be constructed from the following equation:

$$\log \frac{[B]}{[B_o] - [B]} = n_H \log[I] - n_H \log IC_{50} \qquad \text{Equation 22}$$

If the reaction follows mass action principles at equilibrium, the apparent Hill coefficient will be equal to 1. A Hill coefficient significantly different from 1 indicates a more complex interaction between ligand and receptors. This may result from a heterogeneous population of binding sites, a two-step/three component binding system, negative or positive cooperativity between sites or an incorrect definition of non-specific binding sites (Cornish-Bowden & Koshland, 1975).

Computerized analysis: curve fitting

The methods of data analysis described above are mainly for the one binding site model. In the case of multiple binding sites of a radioligand the traditional graphic analysis can no longer deal with the problem, many misinterpretations and misuses of the them have been raised (Norby *et al.*, 1980; Klotz 1982; Munson & Rodbard, 1983; Kermode, 1989). Thankfully the development of the computer has made it possible to calculate complicated binding data using non-linear curve fitting programs. In this study a program called EBDA-LIGAND was used to analyse all the binding data. The LIGAND was originally written by Munson and Rodbard (1980). It has been modified for microcomputers by McPherson (1983). This approach has two major advantages: (i) it uses an

exact mathematical model of the ligand-binding system, thereby, avoiding the possible biases introduced by several commonly used approximations; (ii) it uses a statistically valid, appropriately weighted least square curve fitting algorithm with objective measurement of goodness of fit, thereby avoiding subjective graphical or simplified statistical methods which may introduce bias. There are several other important features which make the analysis more accurate and rapid.

All the the programs in EBDA-LIGAND use a form of weighted non-linear curve fitting. In general weighting is proportional to the inverse of the variance, such as the amount of bound radioligand.

$$\text{Weight} = 1/(\text{calculated bound radioligand})^2 \quad \text{i.e. } (1/Y^2)$$

In order to determine the best model or the best values for the parameters the program evaluates the total squared deviations of the data points from the predicted values (Y_i):

$$SS = \Sigma\ (Y_i - Y_i\ )^2$$

and finds parameter values which minimise SS, i.e., makes the curve run as near to the data points as possible.

When some data points are measured more reliably than others, one must introduce weights (w_i) to offset the tendency for an

unreliable point to unduly influence the location of the curve. Thus, one minimizes

$$SS = \Sigma\, w_i(Y_i - Y_i)^2$$

which is called weighted sum of squares. In this way the points with smaller variance get larger weights.

When additional parameters are added to a model, the goodness of fit will tend to improve, i.e., the residual sum of squares will decrease, simply because of the flexibility of the model. There is a statistical test of whether the increase of goodness of fit for a model with additional parameters is significantly more than we would expect on the basis of chance alone. It is based on the "extra sum of squares" principle.

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2}$$

Here, SS_1 and SS_2 are the residual sums of squares of the deviations of the points to the fitted curve and df_1 and df_2 are the associated degree of freedom for the original model and the model with additional parameters, respectively. The calculated F ratio is then compared to the tabulated value for the F test. One example is the comparison of a fit for one binding site model with a two binding site model. If the calculated F value exceeds the corresponding tabulated value at the probability of less than 0.05, then, the two-site model significantly improved the goodness of fit, and is preferable to the simpler model.

2-3 Results

2-3-1 Determination of the purity of the membrane preparations

A. Morphological examination with electron microscope

Figure 2-1 shows that the ultracentrifugal pellets contained enriched plasma membrane structures in the forms of vesicles and bilayer sheets. Some gap-junctions could be observed.

B. Estimation of the membrane purity with [^{3}H]QNB binding sites as a marker

The saturation analysis of [^{3}H]QNB binding to rat bladder homogenate, supernatant and resuspended ultracentrifugal pellet showed that the maximum specific binding (B_{max}) was 112 ± 17 fmol/mg protein (n = 3) in the homogenate (K_d = 0.063 ± 0.018 nM), 484 ± 37 fmol/mg protein (n = 3) in the membrane fractions (K_d = 0.054 ± 0.014 nM). Only a trace amount of specific binding was observed in the supernatant. It is deduced that the muscarinic receptor density was increased 4.32 times by this membrane separation method.

The specific binding of [^{3}H]α,β-MeATP to the rat bladder homogenate membrane preparations contained high- and low-affinity states. The B_{max} for the high-affinity binding sites was 1235 ± 165 fmol/mg protein (n = 3) in the homogenate (K_d = 10.2 ± 3.3 nM)

and 4793 ± 385 fmol/mg protein (n =3) in the membrane preparations (K_d = 8.5 ± 3.0 nM). The density was therefore increased 3.88 times, which was a little lower than that for [^{3}H]QNB binding. The reason may be that a small amount of specific [^{3}H]α,β-MeATP binding (324 ± 57 fmol/mg protein, n = 3) was still retained in the ultracentrifugal supernatant.

2-3-2 Determination of the optimal volume of washing buffer

Figure 2-2 shows the relationship between the volume of washing buffer and the amount of specific and non-specific binding. It was shown that free [^{3}H]α,β-MeATP was easily washed away. When more than 2 x 5 ml buffer was used the non-specific binding was not reduced as obviously as the specific binding, thus, the optimal ratio between specific and non-specific binding was obtained when 2 x 5 ml washing buffer was used.

2-3-3 Choice of biological buffers

Figure 2-3 shows the specific and non-specific binding of [^{3}H]α,β-MeATP in different buffers and ddH_2O. The non-specific levels in all the groups were similar. No significant difference in specific binding in Tris and HEPES buffers was observed, while the specific binding was much less in PBS buffer than in either Tris or HEPES buffers. Such a phenomenon will be explained by the results from the competitive displacement experiments in this study. The specific binding was higher in ddH_2O than in PBS, but lower than

in Tris and HEPES, which may be due to the pH value of ddH_2O falling outside the optimal range for [^{3}H]α,β-MeATP binding. Thus, both Tris and HEPES are suitable buffers for [^{3}H]α,β-MeATP binding.

2-3-4 Determination of the optimal pH value

The pH value of the incubation media can significantly affect the [^{3}H]α,β-MeATP binding (Figure 2-4). The optimal pH value for the binding was near 7.0, which was a bit lower than that the physiological pH value. It can also be seen that the binding dropped more rapidly in alkaline media.

2-3-5 Reduction of the binding to glass fibre filters and examination of the binding to plastic test tubes

Although the total binding was only about 0.2% to 0.6 % of the total radioactivity added, a small amount of false specific binding was still observed (Figure 2-5). By using the filters which had been soaked in the disodium pyrophosphate solution for 1 hr the non-specific binding was hardly changed while the false specific binding was nearly eliminated (Figure 2-5). The treatment of filters with pyrophosphate did not affect the real specific binding, as can be seen in Figure 2-6. This might be because the reaction mixture was passed through the filters very rapidly, and the specific binding was not displaced. Thereafter, in all [^{3}H]α,β-MeATP binding assays the Whatman GF/B or GF/C glass fibre filters were presoaked in 20 mM disodium pyrophosphate.

In all the tested concentrations no significant loss of [^{3}H]α,β-MeATP was observed after the incubation in plastic test tubes at 30°C for 15 min, which indicates that [^{3}H]α,β-MeATP does not bind to the plastic test tubes used for these experiments.

2-3-6 The relationship between the protein concentration and the binding

Figure 2-7 shows the relationship between the membrane protein concentration in the incubation media and the specific binding. In the range of 2 to 75 μg the specific [^{3}H]α,β-MeATP binding was in a linear relationship with the protein content. However, if the protein content was over 75 μg, the specific binding no longer increased in the same proportion as the protein content. This phenomenon may be partially caused by the reduction of the free radioligand in the incubation media, as it was calculated that in the tubes containing 300 μg protein, 20% of the radioligand was bound to the membrane preparations, thus, the actual concentration of radioligand in these tubes was 8 nM instead of 10 nM. Therefore, when rat bladder membrane preparations were used for the binding assay of P_{2X}-purinoceptors with [^{3}H]α,β-MeATP, it was essential to keep the protein content in the incubation media under 75 μg per assay.

2-3-7 Heat denatur⁓ation of the binding sites

Preincubation of the membrane preparations at different temperatures yielded different amounts of specific binding while the non-specific binding was not affected (Figure 2-8). At 30°C and 40°C, no significant difference in specific binding was observed. At 56°C most of the specific binding was lost, and further, after the membranes were boiled no specific binding was detected.

2-3-8 Effects of EDTA, EGTA and other protease inhibitors

As shown in Figure 2-9 EGTA had no effect on the [^{3}H]α,β-MeATP binding to the bladder membrane preparations in the concentration range tested, while EDTA at concentrations above 5 mM showed mild inhibitory effect on the binding. All the other tested protease inhibitors at the commonly used concentration had no effect on the binding (Figure 2-10), therefore, it is safe to include these inhibitors in the media when necessary.

2-3-9 Binding Na^+•K^+•ATPase

No specific binding of [^{3}H]α,β-MeATP to Na^+•K^+•ATPase was observed.

2-3-10 The effects of Mg^{2+}, Ca^{2+} and Na^+ on the binding

Figure 2-11a,b,c,d shows the behaviours of Mg^{2+}, Ca^{2+}, and Na^+ on the [^{3}H]α,β-MeATP binding to membrane preparations or to the washed homogenate. At 30°C all three cations showed potent

inhibitory effects on the [^{3}H]α,β-MeATP binding to the membranes (Figure 2-11a). At 4°C the inhibitory effect of Mg^{2+} was still shown, while Ca^{2+} slightly enhanced the binding. Na^+ had a tendency to enhance the binding at low concentrations and inhibit the binding at higher concentrations (Figure 2-11b). The effects of the ions on [^{3}H]α,β-MeATP binding to the washed homogenate of rat bladder was in a different pattern. All three ions enhanced the [^{3}H]α,β-MeATP binding at both 30°C and 4°C. In the concentration ranges of 1 to 32 mM for Mg^{2+} and Ca^{2+}, or 10 to 320 mM for Na^+, Ca^{2+} was more potent in increasing the binding than Mg^{2+} and Na^+ (Figure 2-11c,d).

The specific binding of [^{3}H]α,β-MeATP to the rat bladder membrane preparations was saturable in the absence or presence of Mg^{2+}. A typical binding curve (in the buffer with 25 mM $MgCl_2$) is shown in Figure 2-12. Scatchard transformation revealed the existence of two binding sites (Figure 2-13). The K_d values for the high-affinity binding varied between 5.8 and 6.3 nM, while for the low-affinity binding the range was 67 to 104 nM (Table 1). Mg^{2+} ions had a significant inhibitory effect on the binding. The B_{max} for the high-affinity binding state was about 2-fold higher in Mg^{2+} free buffer than that in the buffer with 25 mM $MgCl_2$, while the ratio for the low-affinity binding state was about 4-fold higher in Mg^{2+}-free buffer than that in the presence of 25 mM $MgCl_2$. The K_d values for the high-affinity binding state were slightly reduced with the increase of Mg^{2+} concentration in the buffer, but the K_d values for the low-affinity binding state were

reduced from 104 nM in the Mg^{2+}-free buffer to 67 nM in the buffer with 25 mM $MgCl_2$. The non-specific binding was about 10% of the total binding at the lowest [^{3}H]α,β-MeATP concentration and 50% at the highest [^{3}H]α,β-MeATP concentration. Magnesium ions slightly increased the non-specific binding at lower [^{3}H]α,β-MeATP concentrations (data not shown). The slopes of Hill plots showed that Hill coefficients (n_H) for all high- and low-affinity binding were around 1 (Table 2-1).

2-3-11 Kinetics of the binding

At 30°C the binding of [^{3}H]α,β-MeATP to the bladder plasma membrane preparation was a rapid process, which reached equilibrium in 10 min (Figure 2-14). Kinetic analysis showed that the process was composed of two components: a high-affinity and a low-affinity state (Figure 2-14, inset). The association rate constant of the high-affinity component was 7.64×10^7 M^{-1} min^{-1}, and that of the low-affinity component was 7.31×10^6 M^{-1} min^{-1}. The binding of [^{3}H]α,β-MeATP to bladder membrane was a reversible reaction. After the binding of 10 nM [^{3}H]α,β-MeATP had reached equilibrium, the addition of a high concentration of β,γ-MeATP (100 μM) quickly displaced the bound [^{3}H]α,β-MeATP (Figure 2-15). The dissociation process was also composed of two components: the dissociation rate constant was 0.2896 min^{-1} for the high-affinity binding state, and 0.6348 min^{-1} for the low-affinity binding state (Figure 2-15, inset). The half-life ($t_{\frac{1}{2}}$) of the [^{3}H]α,β-MeATP-receptor complex was 2.31 min for the high-affinity binding sites and 1.09 min for the low-affinity binding sites. Thus, the

dissociation process was also very rapid at 30°C. The K_d values calculated from the kinetic experiment were 3.8 and 86.8 nM, which were in fair agreement with the K_d values obtained from the saturation experiment.

2-3-12 Competitive displacement

All the tested unlabelled ligands showed inhibitory effects on the binding of [^{3}H]α,β-MeATP to a different degree (Figure 2-16a). The competition of the binding by the agonists and antagonist was concentration-dependent. According to the IC_{50} values of the unlabelled ligands in displacing the binding (Table 2-2), in the presence of Mg^{2+} and at 30°C α,β-MeATP was the most potent displacer with an IC_{50} of 91 nM, which was more than 10-fold higher than that of β,γ-MeATP, 25-fold higher than that of suramin, and 320-fold higher than that of ATP. The IC_{50} values of ATP, ADP and 2-MeSATP were in the same order. Adenosine was the least potent among the tested ligands, even at a concentration of 1 mM, only 13% of the specific binding was displaced. In Mg^{2+}-free buffer at 4°C, the concentration-effect curves of all the unlabelled ligands except suramin and adenosine were moved leftward (Figure 2-16b), their increased potencies were shown in Table 2-2. The IC_{50} values of α,β-MeATP, β,γ-MeATP were decreased about 10-fold, while those of ATP, 2-MeSATP and ADP were decreased about 5-fold. The potencies of suramin and adenosine were increased slightly, but not significantly.

All the nucleotides tested was able to displace the specific binding in a competitive manner (Figure 2-16c). However, the potencies of the nucleotides varied; amongst them ATP was the most potent, followed by UTP, and then CTP, TTP, ITP, and GTP (Table 2-3). Adenosine, adenine, and xanthine had almost no effect on the binding (Figure 2-16d). Tripolyphosphate and pyrophosphate showed marked inhibitory effects on the [^{3}H]α,β-MeATP binding with similar potencies, which were higher than all the other tested nucleotides except ATP (Table 2-3). $Na_2H_2PO_4$ showed an obvious inhibitory effect on the binding only at high concentrations (>1 mM) (Table 2-3; Figure 2-16d).

2-4 Discussion

The purity of the membrane preparation is an important factor when quantitative comparisons are made of the receptor binding parameters, especially of the density of the receptor. For the study of P_2-purinoceptor it is very important to use pure membrane preparations to avoid the possible intracellular non-receptor binding sites. It is known that the separation of plasma membrane in smooth muscle is more difficult than in other kinds of tissues such as brain and liver owing to the resistance of muscular tissue to cell disruption methods and an extensive connective tissue network (Evans, 1979). As to the urinary bladder, one successful separation of rabbit bladder membrane has been reported (Batra, 1986a). In our experiments we used a differential centrifugation method to prepare the membrane fraction. The membrane preparation was first morphologically checked with electronic microscopy, and it was found to contain membrane-rich structures. Enzymic and chemical markers were usually then used to determine the purity of the membrane preparations. For the muscle tissue plasma membranes $Na^+ \cdot K^+ \cdot$ATPase and adenylate cyclase are the commonly used markers (Evans, 1979). Markers for specific surface membrane receptors have also been used for the characterisation of plasma membranes, such as [^{3}H]ouabain binding to the membrane-bound $Na^+ \cdot K^+ \cdot$ATPase (Harris *et al.*, 1973; Sachs *et al.*, 1980); [^{3}H]oxytocin to its receptors in myometrial membranes (Matlib *et al.*, 1979). Urinary bladder is partially innervated by cholinergic nerves, thus, the binding sites of [^{3}H]QNB, an antagonist for muscarinic receptors,

have been demonstrated to be a good marker for plasma membranes of urinary bladder (Batra, 1986a; 1986b). In this study the muscarinic receptors were used as a marker to measure the purity of the membrane preparations of rat urinary bladder, and it was found that the [^{3}H]QNB binding sites were increased about 4-fold after the separation process, while the [^{3}H]α,β-MeATP binding sites showed an increase to a similar degree. The parallel increase of binding sites of the two radioligands indicates that [^{3}H]α,β-MeATP mainly binds to the proteins in the membrane and that this method is suitable to separate rat bladder membranes for use in radioligand binding assays.

In radioligand binding studies, the conditions for the assays need to be optimized to ensure the accuracy of the estimated parameters. Non-specific binding should also be well established. Non-specific binding is defined as the binding that occurs in the presence of an excess concentration of unlabelled ligand (usually 100 times the K_d concentration). Under these conditions the non-specific binding may be composed of several components: (a) true non-specific binding to the tissue under study; (b) free radioligand not effectively washed away; and (c) non-specific binding to separation materials. True tissue non-specific binding can rarely be reduced. However, the washing procedure can be evaluated to see if it is effective. In this study, it was found that free [^{3}H]α,β-MeATP can be effectively washed away, 2 x 5 ml of buffer was the optimal volume to wash the glass fibre filters.

Filter-binding of radioligand occurs frequently in many radioligand binding studies. Some radioligand, such as [^{3}H]-phencyclidine, can bind to Whatman GF/B glass fibre filters in a manner indistinguishable from "specific binding" (Maayani & Weinstein, 1980). A stereospecific binding of radiolabelled opiates to glass fibre filters was also described by Pasternak and Snyder (1975). Significant non-specific binding to separation materials can frequently be reduced by including antiadsorbents in the assay system. Examples of such antiadsorbents are albumin or collagen for radioiodinated peptides and *o*-catechol for tritiated catecholamine-binding studies, and polyethyleneimine for muscarinic drugs. [^{3}H]α,β-MeATP also showed some "specific binding" to Whatman glass fibre filters. In rat bladder the density of real specific binding sites are very high, thus, the portion of the false specific binding does not have an obvious effect on the estimation of the final parameters. However, if the binding study is carried out on a tissue which has a low density of receptors or a very small amount of tissue is used, the false specific binding may significantly skew the final results. It was found that soaking the filters in 20 mM pyrophosphate solution nearly abolished that false specific binding, while the real specific binding was not affected. Thus, pyrophosphate can serve as an effective antiadsorbant for [^{3}H]α,β-MeATP.

Biological buffers can sometimes change the estimation of B_{max} or K_d values of the binding, or the selectivity of the ligand for the subtypes of the receptors. In this study, PBS should be avoided, because at the commonly used concentration, it showed

substantial inhibitory effect on the [^{3}H]α,β-MeATP binding. This phenomenon will be discussed below. In the binding assay of β_1- (guinea-pig left atria) and β_2- (guinea-pig uterus) adrenoceptors with (-)-[^{125}I]iodocyanopindolol ([^{125}I]CYP), it was found that the K_d values for [^{125}I]CYP were 2-fold greater in Krebs than in Tris buffer, the B_{max} in the atria was also reduced 2-fold. The mechanism causing the influence of buffer on the binding is unknown, it may be due to the interference of the buffer on the interaction between the agonist and the receptors (McPherson *et al.*, 1985). It has also been observed in the binding study of α-adrenoceptors that glycylglycine buffer increases the apparent affinities of [^{3}H]yohimbine and [^{3}H]rauwolscine in most tissues (Perry & U'Prichard, 1984).

One criterion to distinguish the real specific binding from other binding sites is temperature dependence. Preincubation of the tissue or running the incubation at temperature above 40°C should decrease and ultimately destroy receptor binding. If the binding is maintained or even increased it may suggest a covalent chemical reaction of the radioligand or a breakdown product rather than the reversible receptor binding. In the present study, it was found that the [^{3}H]α,β-MeATP binding sites in rat bladder were temperature sensitive, and were totally denatured in boiling media, which suggests that the binding sites are bioactive structures.

The influence of ions on the radioligand binding assay has

been observed soon after the establishment of this method. In the binding study of opiate receptors it has been found that Na^+ ions (100 mM) decrease the specific binding of agonists and increase that of antagonists (Pert & Snyder 1973; 1974; Pert *et al.*, 1973; Simon *et al.*, 1973). It has been proposed that an opiate receptor exists in two interconvertible states on which Na^+ acts as an allosteric effector. Sodium also regulates receptor binding differentially for agonists and antagonists at α-adrenoceptors (Greenberg *et al.*, 1978; Perry & U'Prichard, 1984), Bradykinin (Innis *et al.*, 1981), histamine H_1 (Chang & Snyder, 1980), and adenosine receptors (Bruns *et al.*, 1980; Goodman *et al.*, 1982). Just how Na^+ regulates synaptic transmission at all of these receptors is quite unclear. Divalent cations regulate numerous receptors including opiate (Pasterak *et al.*, 1975), α-adrenergic (U'Prichard & Snyder, 1980), histamine H_1 (Chang & Snyder, 1980), bradykanin (Innis *et al.*, 1981), dopamine (Usdin *et al.*, 1980, and CCK (Innis & Snyder, 1980) receptors. Unlike the effects of Na^+, which decrease agonist binding, the divalent cations usually increase agonist binding, such an effect may be related to the GTP-binding protein. However, divalent cations have also been observed to decrease the agonist binding to bradykinin receptors (Innis *et al.*, 1981).

The regulatory effects of Mg^{2+} and several other divalent cations such as Mn^{2+} and Ca^{2+} on the binding of P_1-purinoceptors have been known for many years (Bruns *et al.*, 1980; Goodman *et al.*, 1982; Hüttemann *et al.*, 1984). In the binding experiments at A_1-adenosine receptors, Mg^{2+} decreased the binding of [^{3}H]N^6-

cyclohexyladenosine ([^{3}H]CHA) to rat adipocyte membrane in the absence of guanine nucleotide, the binding was increased by Mg^{2+} in the presence of guanine nucleotide (Cooper *et al.*, 1984). Recently the study on P_{2Y}-purinoceptor binding with [^{35}S]ADPβS indicated that without Mg^{2+} the high-affinity binding was almost completely lost (Cooper *et al.*, 1989). The binding of [^{3}H]ATP to rabbit bladder homogenate also showed that a large proportion of the binding sites was magnesium-dependent (Levin *et al.*, 1983). In this study different behaviours of Mg^{2+}, Ca^{2+} and Na^+ ions on the [^{3}H]α,β-MeATP binding to membranes and to the washed homogenate of the bladder were observed, which may indicate that some complicated mechanisms are involved. In membrane binding studies Mg^{2+} showed profound inhibitory effects of [^{3}H]α,β-MeATP binding at both 4°C and 30°C, which indicates that the binding sites are different from ectonucleotidases, because Mg^{2+}-ATP or another divalent ion complex is always the relevant ATP species at the catalytic sites of the enzymes (Cohn, 1990), while the presence of Mg^{2+} interferes with the [^{3}H]α,β-MeATP binding. The influence of Mg^{2+} on purinergic neurotransmission has been reported before. Exposure of guinea-pig bladder to Mg^{2+}-free solution greatly potentiates the responses to ATP and electrical field stimulation (Johns, 1979). In rabbit ear artery, after withdrawal of Mg^{2+} from the perfusate, responses to ATP are potentiated while the responses to adrenaline and noradrenaline are unaffected (Asmawi *et al.*, 1980). An experiment carried out on guinea-pig vas deferens showed that the contractile response to ATP was increased by the removal of Mg^{2+} from the medium (Morishita & Furukawa,

1989). Similar results have also been reported by Fedan *et al.* (1990).

It should be pointed out that the binding sites being examined may not just involve the neurotransmitter recognition moiety, but also the allosterically linked conductance channels and other modulating entities. It has been observed that the activation of P_{2X}-purinoceptors on vascular and visceral smooth muscle cells leads to the opening of non-selective cation channels (see Chapter 1). Since ATP-induced cation influx is a very rapid process, it is possible that the purinoceptor and the regulated ion channel are parts of a pre-existing functional complex, which may be structurally related to the superfamily of multi-subunit receptor channels. Because agonist binding can trigger the ion channels, it is not surprising that the binding of [^{3}H]α,β-MeATP was influenced by cations, which may be part of the physiological regulation mechanism for the P_{2X}-purinoceptor.

The possibility that [^{3}H]α,β-MeATP may bind to ATPases instead of, or as well as, P_{2X}-purinoceptors can be raised. The results from the binding study of [^{3}H]α,β-MeATP with $Na^+ \cdot K^+ \cdot$ATPase indicate that [^{3}H]α,β-MeATP has no, or very weak, affinity with $Na^+ \cdot K^+ \cdot$ATPase. Further evidence against the possibility that the [^{3}H]α,β-MeATP binds to ATPases was that, in autoradiographic localization studies, [^{3}H]α,β-MeATP binding sites are tissue-specific while the ATPases are ubiquitous in cells and tissues (Kreutzberg et al., 1986) (see Chapter 4, 5, and 6). In a study of solubilized membrane preparations from rat vas deferens using

sucrose density gradient centrifugation it was found that the [^{3}H]α,β-MeATP binding sites/detergent complex had a different sedimentation coefficient from the ATPase/detergent complex (see Chapter 7).

Binding of agonists should be clearly reversible as most of their effects are rapidly reversible. α,β-MeATP is an agonist for P_{2X}-purinoceptors, thus its binding to this receptor should be reversible. The dissociation experiment in this study has demonstrated such a property of α,β-MeATP. Another aspect is the time course of the binding process; since the effect of the ligand produced is usually very rapid, so that the binding should be at least as fast as the appearance of the effect. Simple occupancy theory predicts that the dissociation of receptor binding should be as fast as the "washout" of the biological effect. However, in systems exhibiting desensitisation or other complications, this may be not true, because the binding sites might be the desensitized state of the receptor. Both the association and dissociation processes of [^{3}H]α,β-MeATP binding to rat bladder membranes were very rapid, which is in agreement with the observation that the contractile responses of bladder detrusor muscles elicited by α,β-MeATP are very rapid and can be reversed by washing away the ligand (Kasakov and Burnstock, 1983). However, α,β-MeATP has the ability to desensitize P_{2X}-purinoceptor-mediated responses (Kasakov and Burnstock, 1983; Moss and Burnstock, 1985). Whether [^{3}H]α,β-MeATP can also bind to the desensitized receptor remains to be determined.

A receptor is defined or identified in the first place in terms of its pharmacology. Thus the most important criterion in identifying a binding site as a neurotransmitter receptor is the detailed pharmacology of the binding. This means that drugs that are effective in mimicking or blocking the effects of the neurotransmitter should compete for binding at low concentrations, whereas ineffective drugs should compete only at very high concentrations if at all. The displacement experiment in this study proved the specificity of [^{3}H]α,β-MeATP binding to P_{2X}-purinoceptors. In the presence of Mg^{2+} at 30°C which is nearer the physiological condition, the rank order of potency of the unlabelled ligands in displacing the [^{3}H]α,β-MeATP binding is: α,β-MeATP > β,γ-MeATP > suramin > ATP > ADP > 2-MeSATP >> adenosine, which is in good agreement with the potency order of these ligands in their pharmacological actions on P_{2X}-purinoceptors (Burnstock & Kennedy, 1985). P_{2Y}-purinoceptor binding showed a different potency order: 2-MeSATP > ADPβS > ATP > ADP> App(NH)p > α,β-MeATP > β,γ-MeATP (Cooper *et al.*, 1989). This is also in accordance with the criteria for the classification of P_{2Y}-purinoceptors. It should be mentioned that the present displacement experiments were carried out at the radioligand concentration of 10 nM, which approximated to the K_d values for the high-affinity binding, thus the obtained potency order of the cold ligands probably represents their affinities to the high-affinity [^{3}H]α,β-MeATP binding sites.

In Mg^{2+}-free buffer at 4°C, the degradative action of 5'-

nucleotidases on the unlabelled ligands and the interference of Mg^{2+} on the binding were avoided. Thus the potencies of all unlabelled ligands except suramin and adenosine were significantly increased. The rank order of potencies only showed a slight change: α,β-MeATP > β,γ-MeATP > suramin > ATP > 2-MeSATP > ADP >>adenosine. In the presence of Mg^{2+} at 30°C the effects of ATP and ADP in inhibiting the [^{3}H]α,β-MeATP binding increased very sharply when their concentrations were over 10^{-5} M, while in the absence of Mg^{2+} at 4°C, their concentration-effect curves became smoother. This phenomenon may be caused by the degradation of 5'-nucleotidases on these ligands; at lower concentrations nearly all the added ATP and ADP was degraded during the incubation, while at higher concentrations a certain amount of ATP and ADP remained at the end of the incubation (data from the experiment for the determination of ATPase enzymic activities, not shown here). α,β-MeATP and β,γ-MeATP were ATPase resistant, thus their concentration-effect curves remained the same shapes, and were shifted to the left in the absence of Mg^{2+}.

Suramin is a trypanocidal drug which was introduced into therapy in 1920. Recently it was reported that suramin could antagonize the response of mouse vas deferens to α,β-MeATP, and it was suggested as a specific antagonist for P_2-purinoceptors (Dunn & Blakeley, 1988). Our results confirm its affinity for P_{2X}-purinoceptors. However, pharmacological results from our laboratory show that suramin is also an antagonist of P_{2Y}-purinoceptors with an equal affinity (Hoyle *et al.*, 1990). So the

action of suramin is not specific for the P_{2X}-purinoceptors.

Many pharmacological experiments have been carried out to determine the critical structure of ATP in eliciting contractile responses via P_{2X}-purinoceptors (Fedan *et al.*, 1982; 1986; Welford *et al.*, 1987; Howson *et al.*, 1988). Modification of the molecular structure of ATP, such as deletions, substitutions, and isosteric rearrangement, have been performed on the base, ribose, and polyphosphate chain. Both studies on guinea-pig urinary bladder and vas deferens suggest that the tripolyphosphate chain is the critical structure for the potency of the agonist (Lucacsko & Krell, 1982; Fedan *et al.*, 1986; Howson *et al.*, 1988). As to the adenine and ribose, Fedan *et al.*, (1986) suggest that adenine is also involved in the activity of ATP, although to a lesser degree compared with the polyphosphate chain. Howson *et al.*, (1988) consider that both the adenine and ribose are associated with the affinity of ATP to the P_{2X}-purinoceptors.

The results from the present displacement experiments support the hypothesis that the polyphosphate chain is the critical structure for the interaction between ATP and P_{2X}-purinoceptors. However, the phosphate chain is not only responsible for the activity of ATP but also for its affinity. Because adenosine, adenine, and xanthine have little effect in displacing [^{3}H]α,β-MeATP binding, while tripolyphosphate and pyrophosphate showed similar potencies as ATP in displacing the binding. $Na_2H_2PO_4$ is much less potent than either tripolyphosphate or pyrophosphate in displacing [^{3}H]α,β-MeATP binding, which indicates that the binding

of purinergic ligands to P_{2X}-purinoceptors requires a phosphate chain with at least two moieties, that also explains why AMP is not a P_{2X}-purinoceptor agonist.

This study also support the presumption that the increased potencies of ATP analogues with modified polyphosphate chain is not a consequence of greater resistance to ectonucleotidase, but rather a consequence of the function of the modified structures themselves (Welford *et al.*, 1987; Levin *et al.*, 1981). In this study ATP was incubated with the membranes in divalent cation free buffer at 4°C, and under these conditions the degradation of ATP was limited, yet it was still much less potent than β,γ-MeATP in displacing [^{3}H]α,β-MeATP binding.

Adenine in the ATP molecule is also involved in the affinity of ATP to the P_{2X}-purinoceptors because the potency of ATP in displacing [^{3}H]α,β-MeATP binding was about 2-fold higher than that of UTP, and 8- to 20-fold higher than CTP, ITP, TTP, and GTP. These results were consistent with the pharmacological observations. In guinea-pig bladder, although ATP, GTP, and CTP can all cause contractile responses, ATP is more potent than GTP and CTP, while the latter two are equipotent (Lukacsko & Krell, 1982). In guinea-pig vas deferens it has also been found that ATP is more potent than ITP, XTP, and GTP in eliciting contraction (Fedan *et al.*, 1986). Taken together, these findings suggeste that adenine is also an important structure for the affinity as well as the activity of ATP in the interaction with P_{2X}-purinoceptors.

In this chapter the factors which influence the binding of [^{3}H]α,β-MeATP to rat bladder membranes were systematically studied. The properties of the binding sites were also examined. So far, it has been shown that [^{3}H]α,β-MeATP binding sites possesses most of the characteristics of a biological receptor, such as saturability, reversibility, specificity, temperature dependence, influence by pH and ions. Results from competitive displacement with unlabelled purinergic ligands indicate that the binding sites are related to P_{2X}-purinoceptors. It is also suggested that the critical structure of the [^{3}H]α,β-MeATP molecules for the binding to P_{2X}-purinoceptors is the tripolyphosphate chain. These results formed the basis for the further characterization of P_{2X}-purinoceptors in other tissues and species, and also provided important information for the autoradiographic localization of P_{2X}-purinoceptors with [^{3}H]α,β-MeATP.

Figure 2-1 **Transmission electronic micrograph of the membrane fractions obtained by differential centrifugation.**

Membrane fractions were fixed according to the protocol for conventional electron microscopy and embedded in Araldite. Ultrathin (silver colour) sections were cut, stained with uranyl acetate and lead citrate.
Magnification, x 100,000; calibration bar = 500 nm.
Arrow: gap junction

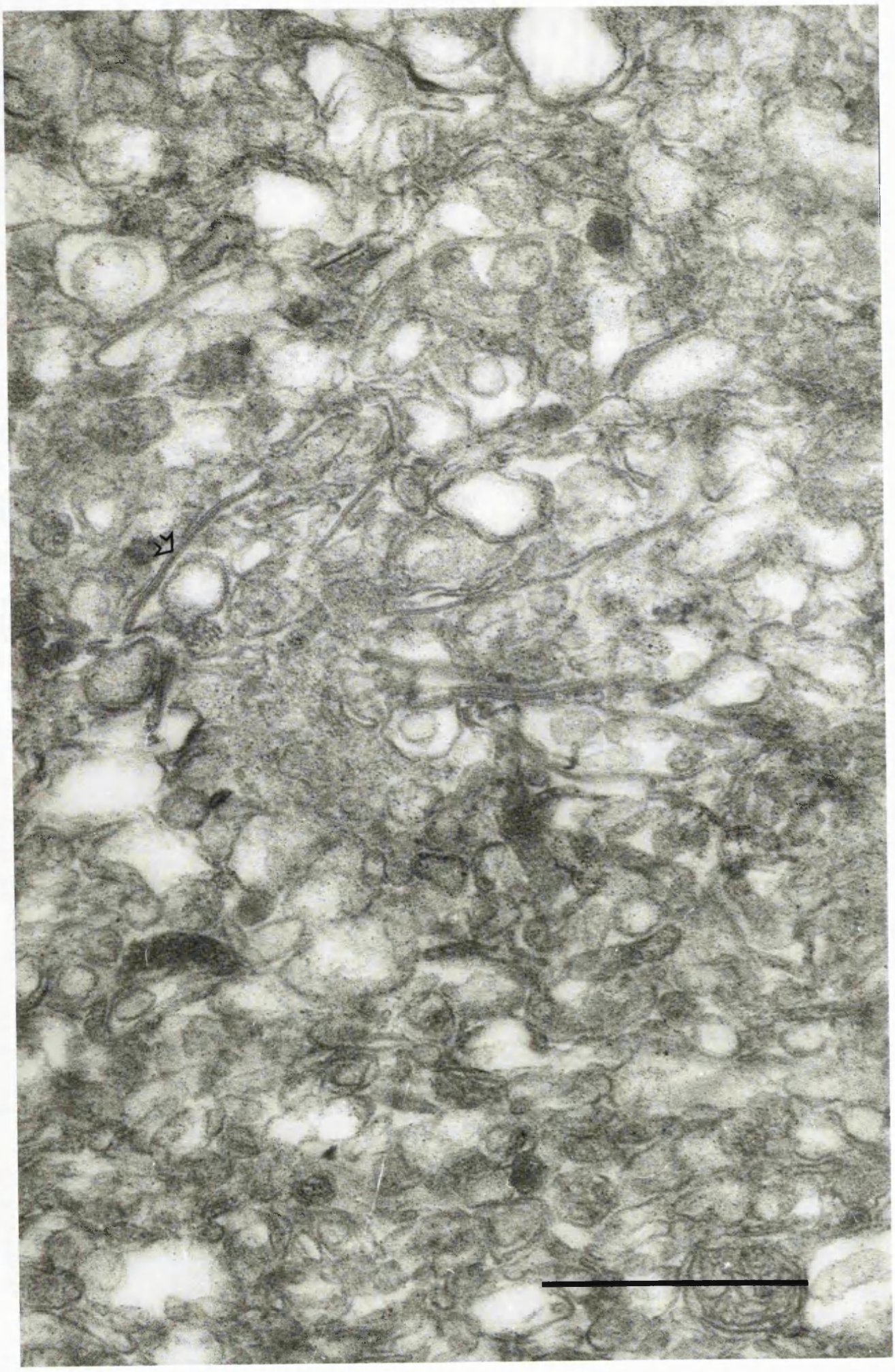


Figure 2-2 **Relationship between the buffer volumes for washing the filters and the specific and nonspecific binding of [^{3}H]α,β-MeATP to rat urinary bladder membranes.**

Membrane preparations were incubated with 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min, passed through double layers of Whatman GF/C filters under vacuum, and then washed with different volumes of ice-cold 50 mM Tris-HCl buffer. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results were from three experiments (each in duplicate).

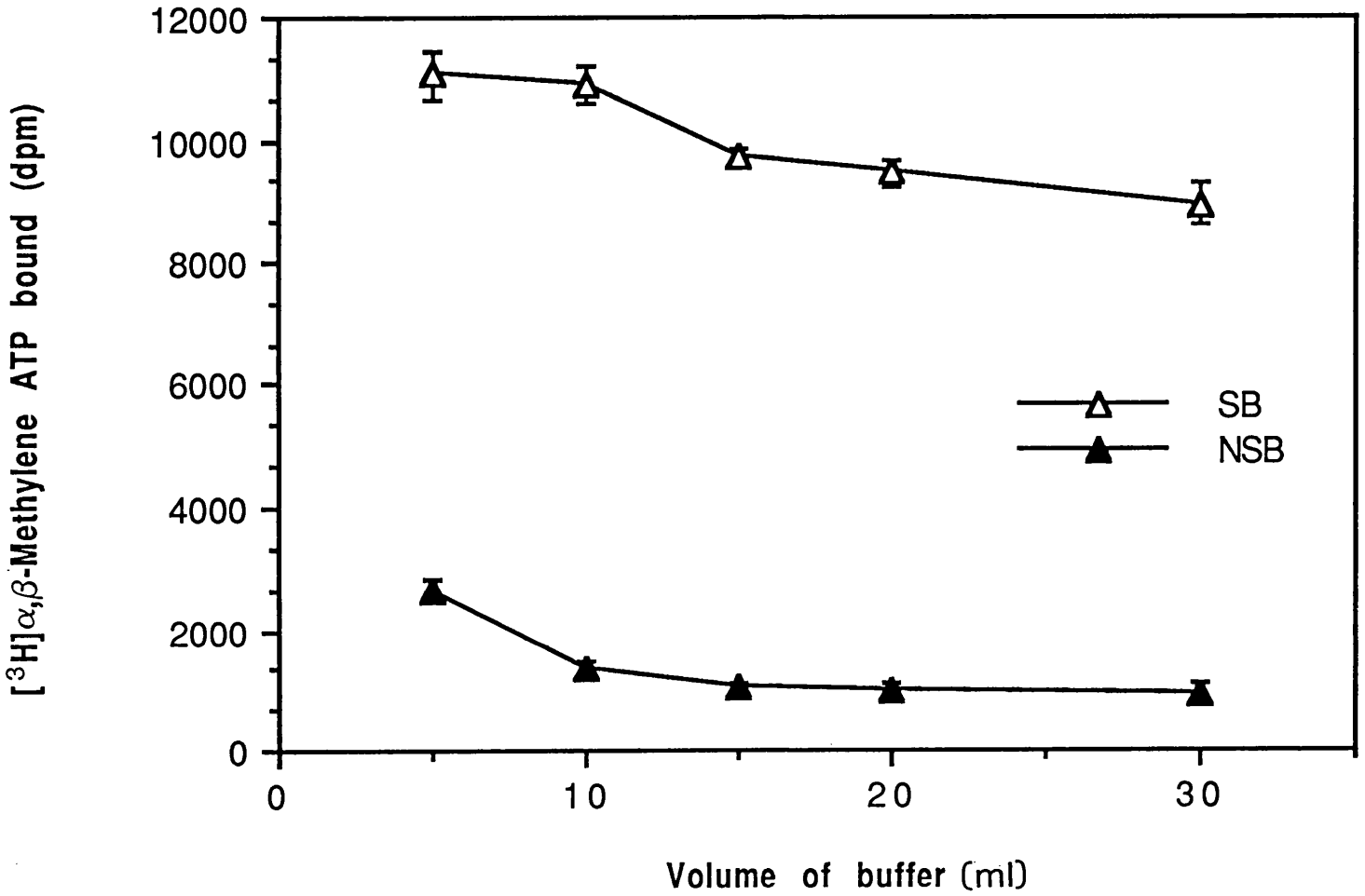

12000
10000
8000
6000
4000
2000
0
0
10
20
30
[3H]α,β-Methylene ATP bound (dpm)
Volume of buffer (ml)
SB
NSB

Figure 2-3 **The effects of different types of buffer on the binding of [^{3}H]α,β-MeATP to rat urinary bladder membranes.**

Membrane preparations were incubated with 10 nM [^{3}H]α,β-MeATP in Tris/HCl, HEPES, phosphate buffer saline (PBS), and double distilled water (ddH_2O) at 30°C for 15 min. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results were from three experiments (each in triplicate).

* $p < 0.05$, *** $p < 0.001$

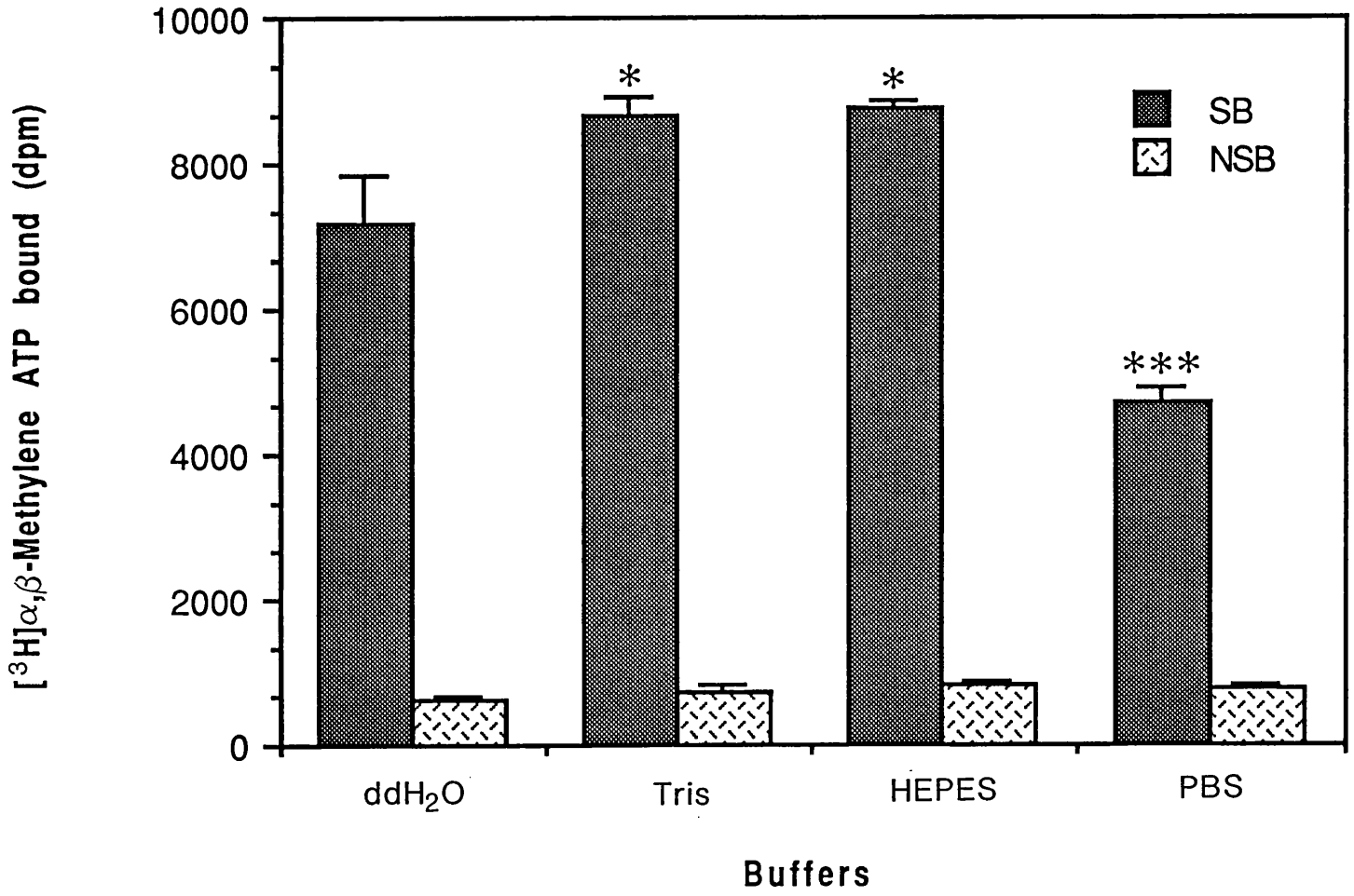

10000
8000
6000
4000
2000
0
[3H]α,β-Methylene ATP bound (dpm)
*
*
***
SB
NSB
ddH2O
Tris
HEPES
PBS
Buffers

Figure 2-4 **The effect of pH values of the incubation solution on the [^{3}H]α,β-MeATP binding to rat urinary bladder membrane preparations.**

Data shown are from six experiments.

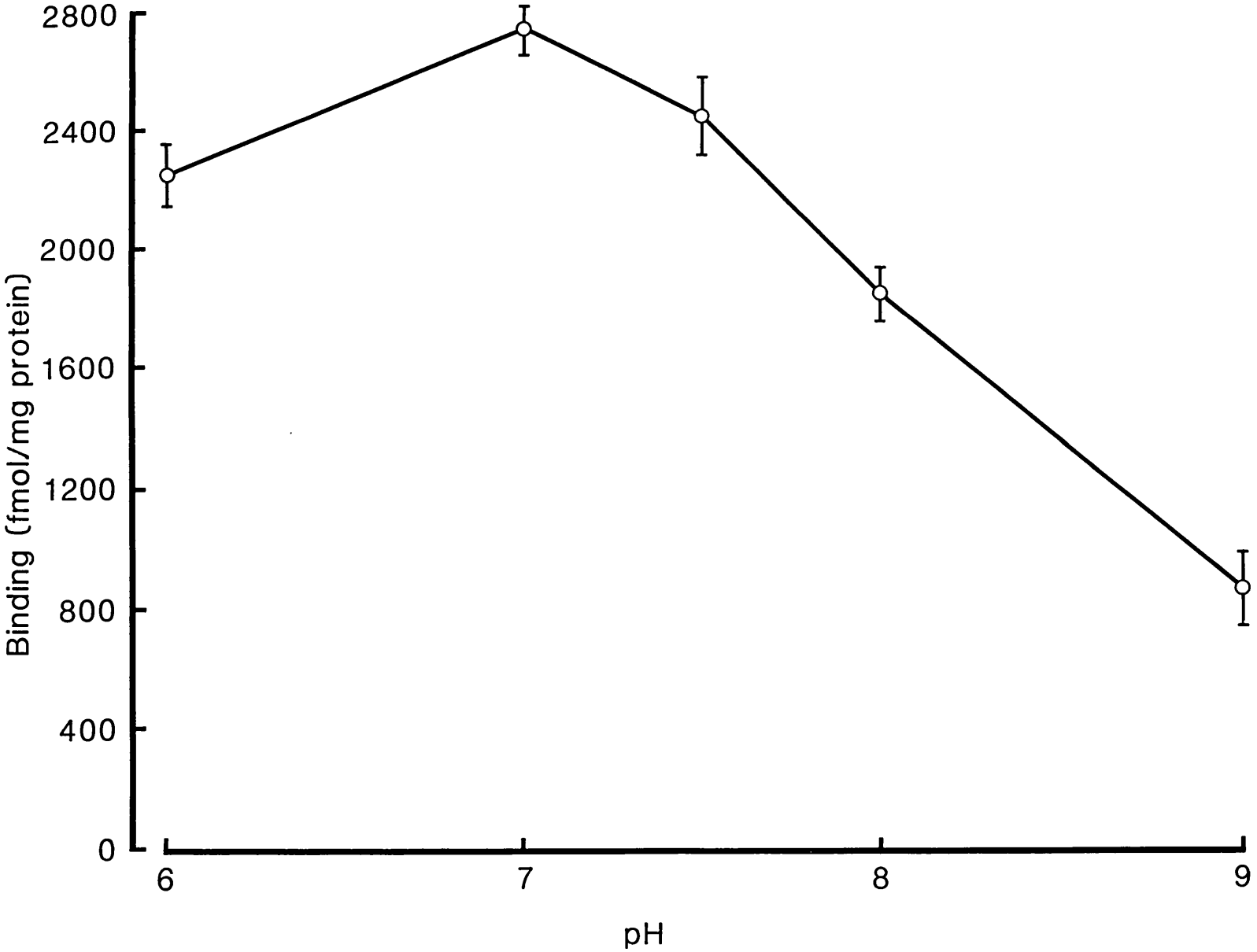

2800
2400
2000
1600
1200
800
400
0
Binding (fmol/mg protein)
6
7
8
9
pH

Figure 2-5 **Effect of the treatment of Whatman GF/C filters with pyrophosphate on the false specific binding of [^{3}H]α,β-MeATP to the filters.**

Serial concentrations of [^{3}H]α,β-MeATP were passed through pyrophosphate-treated or untreated Whatman GF/C filters. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results were from three experiments (each in duplicate).

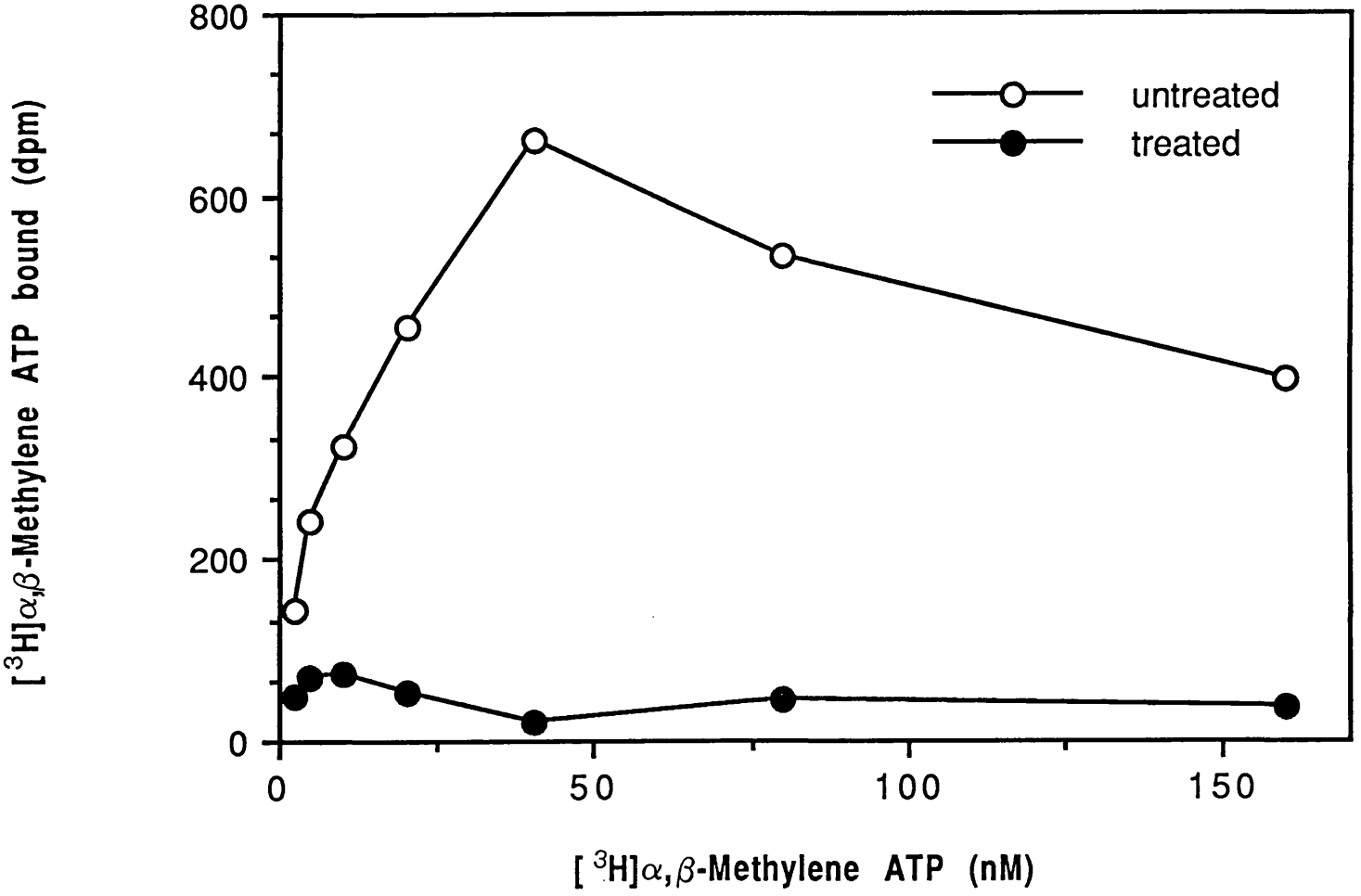

800
600
400
200
0
0
50
100
150
untreated
treated
[3H]α,β-Methylene ATP bound (dpm)
[3H]α,β-Methylene ATP (nM)

Figure 2-6 **Effect of the treatment of Whatman GF/C filters with pyrophosphate on specific binding of [^{3}H]α,β-MeATP to rat urinary bladder membranes.**

Membrane preparations were incubated with 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min and then passed through pyrophosphate-treated or untreated Whatman GF/C filters. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results were from three experiments (each in duplicate).

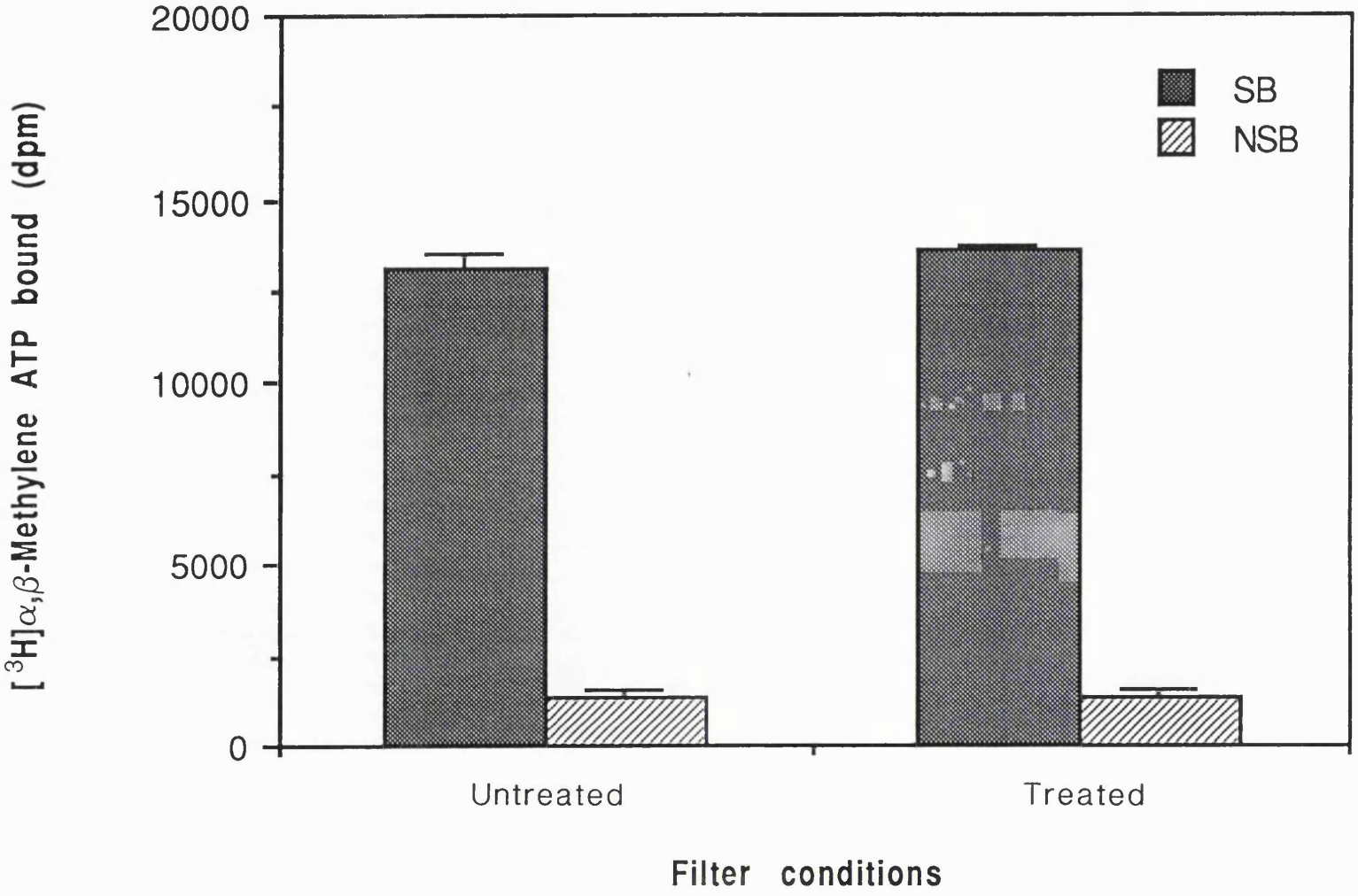

20000
15000
10000
5000
0
SB
NSB
[3H]α,β-Methylene ATP bound (dpm)
Untreated
Treated
Filter conditions

Figure 2-7 **Relationship between membrane protein concentration and specific [^{3}H]α,β-MeATP binding.**

Serial membrane preparations with different protein content were made and incubated with 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results were from three experiments (each in duplicate).

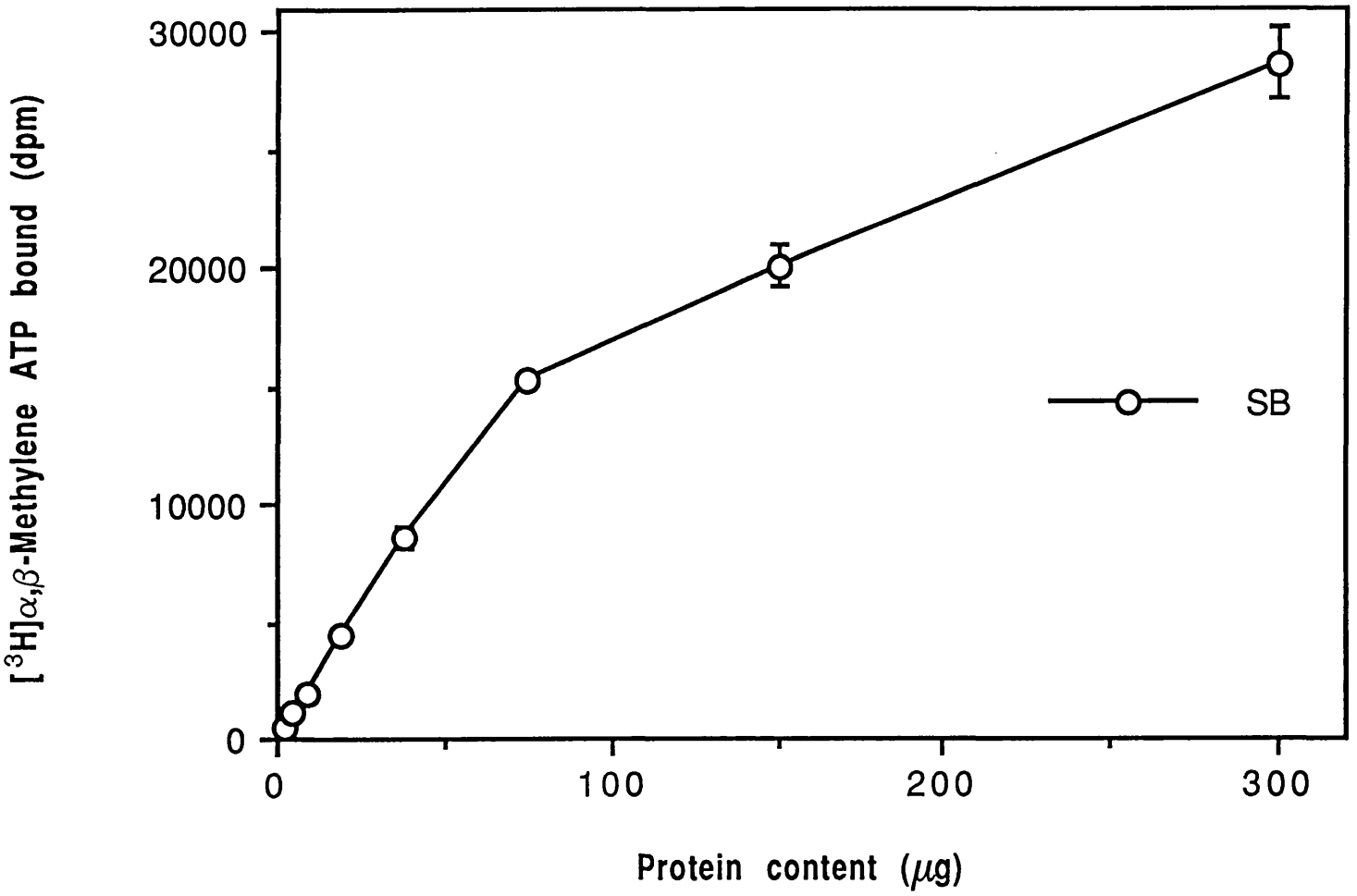

30000
20000
10000
0
0
100
200
300
[3H]α,β-Methylene ATP bound (dpm)
Protein content (μg)
SB

Figure 2-8 **Denaturization of [^{3}H]α,β-MeATP binding sites with heat.**

Rat bladder membrane preparations were preincubated at different temperatures for 20 min and then incubated with 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results were from three experiments (each in triplicate).

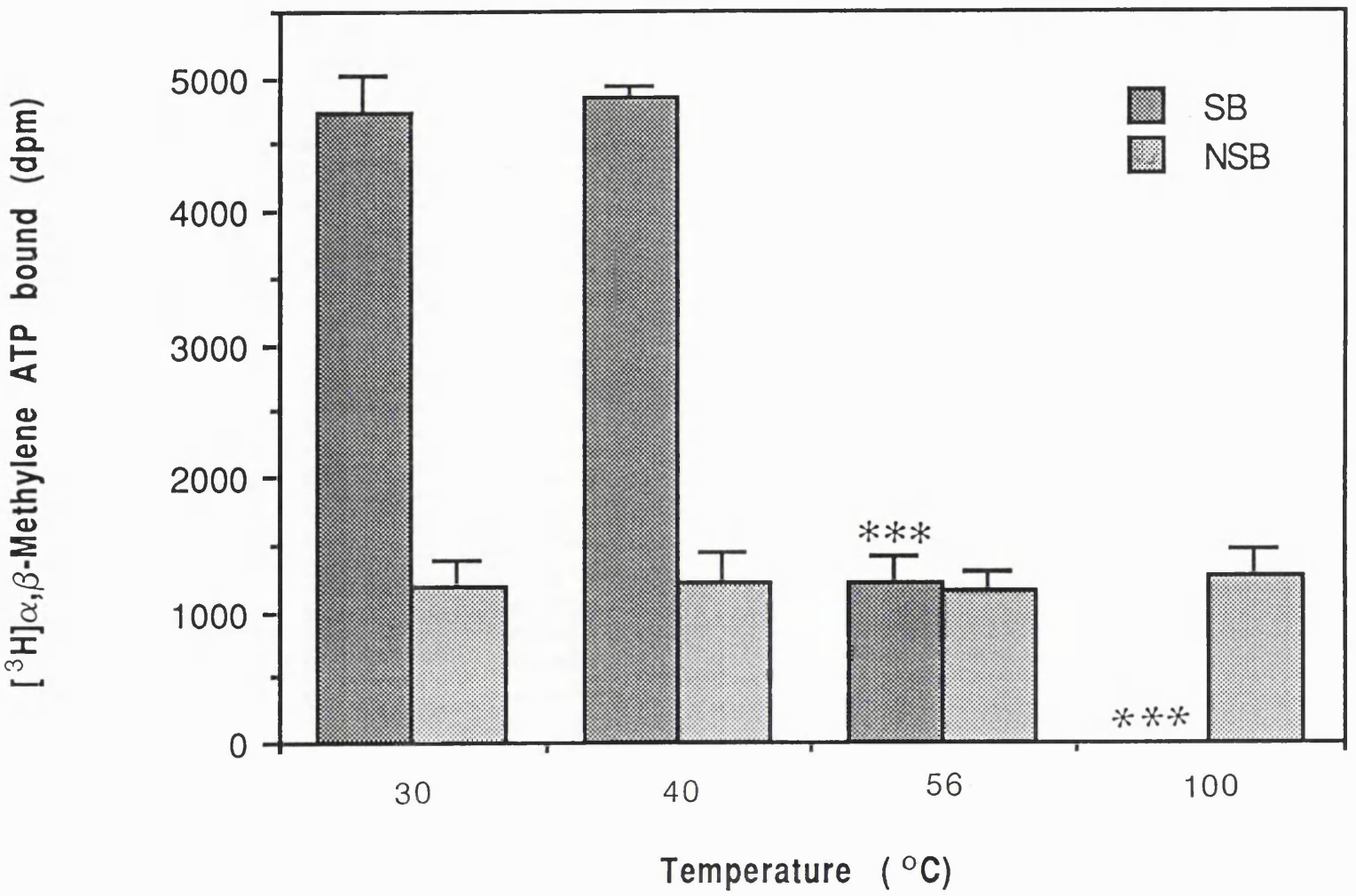

[3H]α,β-Methylene ATP bound (dpm)
5000
4000
3000
2000
1000
0
SB
NSB
***
***
30
40
56
100
Temperature (°C)

Figure 2-9 **Effects of EDTA and EGTA on the [^{3}H]α,β-MeATP binding to rat bladder membranes.**

Rat bladder membranes were incubated in divalent cation-free buffer with 10 nM [^{3}H]α,β-MeATP in the absence or presence of one of the substances at 30°C for 15 min. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results are from three experiments (each in duplicate).

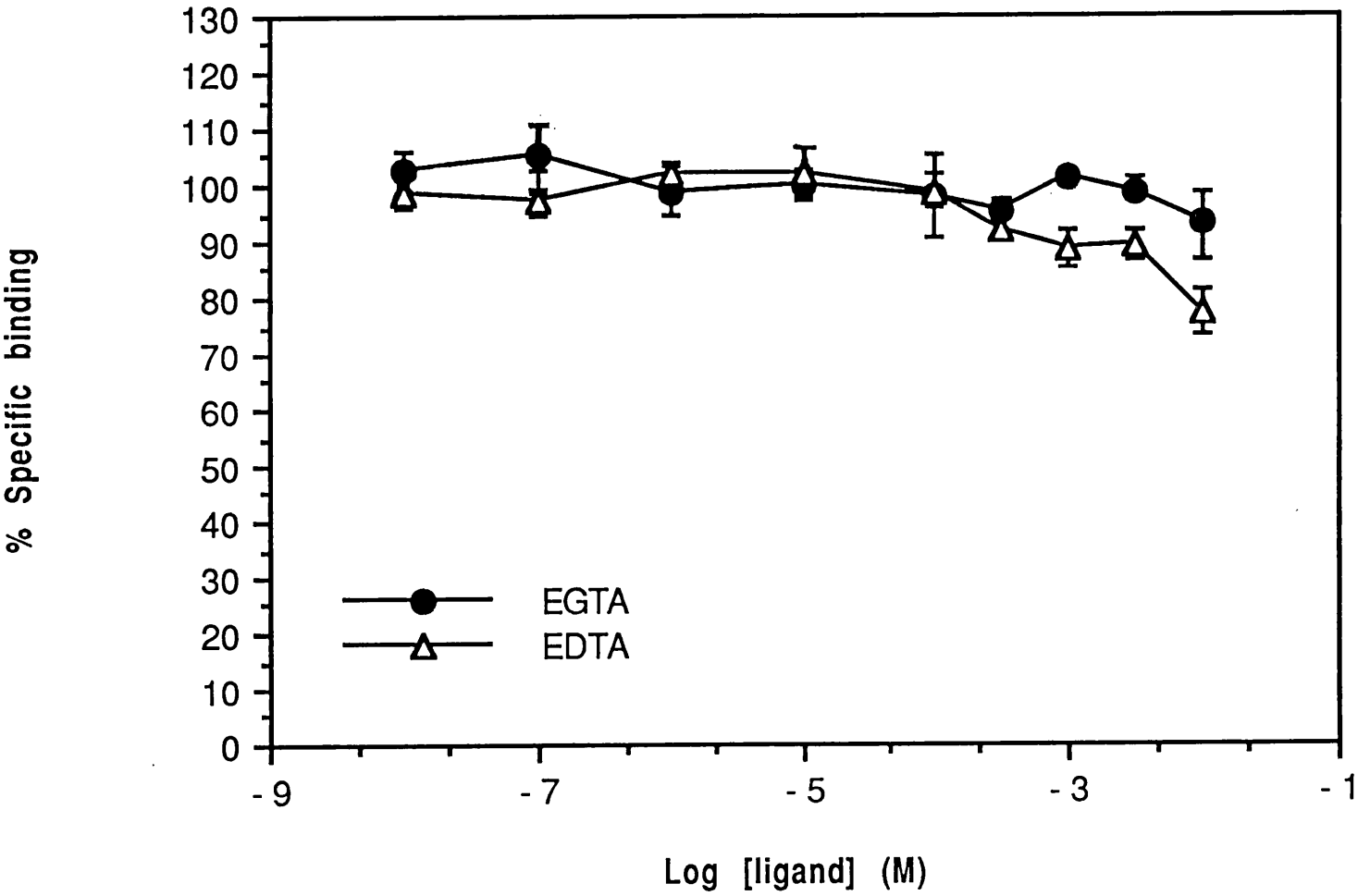

% Specific binding
130
120
110
100
90
80
70
60
50
40
30
20
10
0
- 9
- 7
- 5
- 3
- 1
EGTA
EDTA
Log [ligand] (M)

Figure 2-10 **Effects of protease inhibitors on the [^{3}H]α,β-MeATP binding to rat bladder membranes.**

Rat bladder membranes were incubated in divalent cation-free buffer with 10 nM [^{3}H]α,β-MeATP in the absence or presence of one of the substances at 30°C for 15 min. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. The results are from three duplicated experiments. PMSF, phenylmethylsulfonyl fluoride; Benz, benzamidine; TryInh, soybean trypsin inhibitors; Bacitr, basitracin.

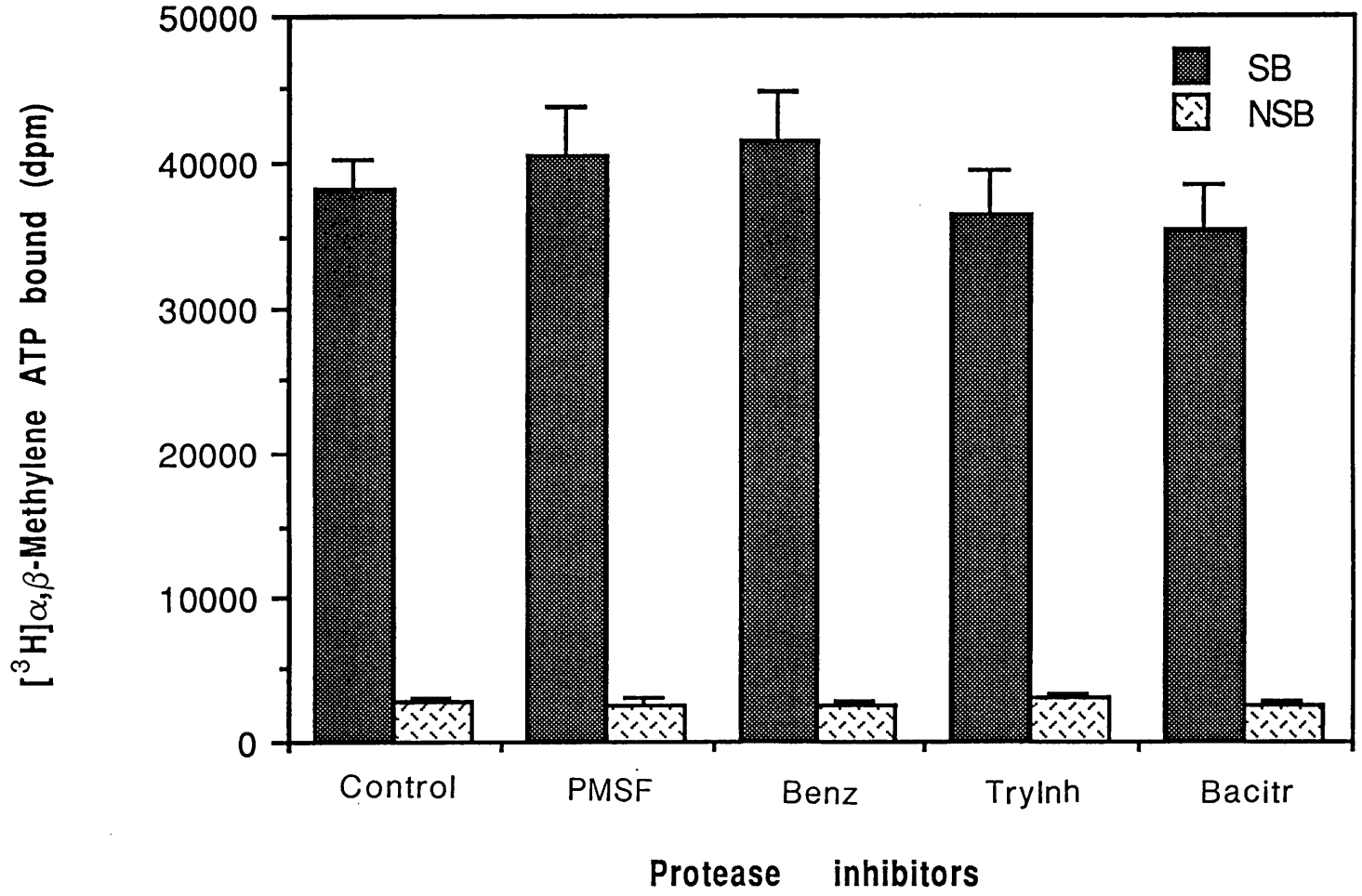
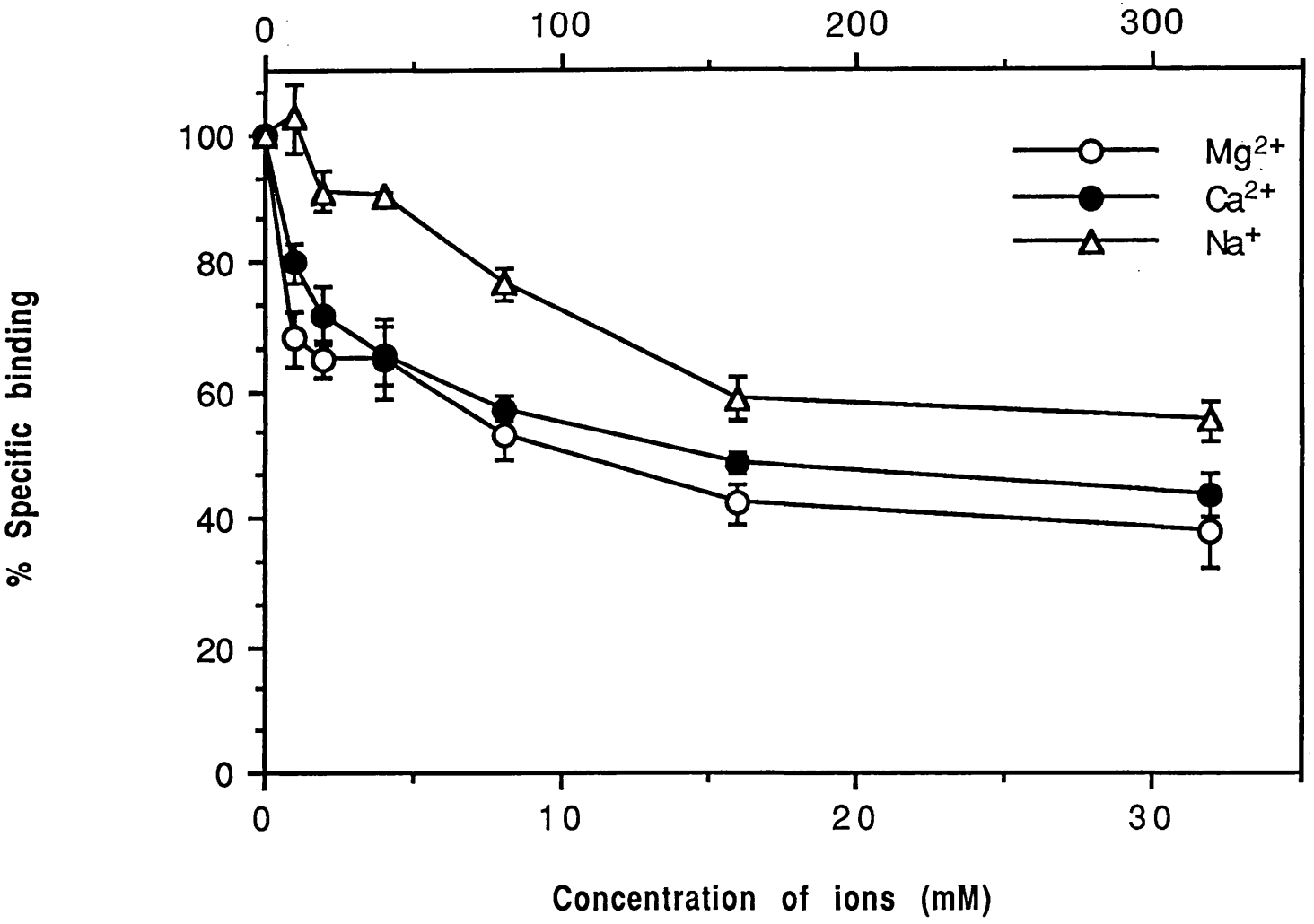
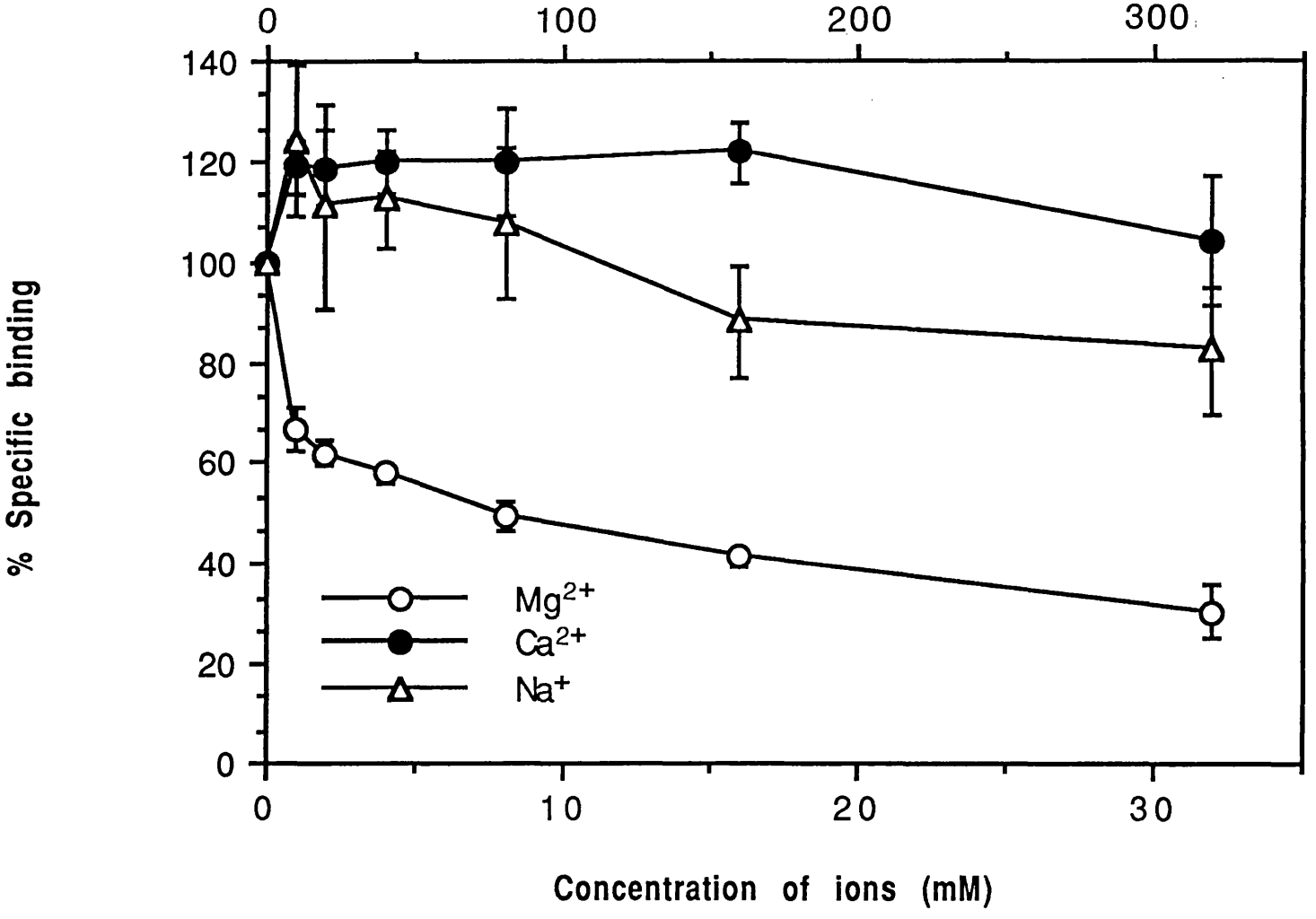
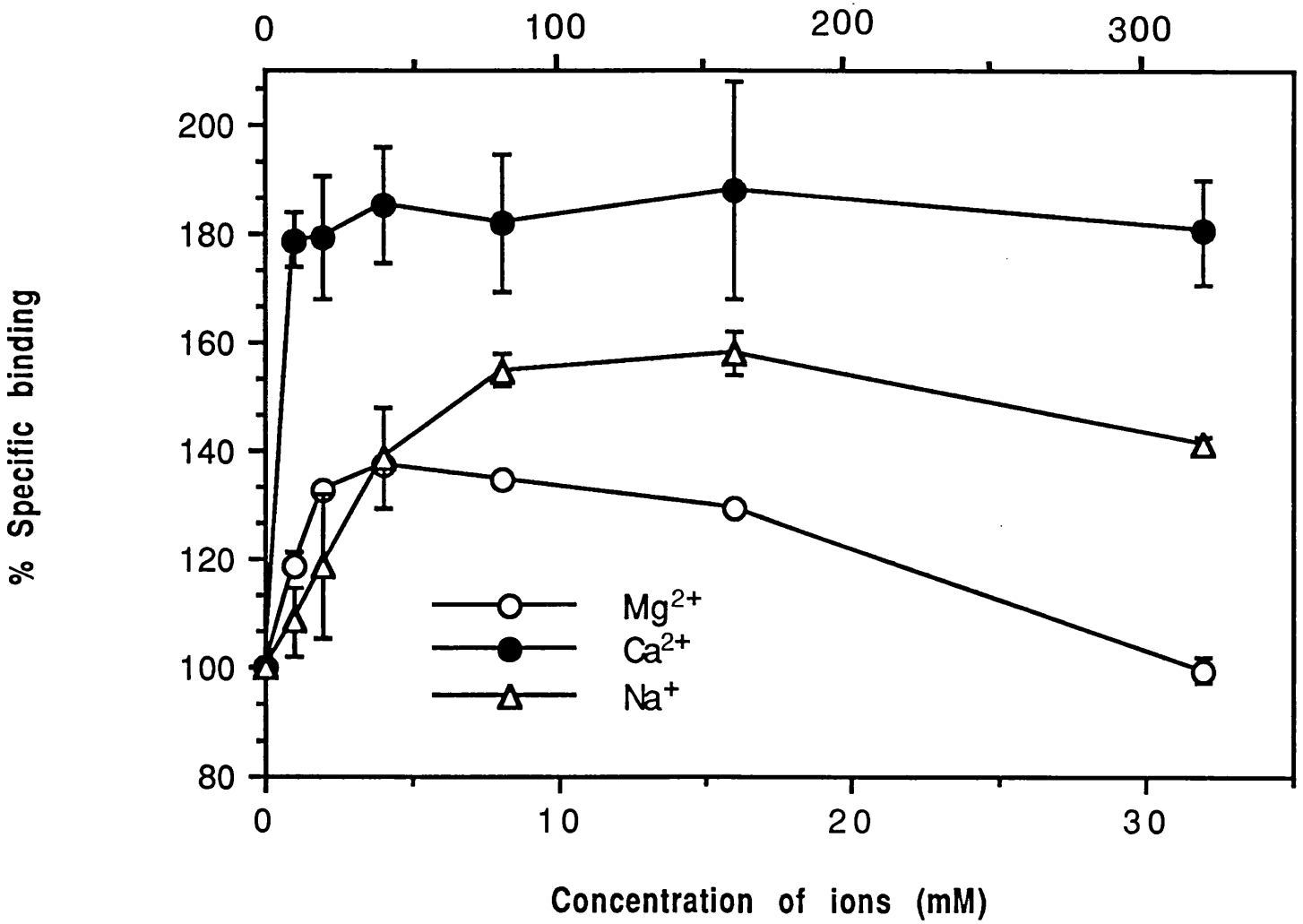

50000
40000
30000
20000
10000
0
SB
NSB
[3H]α1β-Methylene ATP bound (dpm)
Control
PMSF
Benz
TryInh
Bacitr
Protease inhibitors

Figure 2-11 **Effects of Mg^{2+}, Ca^{2+}, and Na^{+} on the [^{3}H]α,β-MeATP binding to rat bladder membranes and the washed homogenate of the bladder.**

Rat bladder membranes or the washed homogenate of the bladder were incubated with 10 nM [^{3}H]α,β-MeATP in the absence or presence of different cations at 30°C for 15 min, or 4°C for 2 hr. Nonspecific binding was determined by including 100 μM β,γ-MeATP in the media. a: Membrane binding at 30°C; b: membrane binding at 4°C; c: the washed homogenate binding at 30°C; d: the washed homogenate binding at 4°C. The results were from five duplicated experiments. * The upper scale of the frame shows the concentration of Na^{+}.


0
100
200
300
100
80
60
40
20
0
0
10
20
30
% Specific binding
Concentration of ions (mM)
Mg2+
Ca2+
Na+


0
100
200
300
140
120
100
80
60
40
20
0
0
10
20
30
% Specific binding
Concentration of ions (mM)
Mg2+
Ca2+
Na+


0
100
200
300
200
180
160
140
120
100
80
% Specific binding
0
10
20
30
Concentration of ions (mM)
Mg2+
Ca2+
Na+

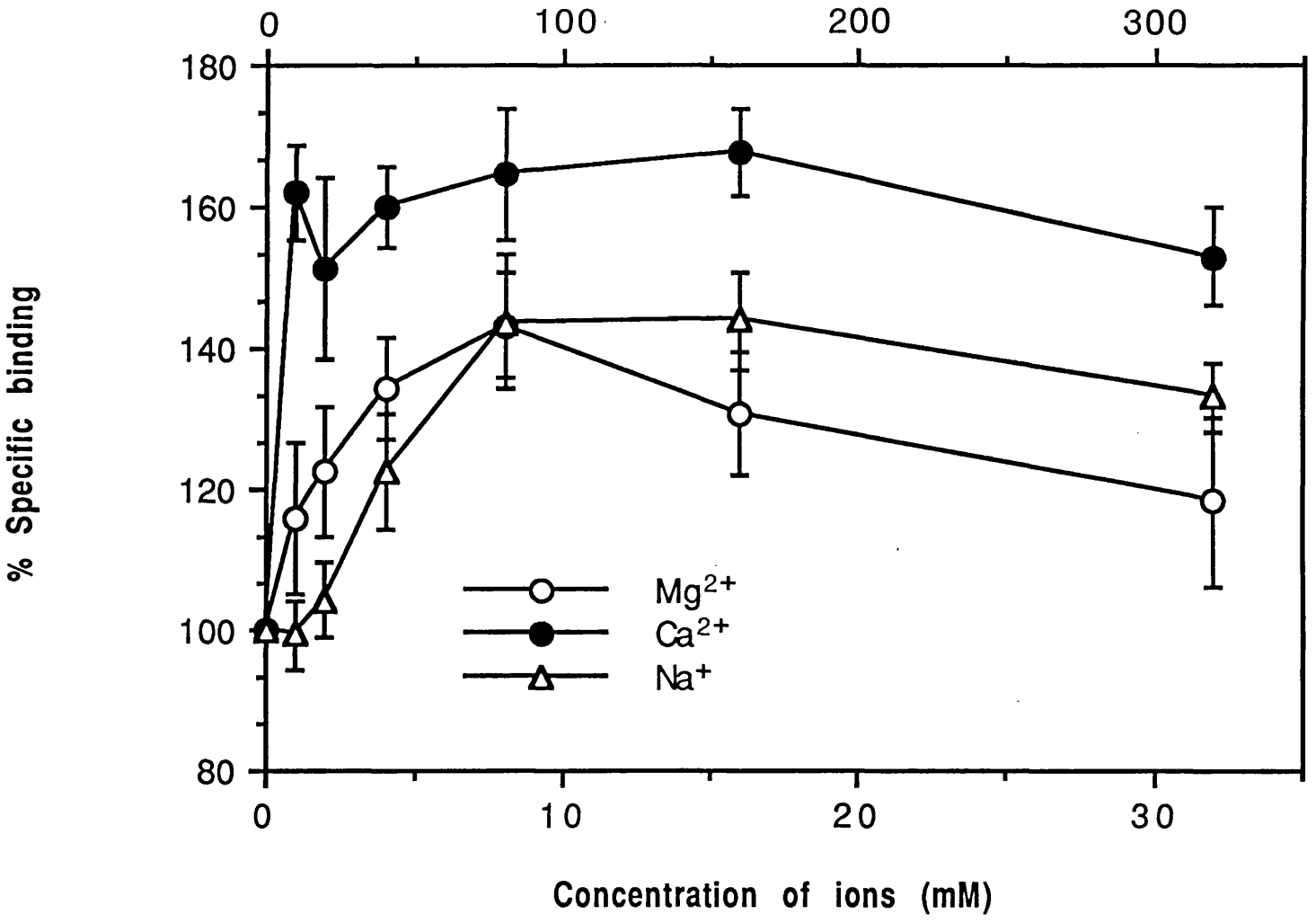

0
100
200
300
180
160
140
120
100
80
0
10
20
30
% Specific binding
Concentration of ions (mM)
Mg2+
Ca2+
Na+

Figure 2-12 **A representative of the saturation assays of [^{3}H]α,β-MeATP binding to rat urinary bladder membrane preparation in 50 mM Tris/HCl buffer with 25 mM $MgCl_2$.**

Incubation was carried out at 30°C for 15 min. Non-specific binding was determined by displacing the binding with 100 μM β,γ-MeATP.

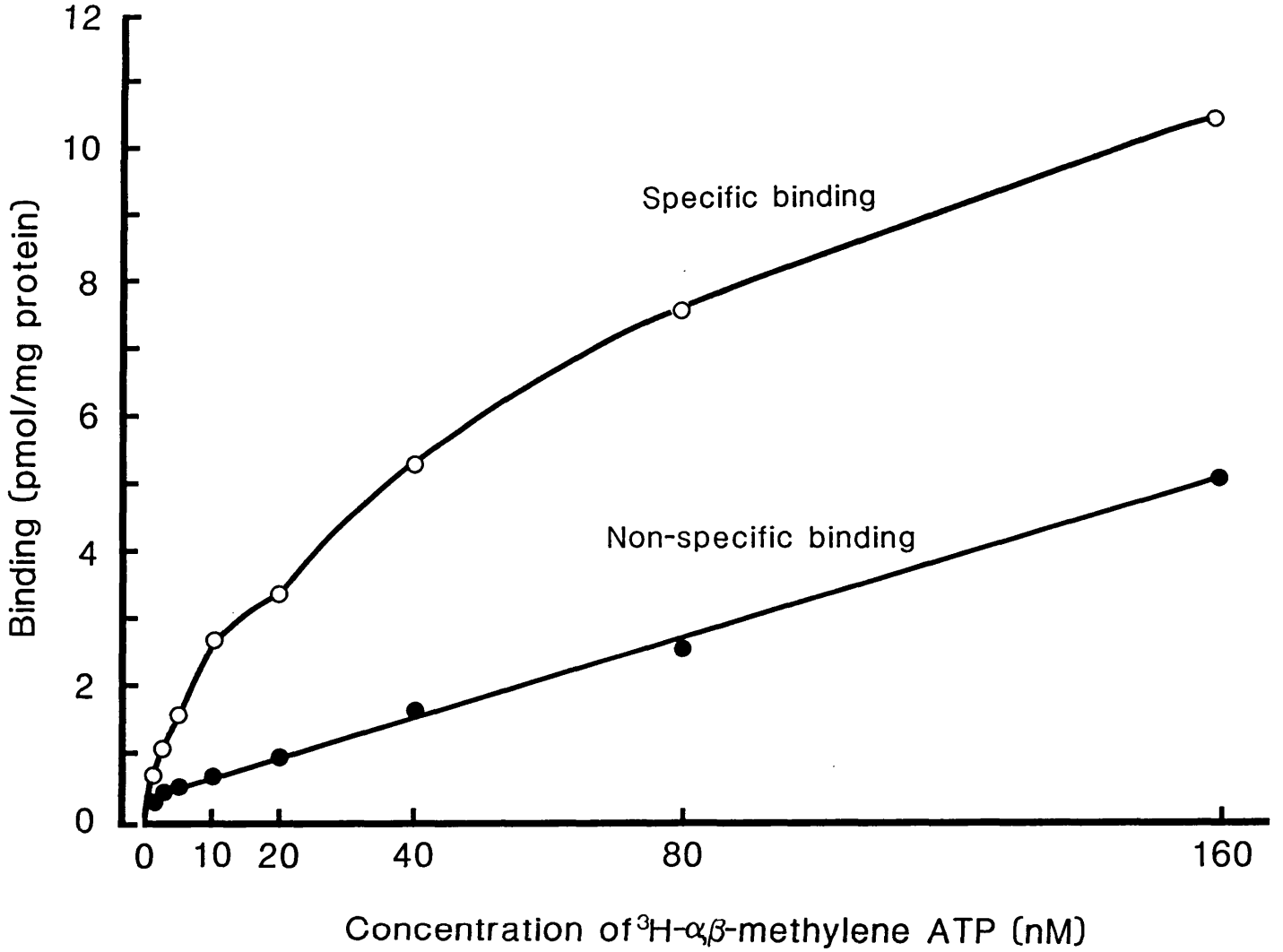

12
10
8
6
4
2
0
Binding (pmol/mg protein)
Specific binding
Non-specific binding
0
10
20
40
80
160
Concentration of ^{3}H-α,β-methylene ATP (nM)

Figure 2-13 **Scatchard transformation of the data from Figure 2-12.**

The curve was fitted with a computer program EBDA-LIGAND. F Test of the weighted residual sum of squares showed that the two binding affinity state model was significantly better than that the model for one binding affinity state.

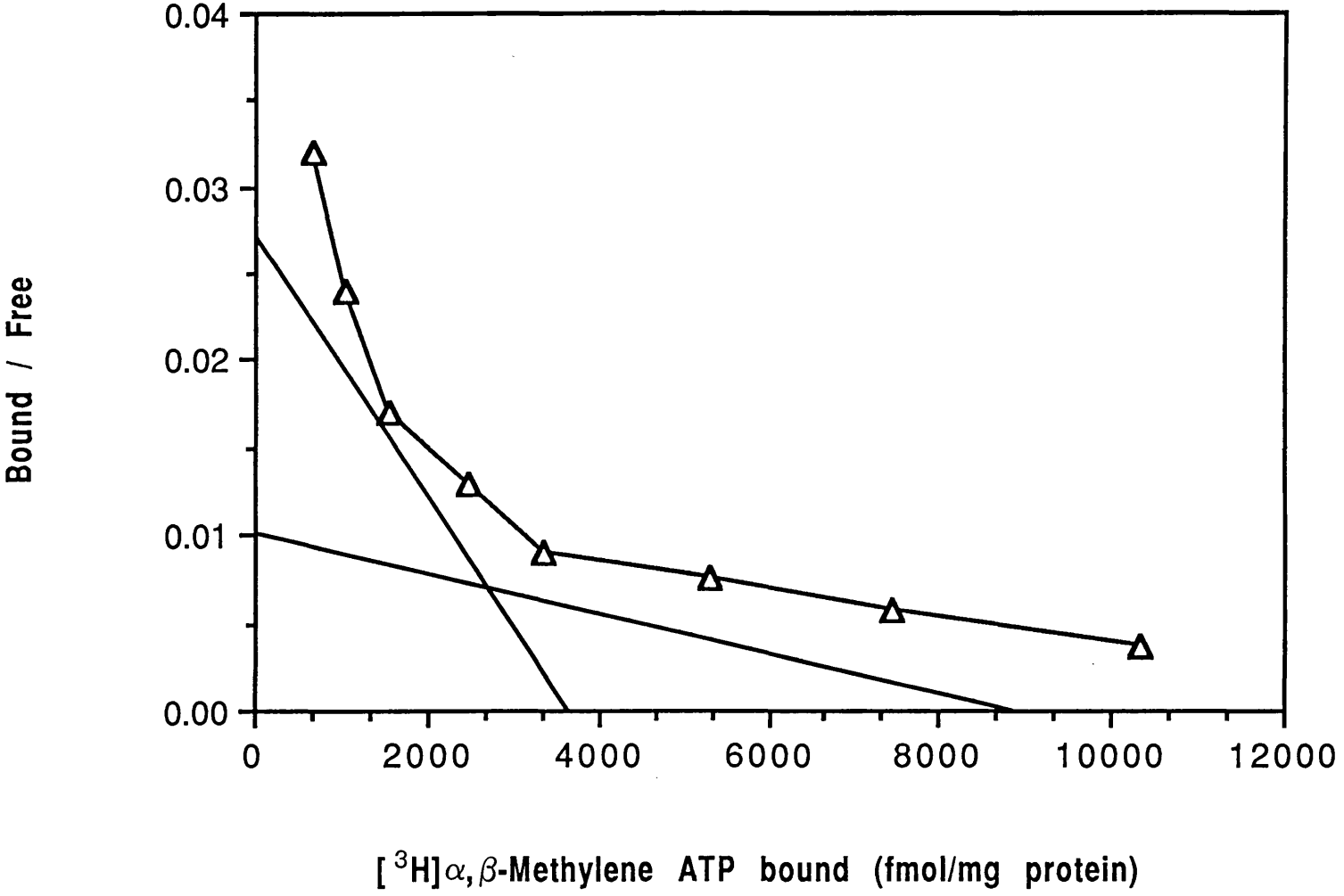

0.04
0.03
0.02
0.01
0.00
Bound / Free
0
2000
4000
6000
8000
10000
12000
[3H] α, β-Methylene ATP bound (fmol/mg protein)

Figure 2-14 **Association curve of [^{3}H]α,β-MeATP (10 nM) binding to rat urinary bladder membrane preparation at 30°C.**

Inset shows the time course after the original data are transformed with pseudo-first-order rate equation for the calculation of association rate constants. Data shown are from seven experiments. [Be]: concentration of receptor-radioligand complex at equilibrium, [B]: concentration of receptor-radioligand complex at the time the reaction was terminated.

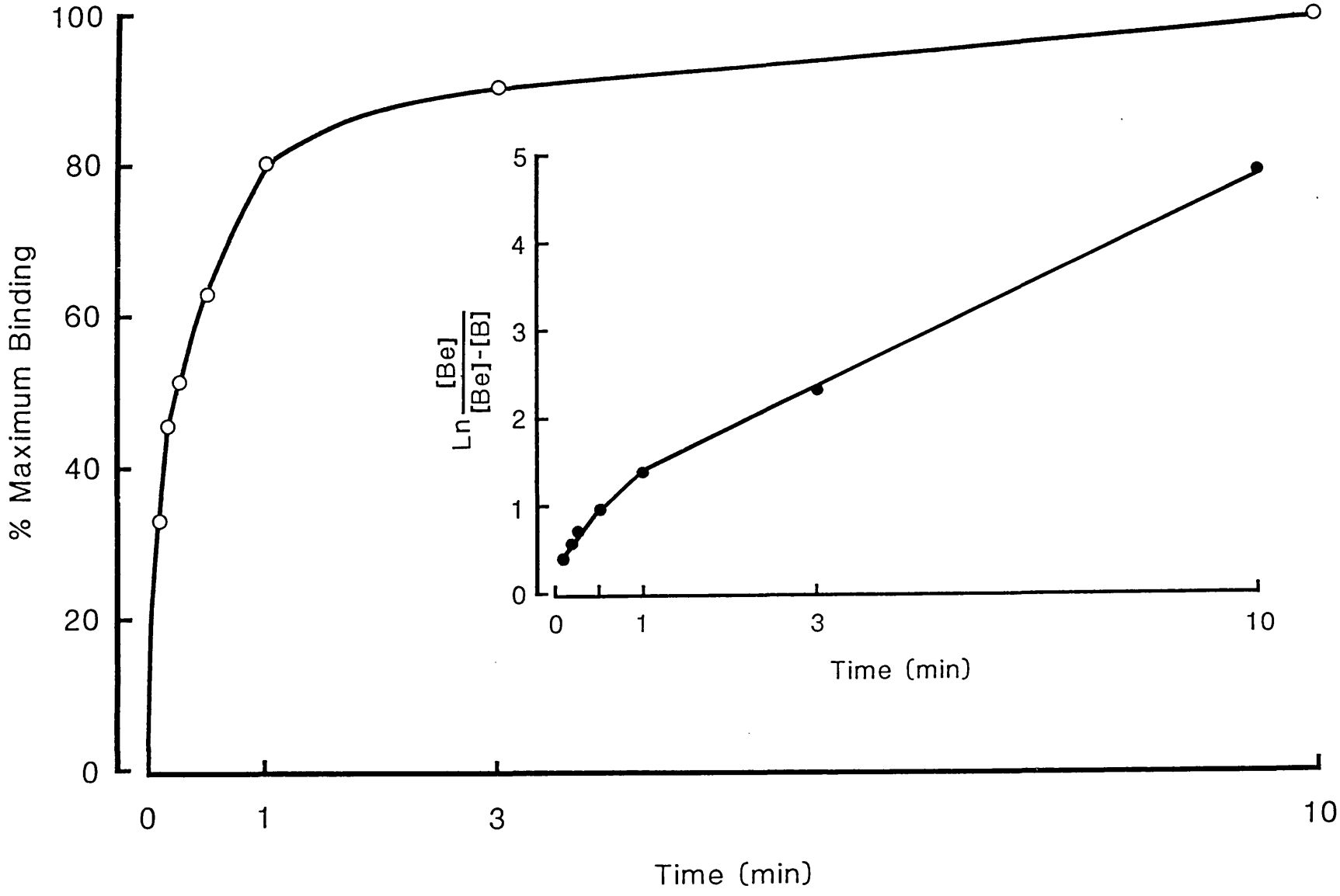

100
80
60
40
20
0
% Maximum Binding
0
1
3
10
Time (min)
5
4
3
2
1
0
Ln [Be]/[Be]-[B]
0
1
3
10
Time (min)

Figure 2-15 **Dissociation curve of [^{3}H]α,β-MeATP (10 nM) binding to rat urinary bladder membrane preparation at 30°C.**

β,γ-MeATP (100 μM) was used to displace the bound [^{3}H]α,β-MeATP after the binding reached equilibrium. Inset shows the time course after the original data were transformed with the equation for dissociation rate constant. Data shown are from seven experiments. [Be]: concentration of receptor-radioligand complex at equilibrium, [B]: concentration of receptor-radioligand complex at the time the reaction was terminated.

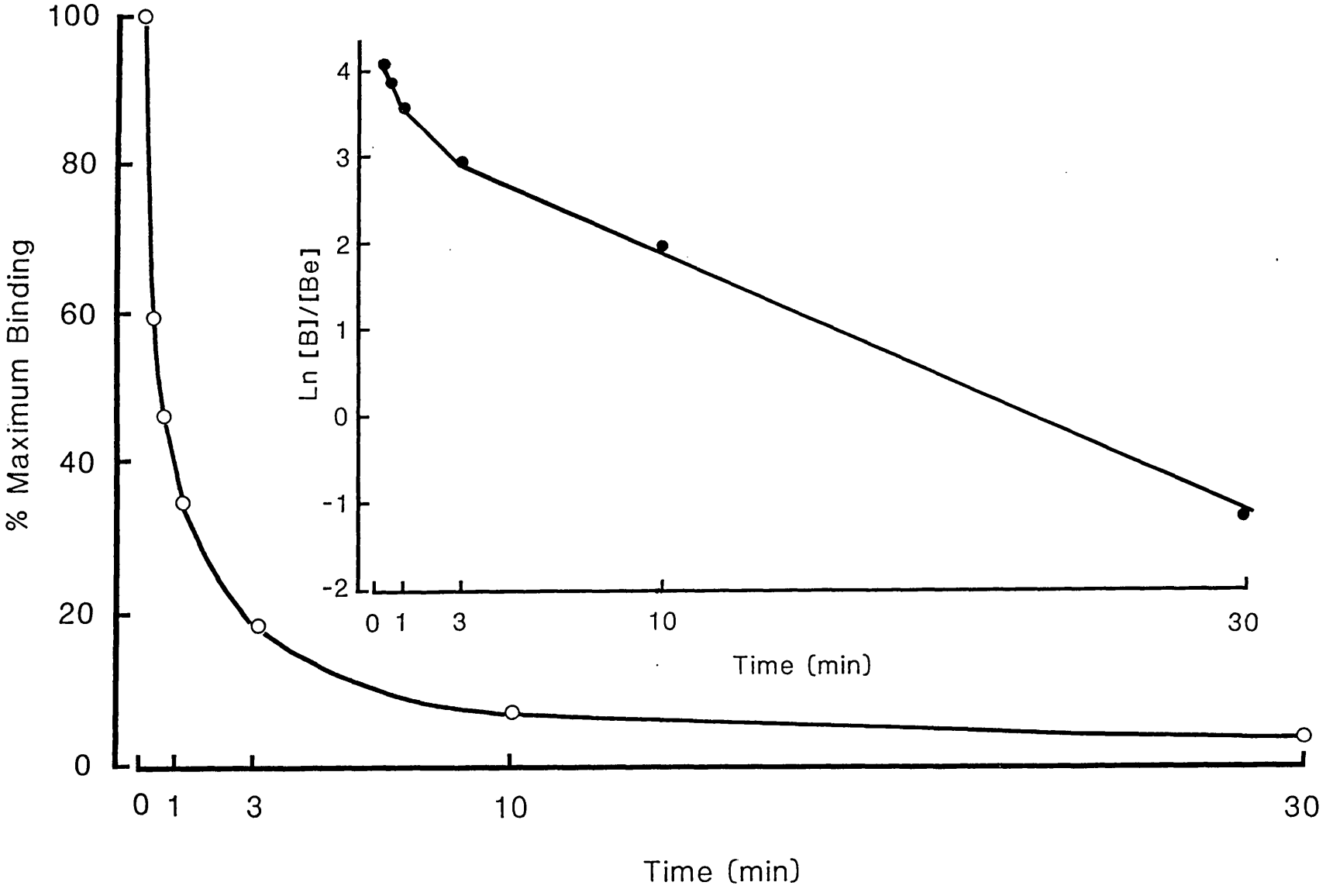
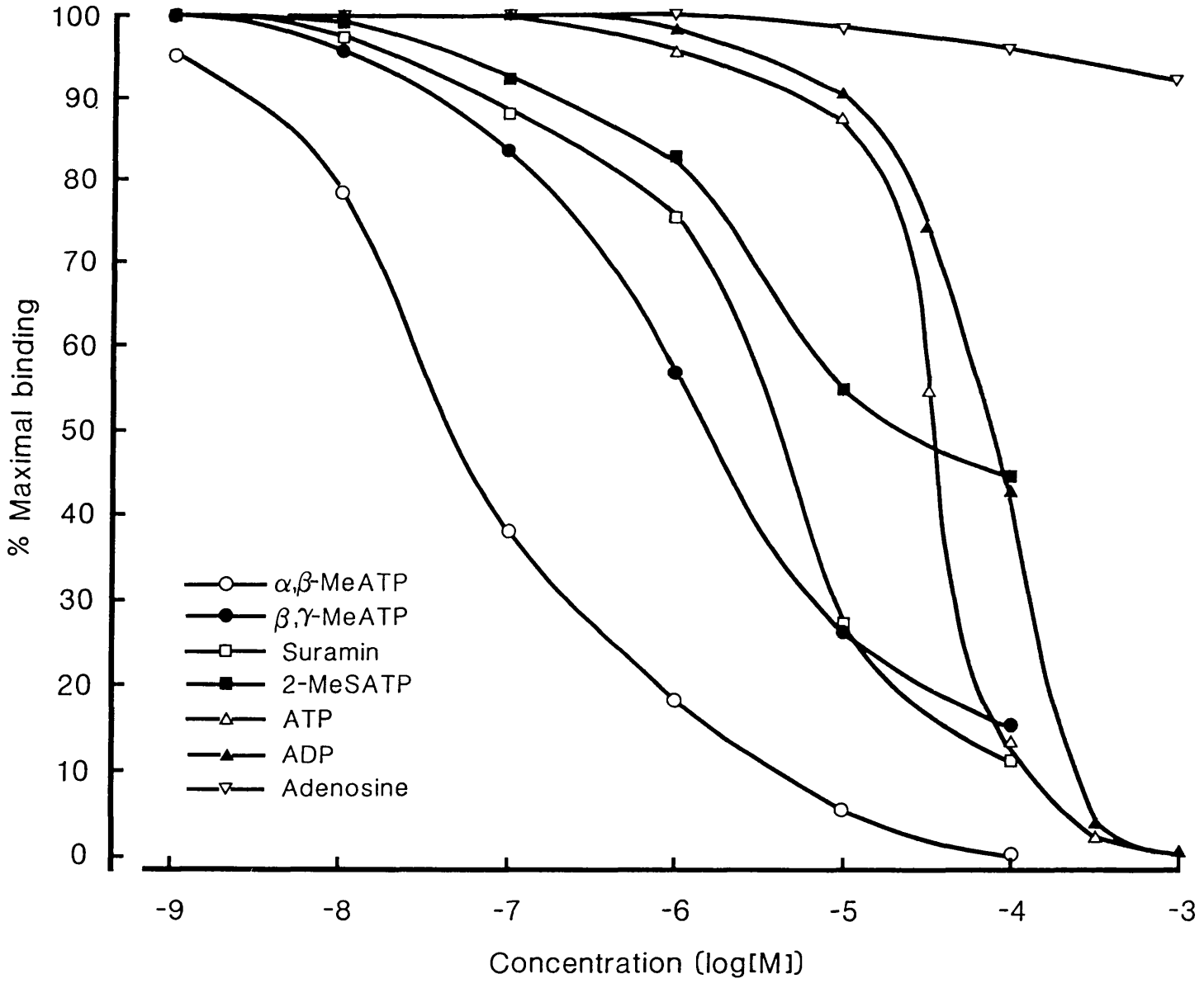
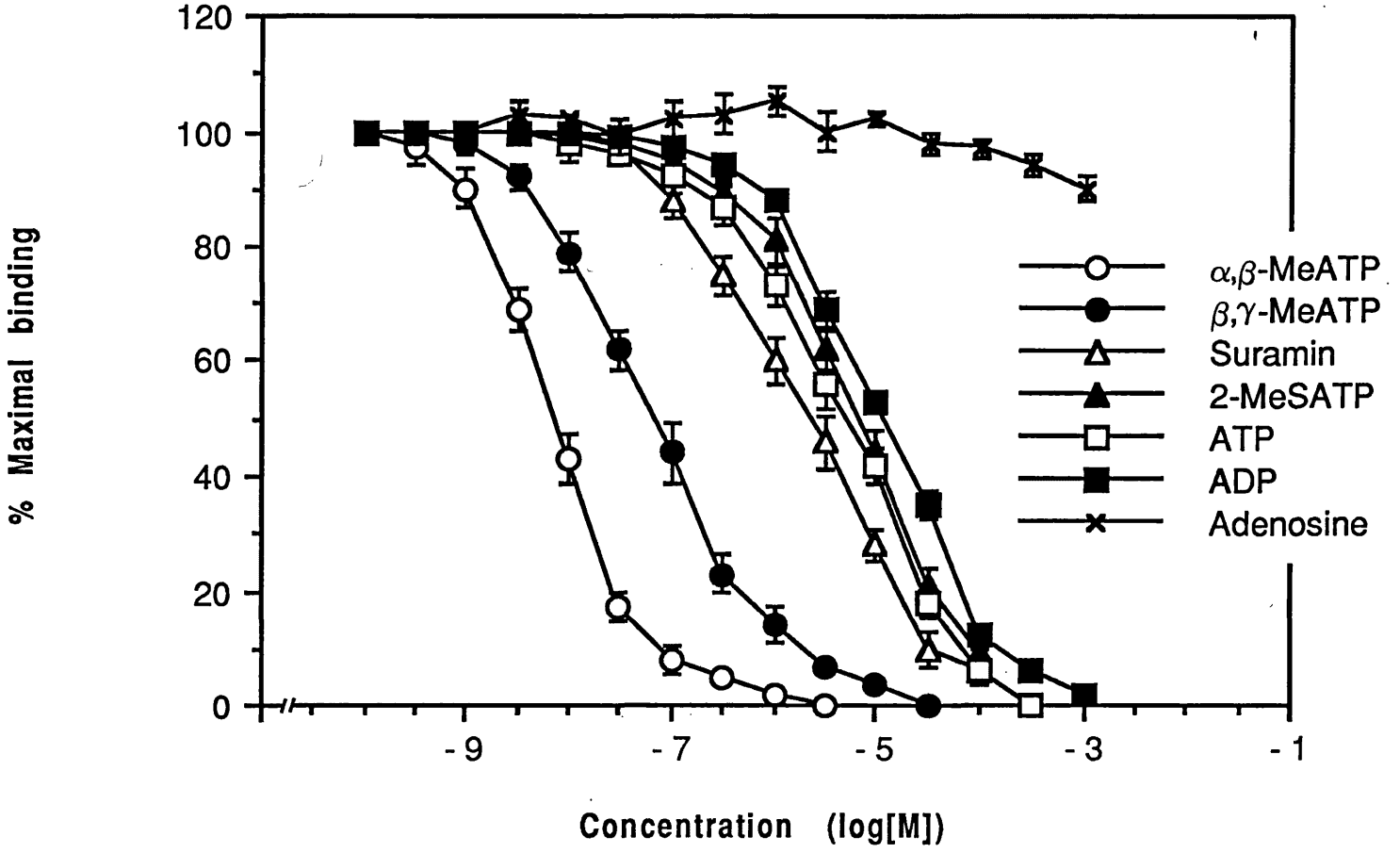

100
80
60
40
20
0
% Maximum Binding
0 1 3 10 30
Time (min)
4
3
2
1
0
-1
-2
Ln [B]/[Be]
0 1 3 10 30
Time (min)

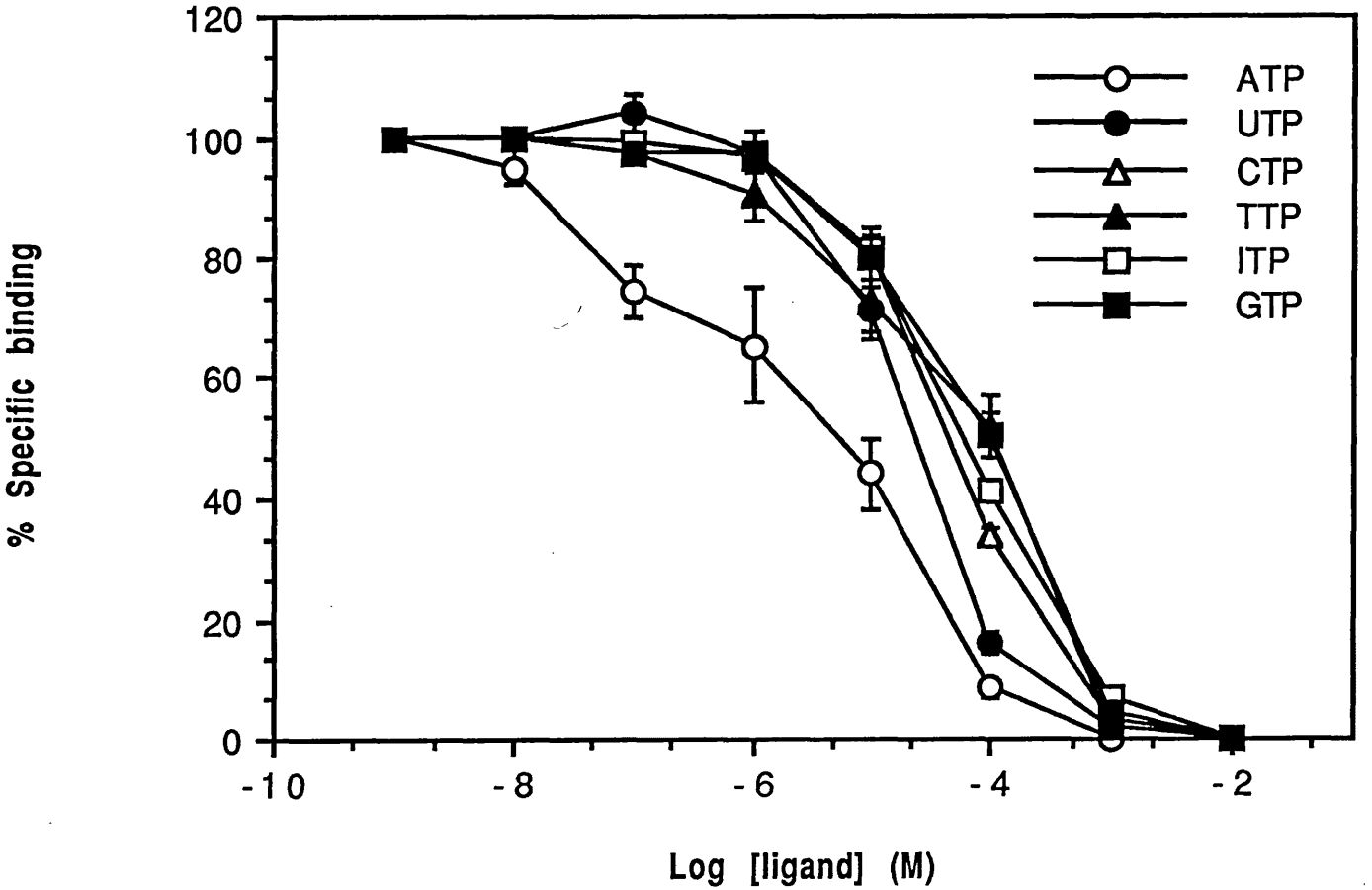
Figure 2-16 **Displacement of [^{3}H]α,β-MeATP binding to rat bladder membranes by unlabelled ligands.**

(a) Rat bladder membranes were incubated with 10 nM [^{3}H]α,β-MeATP in the absence or presence of different unlabelled purinergic ligands in 50 mM Tris/HCl buffer containing 25 mM $MgCl_2$ at 30°C for 15 min; (b) Parallel experiment to (a), but the incubation was carried out in Mg^{2+}-free buffer at 4°C for 2hr; (c) Displacement by other nucleotides: incubation was carried out in Mg^{2+}-free buffer at 4°C for 2 hr; (d) Displacement by other ATP related substances: Incubation was carried out in Mg^{2+}-free buffer at 4°C for 2 hr. Nonspecific binding was determined in the presence of 100 μM β,γ-MeATP.

The results are from four to six experiments (each in duplicate).


100
90
80
70
60
50
40
30
20
10
0
% Maximal binding
α,β-MeATP
β,γ-MeATP
Suramin
2-MeSATP
ATP
ADP
Adenosine
-9
-8
-7
-6
-5
-4
-3
Concentration (log[M])


120
100
80
60
40
20
0
% Maximal binding
-9
-7
-5
-3
-1
Concentration (log[M])
α,β-MeATP
β,γ-MeATP
Suramin
2-MeSATP
ATP
ADP
Adenosine


120
100
80
60
40
20
0
-10
-8
-6
-4
-2
% Specific binding
Log [ligand] (M)
ATP
UTP
CTP
TTP
ITP
GTP

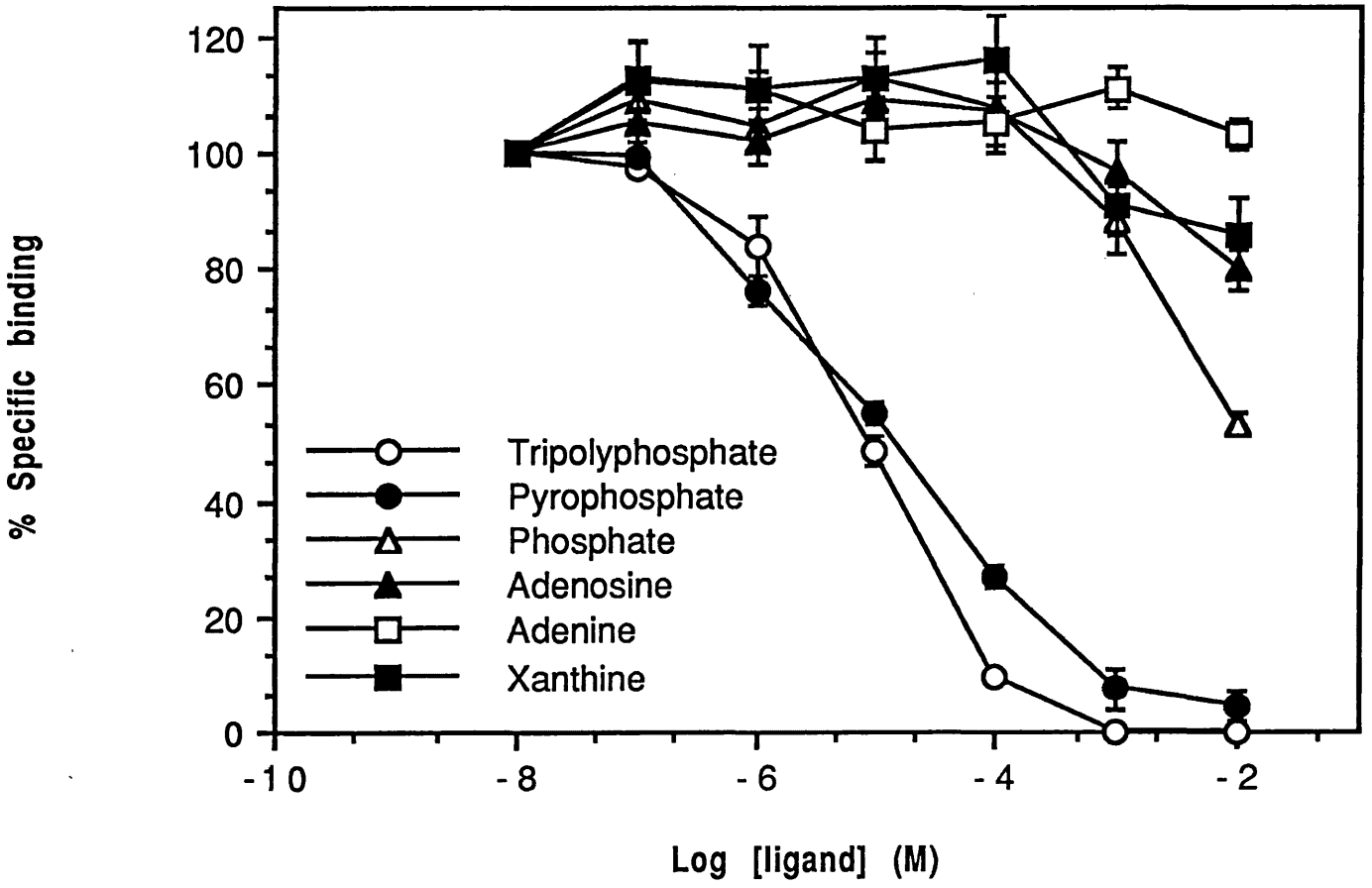

% Specific binding
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log [ligand] (M)
Tripolyphosphate
Pyrophosphate
Phosphate
Adenosine
Adenine
Xanthine

Table 2-1 The maximum binding (B_{max}), dissociation constants (K_d) and Hill coefficients (n_H) of [^{3}H]α,β-MeATP binding to rat urinary bladder membrane preparation in the presence of various concentrations of Mg^{2+}. Means from seven to ten experiments are shown.

	High affinity			Low affinity		
	B_{max} (pmol/mg protein)	K_d (nM)	n_H	B_{max} (pmol/mg protein)	K_d (nM)	n_H
Mg^{2+}-free	8.1±2.1	6.3±1.7	0.99	35.9±8.9	104±37	1.00
2 mM $MgCl_2$	6.1±1.8	6.1±1.9	1.00	24.5±8.5	97±29	1.00
10 mM $MgCl_2$	4.5±1.8	5.9±1.6	1.00	14.3±6.1	83±26	1.01
25 mM $MgCl_2$	3.7±1.0	5.8±2.7	1.07	8.7±2.7	67±15	1.02

Table 2-2 The IC_{50} values of unlabelled purinergic compounds for displacing the binding of [^{3}H]α,β-MeATP (10 nM) to rat bladder membrane preparations in the presence of 25 mM $MgCl_2$ at 30°C for 15 min or in Mg^{2+}-free buffer at 4°C for 2 h.

Ligands	$IC_{50,1}$ (nM) (n=9)	$IC_{50,2}$ (nM) (n=4)	$IC_{50,1}$/ $IC_{50,2}$
α,β-MeATP	91 ± 13	9 ± 3***	10.1
β,γ-MeATP	943 ± 126	83 ± 17***	11.4
Suramin	2,316 ± 437	1,860 ± 232	1.2
ATP	31,466 ± 4351	6,367 ± 577***	4.9
ADP	54,674 ± 6319	11,302 ± 1443***	4.8
2-MeSATP	59,617 ± 6125	7,765 ± 1210***	5.3
Adenosine	>1,000,000 (13%)#	>1,000,000 (19%)#	

Enclosed in the bracket is the percentage of the displaced binding at the maximum tested concentration of adenosine.

*** $P < 0.001$, compared with the IC_{50} values of the unlabelled ligands in the presence of Mg^{2+} ions at 30°C.

Table 2-3 IC_{50} values of unlabelled ligands for displacing the binding of [^{3}H]α,β-MeATP to rat urinary bladder membranes

Unlabelled ligands	IC_{50} (μM)
ATP	7.95 ± 1.50
UTP	20.42 ± 4.35**
CTP	54.24 ± 14.20***
TTP	63.11 ± 19.57***
ITP	68.69 ± 16.75***
GTP	101.86 ± 22.12***
Adenosine	>10,000 n.d.
Adenine	>10,000 n.d
Inosine	>10,000 n.d
Pyrophosphate	13.07 ± 4.22
Tripolyphosphate	9.28 ± 1.01
$Na_2H_2PO_4$	>10,000 n.d.

** $P < 0.01$, *** $P < 0.001$, significantly different from the IC_{50} value of ATP.

Values given as mean ± S.E.M., n = 4.

n.d. Not determined.

Chapter 3: Methodology on autoradiographic localization of [^{3}H]α,β-methylene ATP binding sites and semi-quantitation of autoradiograms

3.1 Introduction

In order to understand how receptors mediate physiological effects in complex organisms, it is important to know where they are localized. Following the development of the radioligand binding technique for identifying receptors biochemically in tissue homogenate or membrane preparations, the most available method for localizing receptors is the autoradiographic localization of specifically bound radioligands at the light microscope level. With the development of the new ligands and new methods, the light microscopic autoradiographic approach offers reliability and flexibility. Like all histochemical methods, it has two significant advantages. One is the greatly increased anatomical resolution, i.e., in the micron range. Another is the tremendous sensitivity in receptor detection, which can be several orders of magnitude greater than that found with biochemical methods.

For gross classification, autoradiographic localization of receptors can be divided into two main categories, i.e., *in vivo* and *in vitro* labelling techniques. Binding *in vivo* provided a first glimpse of receptor distributions. Tests of binding saturability and sterospecificity, use of appropriate pharmacology of blocking agents, and a mode of drug delivery identical to that

used in behavioural studies validated the technique. Although binding *in vivo* established the possibility of autoradiographic receptor localization, it has certain disadvantages. Large quantities of radiolabelled ligand are required to ensure that a sufficient number of receptors are marked for visualization. Unfortunately, much unbound and non-specifically bound ligand remains in the tissue and cannot be washed out, resulting in artefactual or high background labelling. The selection of ligands appropriate for *in vivo* binding is limited to those which cross the physiological barriers to form high-affinity bonds with receptors. Furthermore, after systemic injection, the ligand may be metabolized to an active form other than that intended for the study, or degraded to an inactive form, rendering binding results invalid or unobtainable. However, recently *in vivo* labelling has again become important with the advent of PET (positron emission tomography) scanning of receptors (Maziere *et al.*, 1981; Wagner *et al.*, 1983; Hantraye *et al.*, 1984), which makes it possible to measure receptors *in vivo* in living animals and humans.

The *in vitro* binding of a ligand to slide-mounted sections obviates the use of slides precoated with emulsion for the dry autoradiography of diffusible and non-diffusible ligands. Since wet autoradiography was made possible by the use of a non-diffusible ligand, the early *in vitro* technique was severely limited by the use of a non-diffusible ligand. In 1979, Young and Kuhar (1979) refined the *in vitro* method, adapting it for the use with diffusible as well as non-diffusible substances, which was

achieved by using predried, emulsion-coated coverslips.

Tritium-sensitive film may be used in place of emulsion-coated coverslips as a dry emulsion medium. Receptor distributions represented on the film images are easily quantified by microdensitometry (Palacios *et al.*, 1981; Rainbow *et al.*, 1982; Biegon *et al.*, 1982). Regional receptor densities can be evaluated with computer assistance (Quirion *et al.*, 1981) or by comparison with autoradiographic standards of known radioactivity and protein content (Unnerstall *et al.*, 1982, Miller *et al.*, 1988).

In vitro labelling with a ligand has many advantages over the *in vivo* labelling technique: (a) it is possible to perform biochemical and pharmacological manipulations to selectively alter binding conditions; (b) sections may readily be washed to reduce artefactual and non-specific binding; (c) identically prepared sections can be used for both the characterization of kinetics and pharmacology; (d) by being able to control the conditions of the experiment substances that are rapidly metabolized *in vivo* may be used as a ligand; (e) adjacent tissue sections may be incubated with different ligands to permit the comparison of several receptor types in a single tissue; (f) it is suitable for the study of human tissues, which are usually small biopsy specimens or postmortem tissues.

In the field of localization of purinoceptors, great progress has been made with P_1-purinoceptors, especially the A_1-adenosine receptors, which may be attributed to the availability of

radiolabelled agonists and antagonists (Lewis *et al.*, 1981; Goodman & Snyder, 1982; Goodman *et al.*, 1983; Buckley & Burnstock, 1983; Deckert *et al.*, 1988). P_2-purinoceptor-mediated responses have been observed in many tissues as has been briefly reviewed in Chapter 1. The understanding of P_2-purinoceptor mechanisms with respect to radioligand binding assays is still in its infancy. Several radioligands have been tested (see Introduction of Chapter 2), but no autoradiographic localization of P_2-purinoceptors has ever been carried out before because of the lack of selective and high-affinity radioligands. With the use of [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) as the radioligand we have gained a better understanding about the biochemical characteristics of P_{2X}-purinoceptors. In this chapter the methodology of the autoradiographic localization of [^{3}H]α,β-MeATP binding sites is described. For the first time we were able to see which cell type was specifically labelled, to screen large quantity of tissues, and to detect P_{2X}-purinoceptors in a very small and heterogeneous sample of tissues where it would be impossible to carry out a radioligand binding assay.

3-2 Methods

3-2-1 Slide preparation

Precleaned microscope slides (BDH, Poole, U.K., 76 x 26 mm, 0.8 - 1.0 mm thick, twin-frost) were soaked in a cleaning solution (100 g potassium dichromate was dissolved in 850 ml distilled water, and then 100 ml 98% sulphuric acid was added slowly) overnight. They were subsequently washed with running water for 5 - 7 hr, and then soaked twice in double distilled water (ddH_2O) for 20 min. The cleaned slides were subbed by sequentially dipping twice in a solution which consisted of gelatin (5 g) and chrome alum (chromium potassium sulphate, 500 mg) dissoved in 1000 ml of ddH_2O. The solution was heated, stirred, filtered, and cooled. The dipping was done at room temperature. The coated slides were transferred to an oven to be dried at 37°C. They were then stored in a dust-free environment. The subbing solution was used only once for about 100 slides.

3-2-2 Coverslip preparation

Number 1 microscope glass coverslips (64 x 22 mm) from Chance Propper Ltd. (Smethwick, U.K.) were cleaned overnight by soaking in the above-mentioned sulphuric acid-dichromate solution, and washed using the same procedure as for the slides. They were dried in a 60°C oven and stored in a dust-free environment until used. The coverslips were emulsion-coated by dipping two-thirds of the

coverslip's length in Ilford K5 emulsion. The emulsion was diluted 1:1 with ddH_2O and dissolved at 43°C in a waterbath. After the dipping, one side of the coverslip was wiped clean of emulsion and put on a cool steel plate to dry for 30 min. They were then dried at room temperature for 10 h. They were either used immediately or stored in a light-tight black box at 4°C for a short time (less than two months). The background of the emulsion was checked by developing several coated coverslips.

3-3-3 Tissue preparation

After the animals were sacrificed (the methods used for each kind of animals are stated in the relevant chapters), the tissues of interest were removed immediately and put into modified Krebs solution with the following composition: mM: NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, glucose 7.7, pH 7.4. Samples were trimmed free of adipose and unwanted surrounding tissues and cut to an appropriate size. The tissues were embedded in Tissue Tek, O.C.T. Compound (BDH, Poole, UK) and frozen onto pieces of cork in isopentane precooled in liquid nitrogen. Sections (14 - 15 µm thick) were cut on a Bright cryostat (Huntington, U.K.) or a Reichert-Jung Cryocut 1800 (Cambridge Instruments GmbH, Heidelberg, Germany) at -15 to -25°C depending on the tissue being cut. Sections were picked up from the knife with the subbed slides and thaw-mounted. They were left at room temperature for 30 min and then transferred into a desiccator where they were dried for 24 h at 4°C. They were either used

immediately or stored at -30°C in tightly-sealed boxes.

3-2-4 Receptor labelling

Slide-mounted sections were pre-incubated at 30°C for 10 min in 50 mM Tris/HCl buffer (pH 7.4, buffer A) to leach endogenous ligands and embedding matrix. They were transferred into a jar with 15 ml buffer A containing 10 nM [^{3}H]α,β-MeATP. Isotonic buffer may be selected for incubation and washing so as to prevent any unnecessary stress on tissue morphology. However, cations have been found to influence the [^{3}H]α,β-MeATP binding as described in Chapter 2, thus, incubation was performed in buffer without added cations (Herkenham, 1988). While it is preferable to carry out incubation at physiological temperatures, this may result in a reduction of tissue quality and loss of receptors. Therefore, the incubation was carried out at 30°C for 15 min. Non-specific binding was determined in the presence of 100 μM β,γ-MeATP. At the end of the incubation the slides were washed in ice-cold buffer A for 2 x 2 min and in ice-cold ddH_2O for 1 min to remove salts from slide and tissue section which could cause autoradiographic artefacts. Sections were dried under cold airflow to minimize the diffusion of radioligand, and stored in a desiccator at 4°C.

In preliminary experiments the washing time and the association rate were determined. A group of sections were incubated as described above. At the end of the incubation the sections were washed in ice-cold buffer A for different periods, and then the sections were wiped from the slide with a piece of

Whatman glass fibre filter. The filters were put into scintillation vials and the radioactivities were measured in a Beckman LS6000I scintillation counter. The specific and non-specific binding was plotted against the washing times to obtain the optimal value. The determination of association rate constant was performed by incubating a group of sections for varying periods, and the reaction was terminated by washing. The sections were also wiped and radioactivities were counted. The calculation was done according to the method described in Chapter 2.

3-2-5 Generation of Autoradiograms

Autoradiograms were generated according to the method of Young and Kuhar (1979) (Herkenham, 1984; Kuhar, 1985). An emulsion-coated coverslip was glued to the frosted end of each slide with cyanoacrylate adhesive (RS Components Ltd., Corby, U.K.) so that the coverslip was permanently hinged to the slide and tissue section. After the glue set, the assemblies were tightly bound together with tough tissue paper and were exposed for two weeks (three weeks for the second batch of radioligand) at 4°C. The emulsion was developed in Kodak D-19 (Eastman-Kodak Co., Rochester, New York, U.S.A.) for 3 min at 20°C and fixed in Ilford Hypam (Ilford, Mobberley, U.K.) for 5 min. Having been washed in running water for 30 min the slides were twice soaked in ddH_2O for 5 min, and then the sections were stained with 0.5% toluidine blue. The sections were dehydrated in graded alcohols (from 70% to 100%) and cleared in Histo-Clear (National Diagnostics, Manville,

U.S.A.). The coverslips and the slides were stuck together tightly with D.P.X. Mountant (BDH, Poole, U.K.).

To determine the optimal time of exposure, several sets of testing assemblies with different types of tissue sections were developed at varying periods and the grain densities over different tissues were compared.

3-2-6 Microscopy and photography

The autoradiograms were examined under a Zeiss III-RS (Carl-Zeiss, Germany) photomicroscope with a brightfield/darkfield illuminator. With a Zeiss Ph 1, F10 objective lens for the low power magnification the gross pattern of the grain distribution was discerned. For photography a Zeiss Ph2, Neoflura 16 objective lens was used. Both bright- and dark-field images of the same view were photographed for comparison. Pictures were taken onto Ilford Pan F (ISO 50/18°) fine grain black and white films, which were developed in Acutol FX-14 (Paterson Products, Borehamwood, U.K.) at 20°C for 5 min and fixed in Amfix (Champion Photochemistry Int. Ltd., Brentwood, U.K.) for 10 min.

3-2-7 Semi-quantitation of autoradiograms

The grain densities were counted with a computer-assisted image analysis system. In this study the data were only used for intergroup comparison, no standard was established, and the grain densities were not transformed into such units as fmol/mg tissue

or fmol/mg protein because the tissue sections contained several types of cells. Autoradiographic slides were viewed under a Zeiss photomicroscope with an oil immersion objective lens (Ph 3, Planapo 100/1.3). The images were captured by a Panasonic black-and-white video camera attached to the microscope. Video signals were processed by a Seescan I3000 image processing system using the software Solitaire Plus (Seescan Ltd., Cambridge, U.K.) (Figure 3-2). A special user's task program called GRAIN COUNTING was compiled by the programmer in Seescan Ltd. for counting the autoradiogaphic grains viewed with bright-field illuminator. This program asked the operator to select the background level on each counting to ensure a correct count in cases where the image was too dark or too bright due to the uneven staining of different tissues. After that the program ran automatically and the counting of the grains in one field only took only 30 sec, which enabled us to analyse a large quantity of samples in a short time. On each section, autoradiographic grains in five fields of the same tissue were counted. Five fields over the non-tissue areas were also measured as the background. In each group at least three sections from three animals were studied. The measuring frames were drawn manually to ensure only one kind of tissue was included in the frame. The grain densities were expressed as grains per 1000 μm^{-2}. The specific binding was obtained by subtracting the grain densities over the sections for non-specific binding from those for total binding.

3-2-8 Chemicals

[^{3}H]α,β-MeATP was custom-synthesized by Amersham Int. (Amersham, U.K.). Specific activity was 27 Ci/mmol for the first batch and 19.2 Ci/mmol for the second batch with a chemical purity of 98-99%. α,β-MeATP and β,γ-MeATP were purchased from Sigma Chemicals Ltd. (Poole, U.K.).

3-2-9 Data analysis

Data were expressed as mean ± standard error of means (S.E.M.). The statistical difference in the densities of binding sites was assessed with analysis of variance. The F-protected least significant difference (FLSD) method was used to compare the means, and a probability of less than 0.05 was considered significant.

Figure 3-1 **The procedure of *in vitro* labelling autoradiography.**

Frame 1 shows the preparation of subbed microscope slides. Frame 2 shows the emulsion-coating of the coverslips. Frame 3 shows the *in vitro* labelling of the thaw-mounted tissue sections on slides and the subsequent rinsing to obtain high ratios of specific to non-specific receptor binding. Frame 4 shows the assembly of emulsion-coated coverslips with receptor-labelled tissue to produce autoradiograms. Frame 5 shows the temporary separation of coverslip from tissue and the development of the emulsion. Frame 6 shows the fixing and staining of the tissue and re-apposition of the coverslip and slide for microscopy.

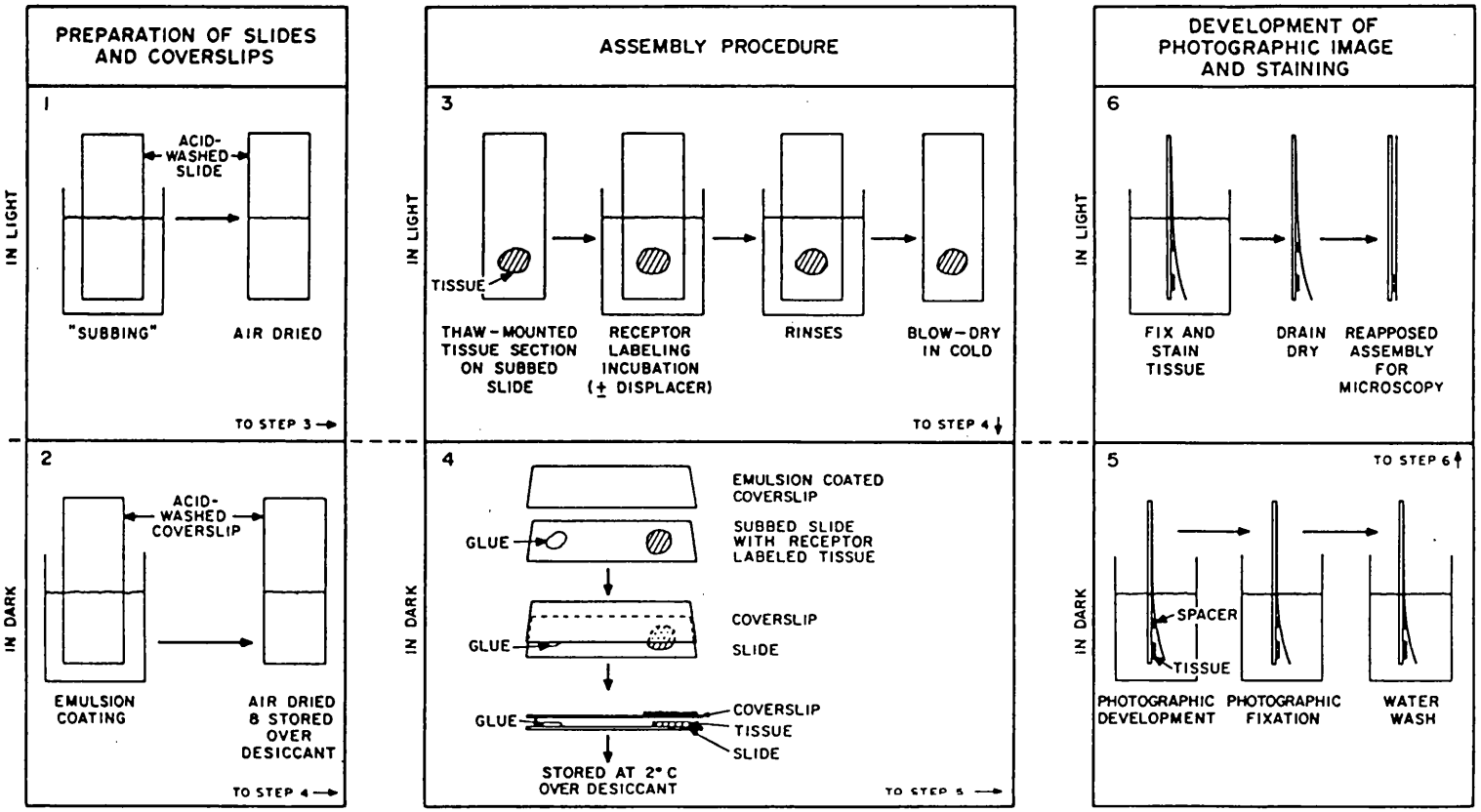
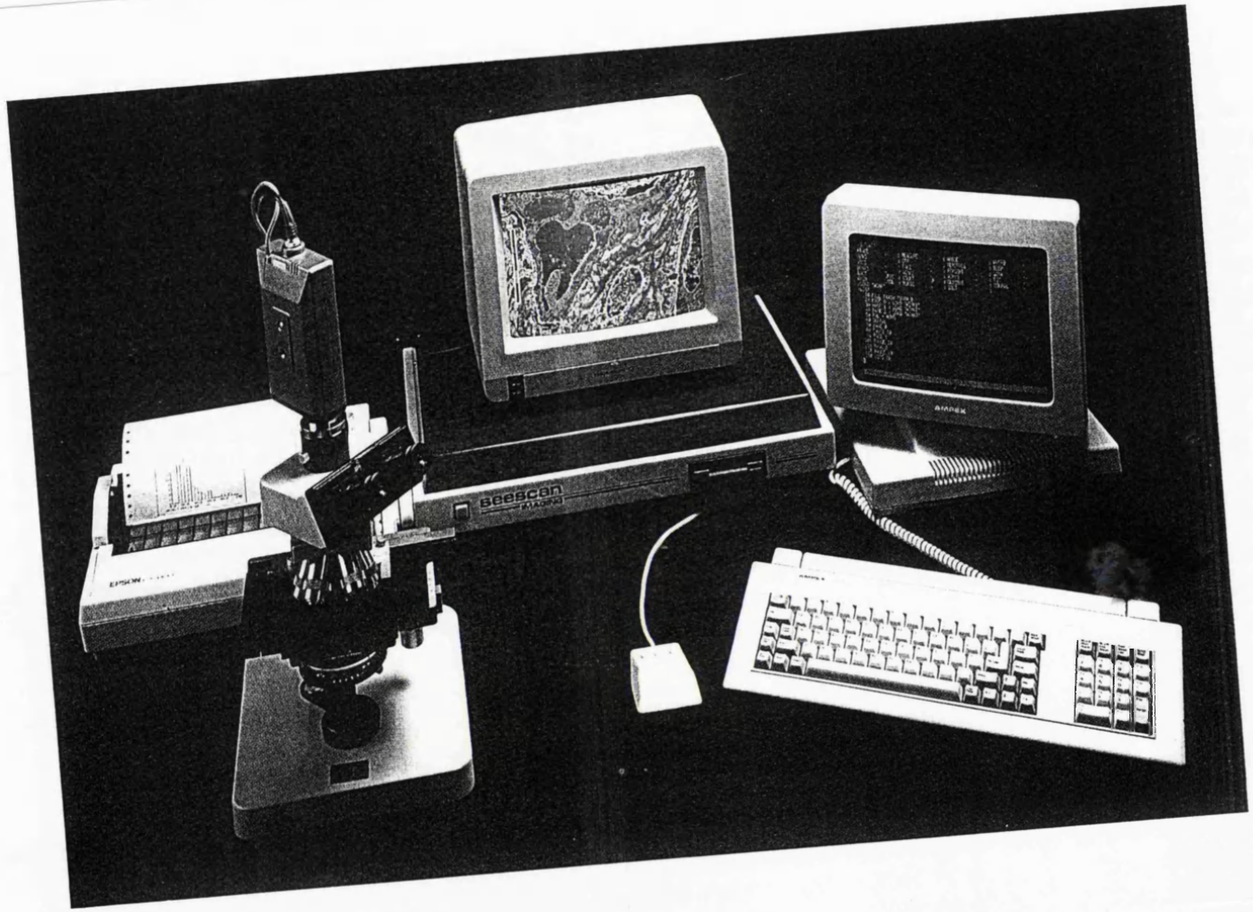

PREPARATION OF SLIDES AND COVERSLIPS
ASSEMBLY PROCEDURE
DEVELOPMENT OF PHOTOGRAPHIC IMAGE AND STAINING
IN LIGHT
IN DARK
1
ACID-WASHED SLIDE
"SUBBING"
AIR DRIED
TO STEP 3
2
ACID-WASHED COVERSLIP
EMULSION COATING
AIR DRIED & STORED OVER DESICCANT
TO STEP 4
3
TISSUE
THAW-MOUNTED TISSUE SECTION ON SUBBED SLIDE
RECEPTOR LABELING INCUBATION (± DISPLACER)
RINSES
BLOW-DRY IN COLD
TO STEP 4
4
EMULSION COATED COVERSLIP
GLUE
SUBBED SLIDE WITH RECEPTOR LABELED TISSUE
COVERSLIP
GLUE
SLIDE
COVERSLIP
GLUE
TISSUE
SLIDE
STORED AT 2° C OVER DESICCANT
TO STEP 5
5
TO STEP 6
SPACER
TISSUE
PHOTOGRAPHIC DEVELOPMENT
PHOTOGRAPHIC FIXATION
WATER WASH
6
FIX AND STAIN TISSUE
DRAIN DRY
REAPPOSED ASSEMBLY FOR MICROSCOPY

Figure 3-2 **An illustration of the set-up of the Seescan image analysis system.**

The image signals are received by the video camera attached to the microscope and transferred to the computer. When the analysis has been completed the results can be shown on the monitor screen or printed out. The mouse can be used to choose the operational commands as well as to draw the measuring frames.


BeeScan
AMPEX

Chapter 4: Species differences in characteristics and distribution of [^{3}H]α,β-methylene ATP binding sites in urinary bladder and urethra of rat, guinea-pig and rabbit

Summary

1. The characteristics of [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) binding sites in the urinary bladder of rat, guinea-pig and rabbit were examined.

2. Receptor binding assay showed that the rat bladder possessed the highest density of specific binding sites, followed by rabbit and guinea-pig bladder.

3. Autoradiographic localization of [^{3}H]α,β-MeATP binding sites in the bladder and urethra of the three species showed that the grain densities in the bladders of rat, guinea pig, and rabbit were parallel to those obtained from receptor binding assay. The grain densities were greatly reduced in the presence of β,γ-methylene ATP (β,γ-MeATP).

4. No significant specific binding was detected in the smooth muscle of rat and guinea-pig urethra, while a very low level of specific binding was observed in the rabbit urethra.

5. Differences in grain densities between different regions (dome, body, and trigone) of the same bladder were also observed, but

they were not as remarkable as those between species.

6. The results of this study demonstrate the species differences of P_{2X}-purinoceptor densities in the urinary bladder, which may reflect differing degrees of purinergic neurotransmitter control of the bladder detrusor muscle.

4-1 Introduction

The parasympathetic nerves supplying the urinary bladder travel in the pelvic nerves, while the postganglionic sympathetic nerves are derived from inferior mesenteric and hypogastric nerves. Stimulation of the pelvic nerves results in a contraction of the bladder body (Langley & Anderson, 1895; Henderson & Roepke, 1934). Stimulation of the hypogastric nerves usually leads to the contraction of the bladder sphincter accompanied by a relaxation of the bladder body. It is well known that cholinergic nerves provide the excitatory innervation of the mammalian bladder, however, as far back as in the last century, an atropine resistant response to nerve stimulation was observed (Langley & Anderson, 1895). Twenty years ago, the non-cholinergic nerves were proposed to utilize ATP or a close analogue as their neurotransmitter (Burnstock, 1972; Burnstock, *et al.*, 1972). So far, purinergic neurotransmission has been observed in many organs and species. The urinary bladder is an intensely studied organ for purinergic neurotransmission, which has been observed in the bladder of rat, guinea-pig (Burnstock *et al.*, 1972, 1978; Brading & Mostwin, 1989; Brading & Williams, 1990), rabbit (Dean & Downie, 1978), mouse (Acevedo & Contreras, 1985, 1989), cat (Theobald, 1983), pig (Sibley, 1984; Fujii, 1988), man (Husted *et al.*, 1983), ferret, and marmoset (Moss & Burnstock, 1985). In contrast, the urethra seems to lack the excitatory P_{2X}-purinoceptor-mediated response, as Hills *et al.* (1984) have observed in the pig urethra. The ATP elicited contractile responses in bladder are suggested to be

mediated by P_{2X}-purinoceptors (Burnstock & Kennedy, 1985), which can be antagonized by $ANAPP_3$ and desensitized by α,β-MeATP.

Many comparative studies have shown that in different species the atropine-resistant component varies relative to the cholinergic component. Usually the atropine-sensitive component of excitatory nerve stimulation increases with increasing frequency of the applied stimulus, and accounts for 40-60% of the contractile responses in rat, rabbit, guinea-pig, marmoset, monkey, ferret, ringtail possum and pig (Burnstock & Campbell, 1963; John & Paton, 1977; Dean & Downie, 1978; MacKenzie & Burnstock, 1984; Maggi *et al.*, 1984; Sibley, 1984; Moss & Burnstock, 1985; Craggs & Stepheson, 1986; Levin *et al.*, 1990). In cats the cholinergic component may account for only 20% of the neurogenic responses (Langley & Anderson, 1895; Craggs & Stepheson, 1982), while in human bladder, more than 80% of the neurogenic response is cholinergic (Hoyle *et al.*, 1989), although some have claimed that it is wholly cholinergic (Sibley, 1984). In baboons and rhesus monkeys the cholinergic component may also dominate, accounting for over 90% of the contractile responses evoked by nerve stimulation (Brindley & Craggs, 1975; Craggs & Stepheson, 1986). Despite such striking differences in the proportions of the non-cholinergic innervation, there is as yet no direct evidence which compares P_{2X}-purinoceptor densities among different species.

The aim of the study reported in this chapter was to

determine whether there are species differences in P_{2X}-purinoceptor densities in rat, guinea-pig and rabbit bladders and urethrae. In addition to receptor binding assays, autoradiographic localization of [^{3}H]α,β-MeATP binding sites in urinary bladder and urethra of rat, guinea-pig and rabbit was also carried out.

4-2 Materials and Methods

4-2-1 Radioligand binding assay

In this study the insoluble components of the homogenate of the bladder (the "washed homogenate") were used instead of membrane preparations because the procedure we used for the separation of rat bladder membranes may produce different yields of membranes for guinea-pig or rabbit bladder, which would skew the final estimation of binding site densities. The washed homogenate was prepared as follows: male Wistar rats (200 - 250 g) and male guinea-pigs (300 - 350 g) were killed by asphyxiation with CO_2 and male New Zealand rabbits (2.5 kg) were killed by overdose of Sagatal (RMB Animal Health Ltd., Dageham, U.K.); urinary bladders were removed immediately and placed in modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_3$ 0.6, $NaHCO_3$ 16.3, NaH_2PO_4 1.4, glucose 7.7, pH 7.4); they were trimmed free from surrounding adipose and connective tissues, minced and homogenized in 5 ml 50 mM Tris/HCl buffer (pH 7.4, buffer A) with a motor-driven glass homogenizer at top speed for 30 sec. The homogenate was passed though double layers of nylon mesh and centrifuged at 105,000 x *g* for 50 min at 4°C in a MSE Europa M-50 ultracentrifuge. The pellets were carefully washed with ice-cold buffer A and suspended in buffer A for the binding assay. Protein concentration was measured with the method of Lowry *et al.* (1951) and bovine serum albumin was used as standard.

The basic procedure for the binding assay has been described in Chapter 2. The washed homogenate was incubated with serial concentrations of [^{3}H]α,β-MeATP for 2 h at 4°C. The non-specific binding was determined in the presence of 100 μM β,γ-methylene ATP (β,γ-MeATP). Each experiment was carried out in duplicate.

4-2-2 Autoradiography

The basic procedure for the autoradiography is presented in Chapter 3. The animals were killed as described above. Small pieces of bladder tissue (rat and guinea-pig bladders, 2 x 2 mm, rabbit bladders, 3 x 3 mm) were cut from the dome, fundus and trigone areas. Urethras were dissected free from surrounding tissues and cut into segments of about 3 mm in length. Sections (15 μm thick) were cut on a Bright cryostat. Slide-mounted sections were pre-incubated at 30°C for 10 min in buffer A. Incubation was carried out in buffer A containing 10 nM [^{3}H]α,β-MeATP at 30°C for 15 min. Non-specific binding was determined in the presence of 100 μM β,γ-MeATP. The emulsion was developed after two weeks' exposure at 4°C.

Autoradiograms were analyzed with a Seescan image analysis system. On each section, autoradiographic grains in fifteen fields were counted: five over smooth muscle, five over epithelium and five over the non-tissue areas as background. In each group at least three sections from three animals were studied. The average measured area of each field was around 700 μm^2.

4-2-3 Chemicals

[^{3}H]α,β-MeATP was custom-synthesized by Amersham International, Amersham, U.K. Specific activity was 27 Ci/mmol with a chemical purity of 98-99%. β,γ-MeATP was purchased from Sigma Chemicals Ltd., Poole, U.K.

4-2-4 Data analysis

See Chapters 2 and 3.

4-3 Results

4-3-1 Receptor binding assay

The binding processes in all samples were saturable. A typical saturation curve of the washed homogenate of guinea-pig bladder detrusor is shown in Figure 4-1, and the corresponding Scatchard plot is shown in Figure 4-2. Scatchard analysis reveals that the binding sites in the washed homogenate of bladders of all species contain high- and low-affinity states (Table 4-1). The K_d values for the low-affinity state were about 30 to 40 times higher than those for the high-affinity state. The receptor densities of both high- and low-affinity states in rat bladder were the highest amongst the three species, the second highest were those in rabbit bladder, and the guinea-pig bladder had the lowest densities of P_{2X}-purinoceptors. The K_d values for both high- and low-affinity states in the rat and guinea-pig bladder washed homogenate were not significantly different, while the K_d values for both the high- and low-affinity states of rabbit bladder washed homogenate were significantly lower than those of rat and guinea-pig bladders.

4-3-2 Autoradiographic localization and semi-quantitation

Autoradiograms showed that a significant number of grains were distributed over the smooth muscle in urinary bladder of rat, guinea-pig and rabbit (Figure 4-3). The smooth muscle of the

arterioles within the bladder walls was also labelled. Grains were sparsely distributed over the epithelium, the submucosa and the connective tissues between the smooth muscle bundles, the serosa and the fibrous adventitia. Specific grain densities over the smooth muscle were markedly reduced in the presence of β,γ-MeATP (Figure 4-3). In the autoradiograms of rat, guinea-pig and rabbit urethra, very low densities of grains could be seen over the smooth muscle (Figure 4-4). Further, only a small amount of the binding was displaced by β,γ-MeATP (Figure 4-4).

Table 4-2 shows the relative densities of specific [^{3}H]α,β-MeATP binding sites, which were determined by subtracting grain densities representing non-specific binding from those representing total binding, over the smooth muscle of different regions of urinary bladder and urethra from rat, guinea-pig and rabbit. Amongst the three species tested, the smooth muscle in rat urinary bladder possessed the highest density of specific binding sites. The differences between all the three regions of rat bladder with the respective regions of guinea-pig and rabbit bladders were highly significant ($P < 0.001$). The rabbit bladder had the second highest density of specific binding sites, which were significantly higher than those of guinea-pig ($P < 0.001$).

Some degrees of difference in receptor densities in different regions of the same bladder were also observed (Table 4-2), but they were not as obvious as those between the species.

In both rat and guinea-pig urethra, although the grain densities over the smooth muscle were relatively higher than those over the epithelia and connective tissues, they were much lower than those over the smooth muscle in bladder, and no significant specific binding was detected. Only the rabbit urethra smooth muscle showed a significantly higher density of specific binding sites.

The grain densities over the epithelia of both bladder and urethra were very low, ranging from 7 to 62 grains per 1000 μm^{-2} on the sections for total binding, and no specific binding was observed.

4-4 Discussion

Although the amplitude of a neurogenic response depends on many links in the neurotransmission chain (such as the density of innervation, the quantity of the neurotransmitter released per action potential, the anatomical structure of the synapse-target cell junctions, the efficiency of the degradation enzymes of the neurotransmitter, the efficiency of the second messenger system, and the state of the ion channels), the densities and affinity of the receptors can be used as important indices to assess a certain neurogenic response. The establishment of a radioligand binding assay and of autoradiographic localization of P_{2X}-purinoceptors enables us to carry out comparative studies to estimate the importance of purinergic neurotransmission in different tissues and different species. According to previous pharmacological experiments, purinergic neurotransmission in the bladder of rodents is more pronounced than that in higher species, such as pig and man. In this study, the P_{2X}-purinoceptor densities, affinities, and localization were compared between the three most widely used models for purinergic neurotransmission: the bladders of rat, guinea-pig and rabbit. The washed homogenate of the bladder detrusor were chosen for the binding assay instead of the purified membrane because the degree of purification differs from the bladder of one species to another when the same separation parameters are used. The binding results showed that although the bladders of all three species showed potential responses to exogenous ATP and its analogues and to the nerve stimulation of

the purinergic component, there do exist differences in P_{2X}-purinoceptor densities and affinities between the species, which may in some sense reflect that there is some degree of difference in purinergic responses amongst the bladders of the three species. Thus, such a study can be extended to more tissues and species, and can be compared with the results from morphological, pharmacological and electrophysiological experiments. The receptor binding assay and autoradiography on human bladder body did show that the P_{2X}-purinoceptor density was much lower than the densities in rat, guinea-pig, and rabbit bladder, and in some samples no P_{2X}-purinoceptors were detected at all (see Chapter 5). In normal human bladder it has been shown that the excitatory purinergic responses are small or absent (Sjögren *et al.*, 1982; Hoyle *et al.*, 1989). It has been observed that purinergic responses are confined mainly to the trigone region of the human bladder (Brading, 1990).

As has already been observed in binding studies on membrane preparations of rat bladder (see Chapter 2), the [^{3}H]α,β-MeATP binding to the washed homogenate of the bladder detrusor of rat, guinea-pig, and rabbit are also composed of a high- and a low-affinity components. So far we are still unable to distinguish whether these two components are two-affinity states of the same receptor or whether they are two different receptors. Further employment of receptor purification and other molecular biology techniques should help to resolve the problem.

Although ATP exists intracellularly in every cell type, autoradiographic localization showed the specific binding of [^{3}H]α,β-MeATP was only over the smooth muscle cells of the bladder in this study, where the physiological function of P_{2X}-purinoceptors is expressed. With the assistance of an image-analysis system, the autoradiograms were semi-quantitatively studied. Differences in the grain densities in the bladders of different species were displayed. The results are in agreement with those from the radioligand binding assay. In this experiment only one concentration of radioligand was used for the autoradiography, thus, the measured grain densities are a relative estimate of the receptor densities. When only a small amount of tissue is available or the tissue is composed of many kinds of cells, and it is impossible to carry out receptor binding assay, the results from the semi-quantitative autoradiography are still of great value, especially when a saturation concentration of radioligand is used.

Regional differences in innervation and adrenoceptor and cholinoceptor densities in the urinary bladder have been reported by Levin *et al.* (1980). They found that the rabbit bladder body preferentially responded to muscarinic, β-adrenergic agonists and ATP, while the bladder base preferentially responded to α-adrenergic agonists. Radioligand binding assays revealed that the bladder body possesses higher densities of muscarinic receptors and β-adrenoceptors, whereas the bladder base has a higher density of α-adrenoceptors. An *in vitro* study showed that the trigone area of human bladder has non-adrenergic, non-cholinergic responses

(Speakman *et al.*, 1988). In our autoradiographic study, regional differences in P_{2X}-purinoceptor densities of the bladder were also displayed, but the differences were not so striking, thus, it is difficult to predict if there is any difference in the regional control of purinergic neurotransmission in the bladder just based on the autoradiographic results.

Non-adrenergic, non-cholinergic nerve-mediated relaxation has been observed in the urethra from pig, rabbit and man (Andersson *et al.*, 1983; Klarskov *et al.*, 1983; Klarskov, 1987), and in bladder neck from pig (Klarskov *et al.*, 1983; Hills *et al.*, 1984; Klarskov, 1987). In guinea-pig urethra (Callahan & Creed, 1981) and pig bladder neck (Hills *et al.*, 1984) ATP was found to induce relaxation. However, since α,β-MeATP had no effect on pig bladder neck, and the response was blocked by 8-phenyltheophylline, a P_1-purinoceptor antagonist, it is concluded that the inhibitory action of ATP was mediated by P_1-purinoceptors. In the present study the specific labelling over the urethral smooth muscles from both rat and guinea-pig was not significant, which may imply a lack of P_{2X}-purinoceptors in the urethra of rat and guinea-pig. However, in rabbit urethra smooth muscle, a small amount of specific [^{3}H]α,β-MeATP binding was observed, which may mediate the non-adrenergic, atropine-resistant contractile responses reported to be present in urethral muscle from rabbit (Mattiasson *et al.*, 1985; Chen, 1990). Further investigations are required to assess their physiological significance.

In conclusion, both the receptor binding assay and the autoradiographic localization of P_{2X}-purinoceptors shows that species differences in receptor densities and affinities exist in urinary bladder. The comparative studies with urethra prove that the distribution of the P_{2X}-purinoceptor is heterogeneous and consistent with what is known of its physiological functions.

Figure 4-1 **A representative of the saturation curves of the specific [^{3}H]α,β-methylene ATP binding to the washed homogenate of guinea-pig bladder.**

Incubation was carried out at 4°C for 120 min. Non-specific binding was determined in the presence of 100 μM β,γ-methylene ATP.

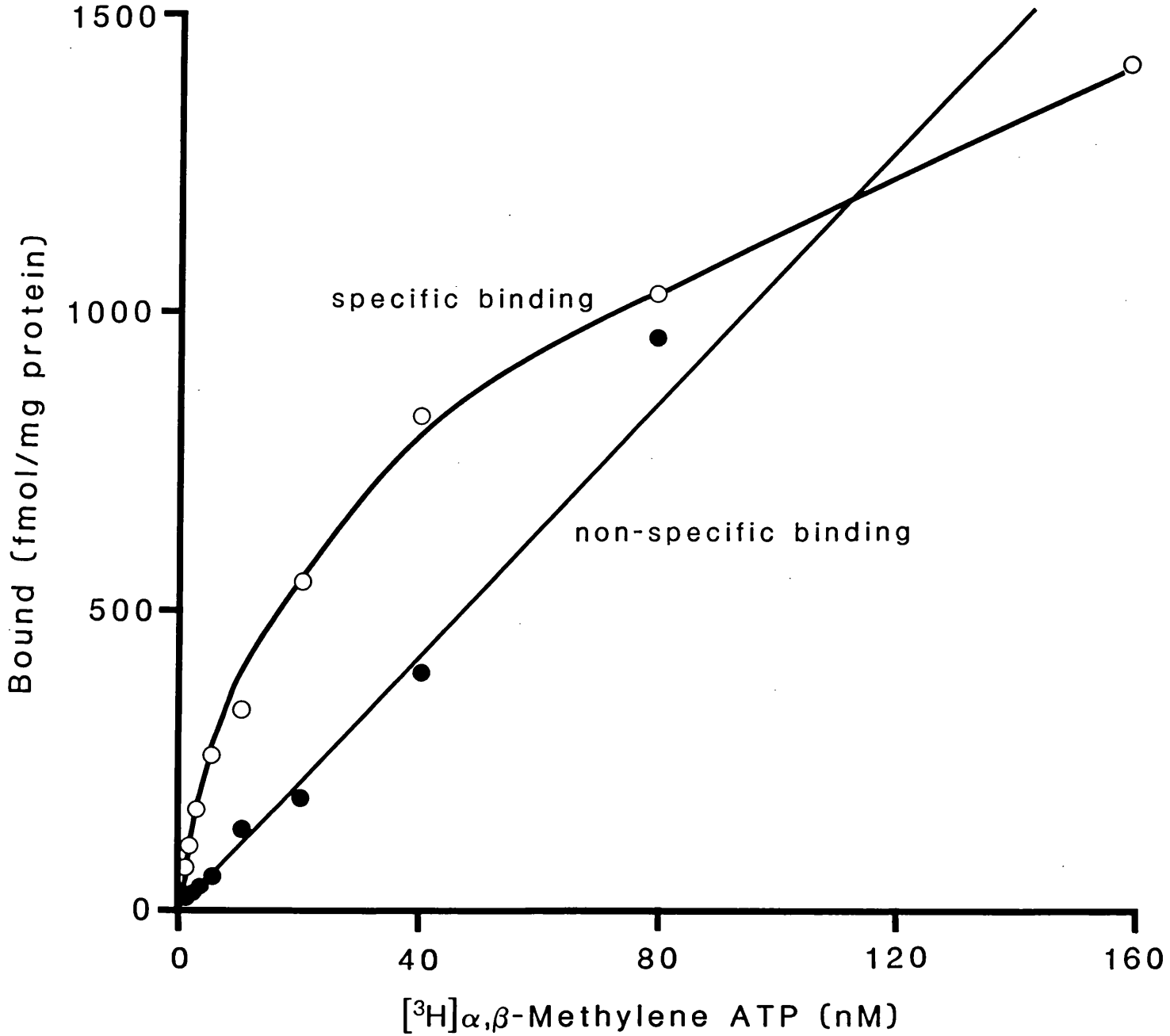

1500
1000
500
0
Bound (fmol/mg protein)
specific binding
non-specific binding
0
40
80
120
160
[3H]α,β-Methylene ATP (nM)

Figure 4-2 **A representative of the Scatchard plots of the specific [^{3}H]α,β-methylene ATP binding to the washed homogenate of guinea-pig bladder.**

The two straight lines which represent two affinity states were fitted by a computer programme EBDA-LIGAND.

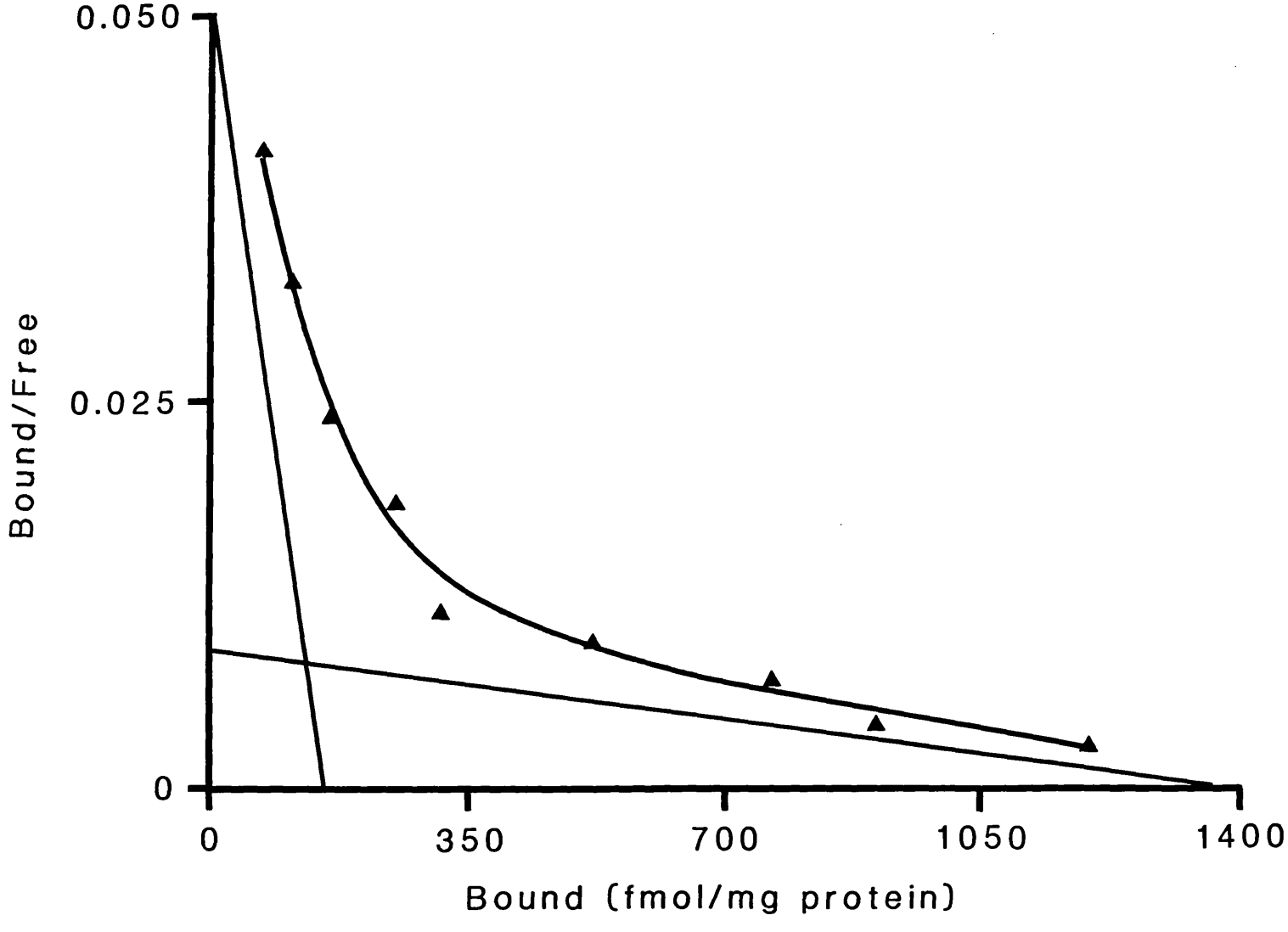
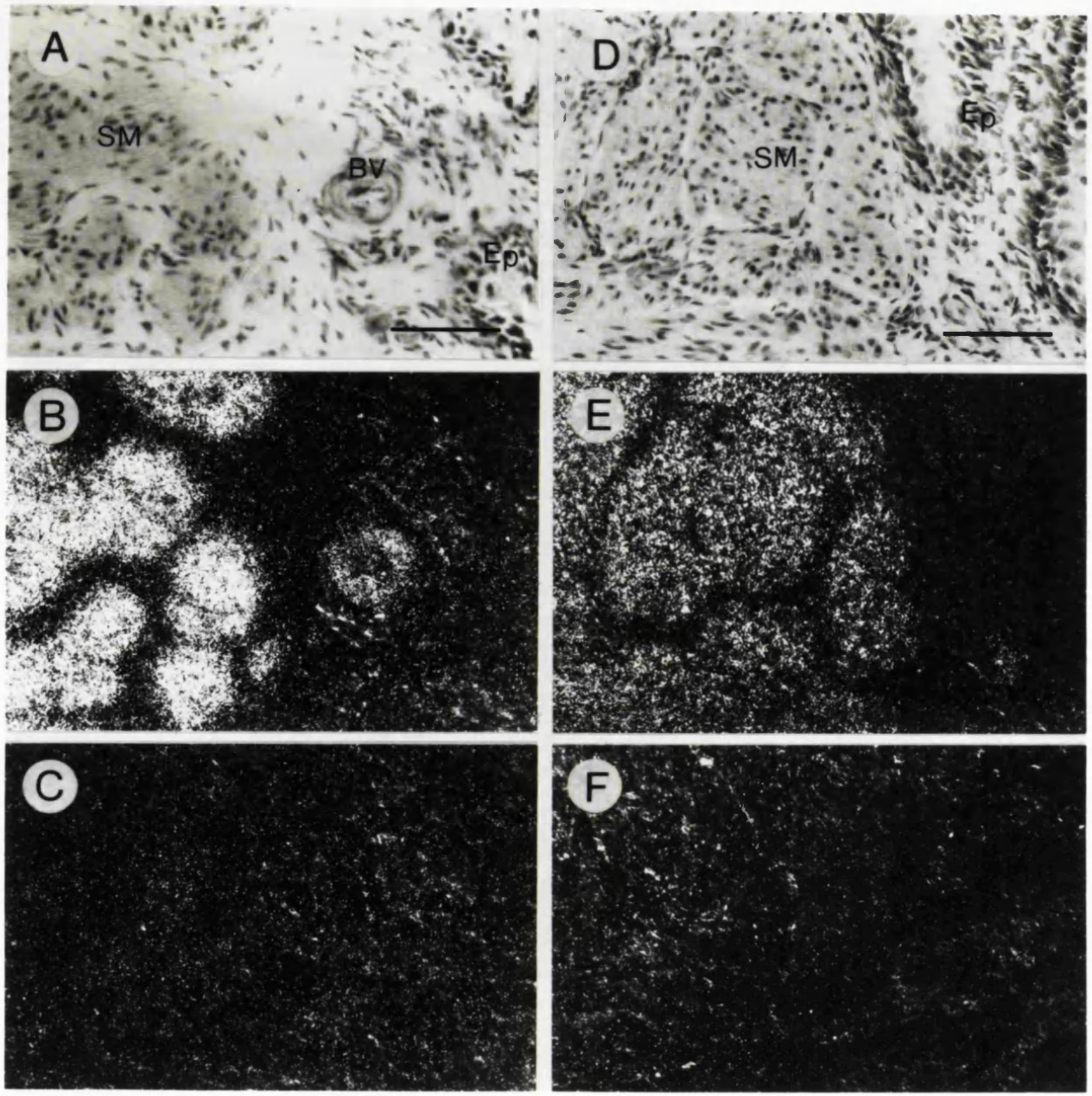

0.050
0.025
0
Bound/Free
0
350
700
1050
1400
Bound (fmol/mg protein)

Figure 4-3 **Autoradiographs of urinary bladder of rat, guinea-pig and rabbit.**

(A), (D), and (G) Bright-field views of the sections of rat, guinea-pig, and rabbit bladder bodies (stained with 0.5% toluidine blue). (B), (E), and (H) Dark-field views of the same fields of (A), (D), and (G). The grains represent the total binding of 10 nM [^{3}H]α,β-methylene ATP to the sections after exposure for 2 weeks. (C), (F), and (I) Dark-field views of the sections adjacent to (A), (D), and (G). The grains represent the non-specific binding of 10 nM [^{3}H]α,β-methylene ATP to the sections after displacement with β,γ-methylene ATP (100 μM). BV, blood vessel; Ep, epithelium; SM, smooth muscle. Calibration bar = 100 μm.


A
SM
BV
Ep
D
SM
Ep
B
E
C
F

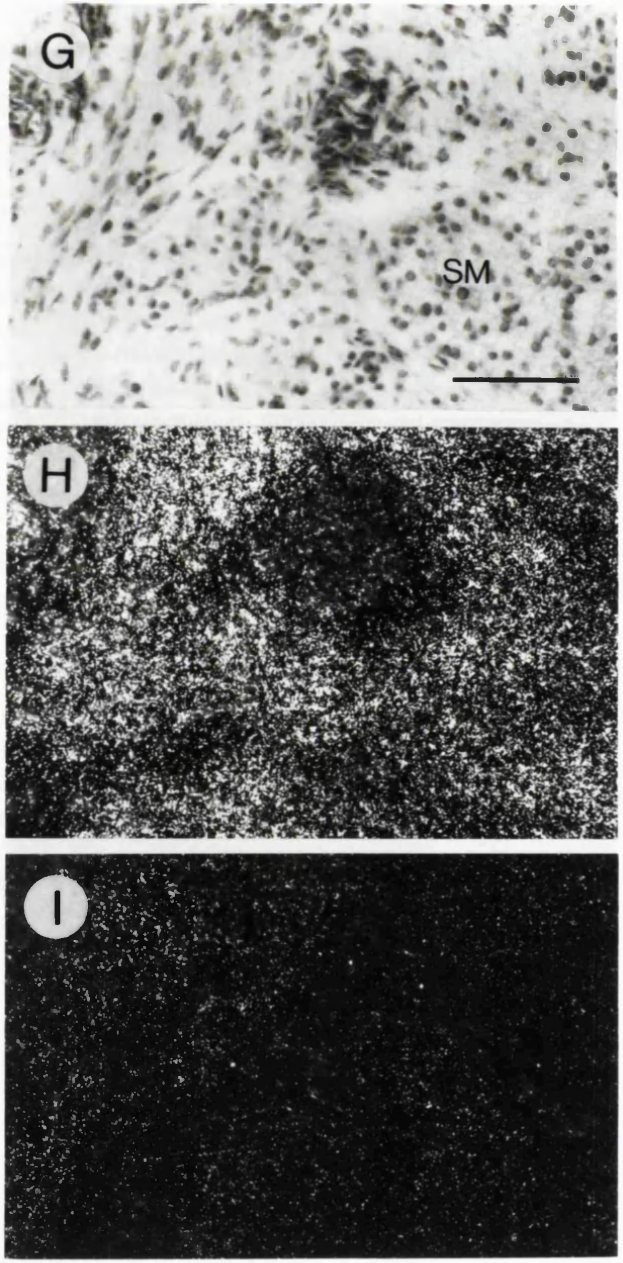

G
SM
H
I

Figure 4-4 **Autoradiographs of urethra of rat and rabbit.**

(A) and (D) Bright-field views of the sections of rat and rabbit urethra (stained with 0.5% toluidine blue). (B) and (E) Dark-field views of the same fields of (A) and (D). The grains represent the total binding of 10 nM [^{3}H]α,β-methylene ATP to the sections after exposure for 2 weeks. (C) and (F) Dark-field views of the sections adjacent to (A) and (D). The grains represent the non-specific binding of 10 nM [^{3}H]α,β-methylene ATP to the sections after displacement with β,γ-methylene ATP (100 μM). Note that there is no significant difference in grain densities between (B) and (C), while the grain densities between (E) and (F) are significantly different ($P<0.01$). Ep, epithelium; SM, smooth muscle. Calibration bar = 100 μm.

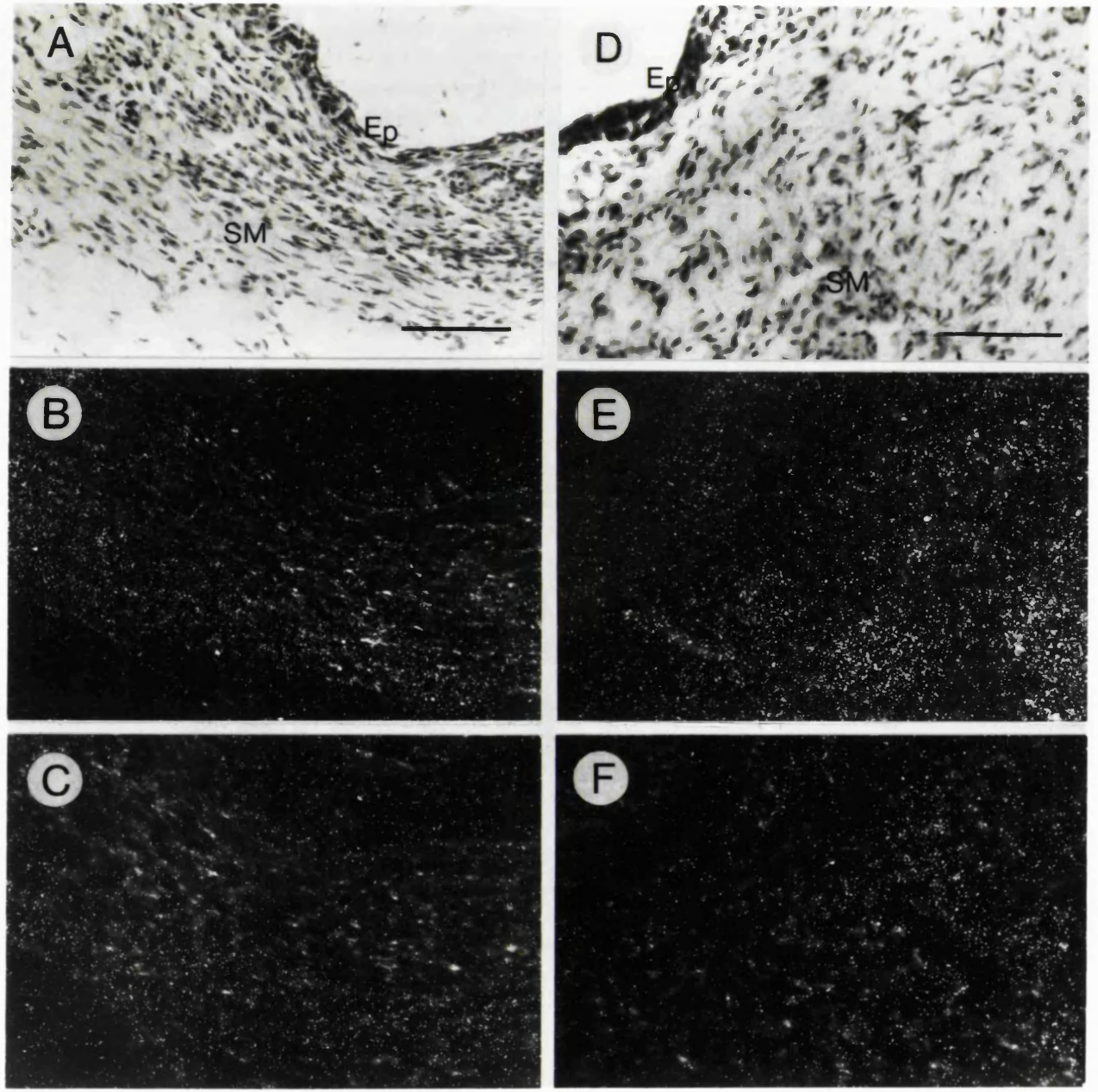

A
Ep
SM
D
Ep
SM
B
E
C
F

Table 4-1 **The maximum specific binding sites (B_{max}, pmol/mg protein) and apparent dissociation constants (K_d, nM) of [^{3}H]α,β-MeATP binding to the washed homogenate of rat, guinea-pig, and rabbit urinary bladder.**

Each experiment was carried out in duplicate and the experiment numbers are indicated in the brackets.

Species	High-affinity state		Low-affinity state	
	B_{max}	K_d	B_{max}	K_d
Rat (n=6)	1.50±0.30***	2.45±0.28	4.46±0.79***	78.8±14.7
Guinea-pig (n=5)	0.19±0.05	1.83±0.72	1.45±0.21	80.4±17.8
Rabbit (n=4)	0.84±0.20***	0.86±0.08^{+++}	3.35±1.10**	29.3±3.8^{+++}

*** P<0.001, ** P<0.01, significantly different from the receptor densities of guinea-pig bladder;

$^{+++}$ P<0.001, significantly different from the K_d values of the binding to rat and guinea-pig bladders.

Table 4-2 **Grain densities (grains 1000 μm^{-2}) representing the specific binding of [^{3}H]α,β-MeATP (10 nM) in smooth muscles of rat, guinea-pig and rabbit urinary bladder and urethra.**

Data are obtained by calculating the differences between the [^{3}H]α,β-MeATP labelling with and without the displacement by β,γ-MeATP (100 μM) and expressed as mean ± S.E.M. of nine sections from three animals.

	Bladder			Urethra
	Dome	Body	Trigone	
Rat	761±42***	757±23***	869±29***	23±17
Guinea-pig	275±25	242±17	319±29	41±23
Rabbit	439±29^{+++}	502±46^{+++}	413±37^{+++}	90±22$^{\#\#}$

*** P<0.001, significantly different from the relevant regions of guinea-pig and rabbit bladders;

$^{+++}$ P<0.001, significantly different from the relevant regions of guinea-pig bladder

$^{\#\#}$ P<0.01, significantly different from the non-specific binding.

Chapter 5: Characterization and Autoradiographic Localization of [^{3}H]α,β-Methylene ATP Binding Sites in Human Urinary Bladder

Summary

1. The characteristics of [^{3}H]α,β-MeATP binding sites were determined in the washed homogenate and membrane preparations of human urinary bladder. Autoradiographic localization of [^{3}H]α,β-MeATP binding sites in the bladder was also carried out.

2. The samples were obtained from male patients aged 56 - 79 years. Six out of the sixteen samples in the binding assay and three out of seven samples in the autoradiographic localization study showed specific [^{3}H]α,β-MeATP binding, possible explanations for this are discussed.

3. The binding process was saturable and the specific binding sites were composed of high- and low-affinity components. The specific binding to membrane preparations was reduced in the presence of Mg^{2+} in the incubation medium.

4. Competitive displacement experiments showed that the potency order of the unlabelled ligands to displace the [^{3}H]α,β-MeATP binding was α,β-MeATP > β,γ-MeATP > suramin > 2-MeSATP > ATP > ADP >> adenosine, which indicate that the binding sites are, or are linked to, P_{2X}-purinoceptors.

5. Autoradiographic localization showed that the specific [^{3}H]α,β-MeATP binding sites were only on the smooth muscle of the bladder. The autoradiographic grain density was lower in the presence of Mg^{2+}.

6. These results suggest that P_{2X}-purinoceptors exist in human urinary bladder, although at a lower density than in rodent urinary bladder.

5-1 Introduction

Excitatory purinergic neurotransmission has been observed in the urinary bladder of many species (see Chapter 4). There are conflicting reports as to whether purinergic neurotransmission exists in human urinary bladder. Sibley (1984) reported that atropine completely abolished nerve-mediated contractions in human urinary bladder muscle and claimed that an excitatory non-adrenergic, non-cholinergic (NANC) component was not present in human urinary bladder. However, other researchers have demonstrated atropine-resistant responses (Hindmarsh *et al.*, 1977; Cowan & Daniel, *et al.*, 1983; Husted *et al.*, 1983; Hoyle *et al.*, 1989; Ruggieri *et al.*, 1990). Husted *et al.* (1983) showed that ATP could induce concentration-dependent contractions in isolated preparations from the human bladder, and that these responses could be blocked by nifedipine. A report from our laboratory also showed that some human detrusor muscle strips respond to ATP, α,β-methylene ATP (α,β-MeATP), and P^1,P^6-diadenosine hexaphosphate (A6PA) (Hoyle *et al.*, 1989). Moreover, the responses to ATP, A6PA and non-cholinergic nerve stimulation were blocked following desensitization by α,β-MeATP. Another report showed that α,β-mehtylene ATP, β,γ-methylene ATP, ADPβS and ATP all elicited contractile responses in human urinary bladder to a certain amplitude, and suramin dose-dependently antagonized the bottom part of the α,β-methylene ATP concentration-response curve (Palea *et al.*, 1992). Exogenously applied ATP has been shown to depolarize dispersed human detrusor cells in a similar fashion to

detrusor cells from pig and guinea-pig (Inoue & Brading, 1991). Taken together, these facts indicate that purinergic neurotransmission probably does exist at least in some regions of the human urinary bladder, mediating detrusor contraction via P_{2X}-purinoceptors.

In order to obtain some knowledge about the characteristics of P_{2X}-purinoceptors in the human urinary bladder, radioligand binding assays and autoradiographic localization were employed in this study, using [^{3}H]α,β-MeATP as the radioligand, which will provide direct evidence for the existence of P_{2X}-purinoceptors in human urinary bladder.

5-2 Materials and methods

5-2-1 Radioligand binding assay

Specimens of the human urinary bladder were obtained from 16 male patients (aged 61 to 79) undergoing cystectomy for carcinoma of the bladder. Specimens were cut from the bladder fundus where the tissues were macroscopically normal. The blocks of bladder wall were trimmed free of mucosa and adipose tissue in cold Krebs solution, and membrane preparations and the washed homogenate were prepared as described in Chapter 2.

According to the results from the radioligand binding assay on rat urinary bladder, Mg^{2+} was found to have a substantial influence on the binding of [^{3}H]α,β-MeATP to the membrane preparation at 30°C. Thus, in this experiment the saturation analysis on the membrane preparations was carried out in Mg^{2+}-free buffer and in buffer containing 25 mM $MgCl_2$, for comparison. The saturation analysis on the washed homogenate of the bladder was carried out in the absence of Mg^{2+}. The washed homogenate (protein content from 100 to 180 μg per assay) or membrane preparations (protein content from 30 to 50 μg per assay) was incubated with serial concentrations of [^{3}H]α,β-MeATP at 30°C for 15 min. Non-specific binding was determined in the presence of 100 μM β,γ-MeATP.

Further characterization of [^{3}H]α,β-MeATP binding sites on

human bladder membranes was achieved by competitive displacement experiments using several unlabelled ligands. These experiments were carried out in Mg^{2+}-free buffer at 4°C. The total binding was determined in the absence of the non-labelled ligands, which was about 2 - 3% of the added radioactivity. The following ligands were tested: α,β-MeATP, β,γ-MeATP, 2-MeSATP, suramin, ATP, ADP, and adenosine.

5-2-2 Autoradiography

The samples of human bladder in this experiment were biopsy chips from seven patients (male, aged from 56 to 72) undergoing cystoscopy. Sections 15 μm thick were cut on a Bright cryostat at -25°C. Other procedures were that same as those described in Chapter 3.

5-2-3 Chemicals

[^{3}H]α,β-MeATP was custom synthesized by Amersham International (Amersham, U.K.) with a specific activity of 27 Ci/mmol and chemical purity of 98-99%. β,γ-MeATP, ATP, ADP, adenosine were purchased from Sigma Chemicals (Poole, U.K.). 2-MeSATP was from Research Biochemicals Inc. (Natick, U.S.A.). Suramin was a gift from Bayer AG (West Sussex, U.K.)

5-2-4 Data analysis

See Chapters 2 and 3.

5-3 Results

5-3-1 Saturation analysis

Six out of the sixteen samples showed specific [^{3}H]α,β-MeATP binding. The binding process was saturable. A saturation curve on the washed homogenate of the bladder is shown in Figure 5-1, and the corresponding Scatchard plot is shown in Figure 5-2. Scatchard analysis revealed the binding sites to contain high- and low-affinity components. The maximum binding (B_{max}) was 105 ± 43 fmol/mg protein (n = 6) for the high-affinity component, and 765 ± 277 fmol/mg protein (n = 6) for the low-affinity component. The dissociation constant (K_d) was 2.2 ± 0.7 nM (n = 6) for the high-affinity component and 57 ± 16 nM (n = 6) for the low-affinity component.

The specific binding of [^{3}H]α,β-MeATP to the bladder membrane preparation was also saturable both in the presence and absence of Mg^{2+}. In the absence of Mg^{2+} the B_{max} for the high-affinity component was 747 ± 230 fmol/mg protein (n = 6) with a K_d value of 6.9 ± 2.4 nM (n = 6), while the B_{max} for the low-affinity component was 2253 ± 775 fmol/mg protein (n = 6) with a K_d value of 53 ± 19 nM (n = 6) . In the presence of 25 mM $MgCl_2$, the specific binding was substantially inhibited, the B_{max} for the high affinity component was reduced to 185 ± 56 fmol/mg protein (n = 6) and the affinity was increased (the K_d was reduced to 2.7 ± 0.7 nM, n = 6). The B_{max} for the low-affinity component was

reduced to 618 ± 228 fmol/mg protein (n = 6) and the affinity was not much affected (K_d = 64 ± 12 nM, n = 6).

The non-specific binding of [^{3}H]α,β-MeATP in the washed homogenate was high above the specific binding (Figure 5-1), ranging from 55% at the lowest radioligand concentration to 92% at the highest radioligand concentration. However, the proportion of non-specific binding in the membrane preparation was lower than that in the washed homogenate, ranging from 21 to 68% in the experiment in the absence of Mg^{2+}, and from 50 to 88% in the experiment in the presence of Mg^{2+}. Mg^{2+} ions could slightly increase the non-specific binding, but this was not significant.

5-3-2 Competitive displacement

Competitive displacement experiments (Figure 5-3) showed that α,β-MeATP was the most potent ligand to displace the binding (IC_{50}, 0.012 ± 0.004 μM, n = 6), followed by β,γ-MeATP (IC_{50}, 0.09 ± 0.03 μM), suramin (IC_{50}, 0.18 ± 0.04 μM) and 2-MeSATP (IC_{50}, 0.36 ± 0.08 μM). The IC_{50} values for ATP and ADP were 1.6 ± 0.3 μM and 4.1 ± 1.3 μM. Adenosine was the weakest ligand to displace the binding, only 12% was displaced at a concentration of 1 mM.

5-3-3 Autoradiography

Specific binding was observed in the autoradiograms from three of the seven bladder biopsy chips. It is shown that the

specific binding sites were over the smooth muscle of the bladder (Figure 5-4). The grain densities were greatly reduced in the presence of 100 μM β,γ-MeATP. In the sections incubated in Mg^{2+}-free buffer (Figure 4A,B,C) the specific grain density was higher than that with 25 mM $MgCl_2$ (Figure 4D,E,F) (87 ± 33 vs. 52 ± 22 grains/1000 μm^2), which was in accordance with the radioligand binding assay. No specific binding site was observed over the epithelium (pictures not shown).

5-4 Discussion

The binding characteristics of [^{3}H]α,β-MeATP in the washed homogenate and membrane preparations of human bladder were similar to those from rat, guinea-pig and rabbit urinary bladder, in that the binding sites have high- and low-affinity components, the K_d values are similar, the B_{max} is reduced in the presence of Mg^{2+} in the incubation media, and the grains which represent the specific binding were only over the smooth muscle. However, only 38% of human bladder samples in the binding study and 43% in the autoradiographic localization study showed specific [^{3}H]α,β-MeATP binding, while all rat, guinea-pig and rabbit bladder tested showed specific binding. The density of the specific binding sites is lower in human bladder than in rat, guinea-pig and rabbit bladder and the individual variance is rather wide (as can be seen from the large standard error of the B_{max}). These results are in accordance with those from pharmacological experiments, because it has been found that in those presumably normal specimens of human bladder, only some showed the atropine-resistant responses (Husted *et al.*, 1983; Hoyle *et al*, 1989; Ruggieri *et al.*, 1990). There may be regional differences in the distribution of P_{2X}-purinoceptors in the human bladder, for example, it has been reported that the trigone has more prominent purinergic responses compared with other regions (Brading, 1990). It should also be mentioned that all samples used in this study were from elderly male patients. Some reports indicate that bladders from female and young subjects possess a larger proportion of atropine-resistant responses (Cowan

& Daniel, 1983; Hoyle *et al.*, 1989). It has also been reported that functionally disturbed bladders are more sensitive to ATP and have larger atropine-resistant responses (Sjögren *et al.*, 1982; Husted *et al.*, 1983). At present only a handful of papers are available on the non-adrenergic, non-cholinergic neurotransmission in human urinary bladder. Further comparative studies on effects of sex and age differences, and on normal and functionally disturbed bladders may result in more information which may be of clinical significance.

Although the density of [^{3}H]α,β-MeATP binding sites in human bladder was much lower than those in rodent bladder (see Chapter 4), it is still much higher than the densities of the classic neurotransmitter receptors. One may question why, in normal human urinary bladder, purinergic neurotransmission does not appear to be very important, but a high density of [^{3}H]α,β-MeATP binding sites exists. A definite answer to this question is not available at present. One postulation is that the rapid degradation of the endogenous ligand ATP and the low affinity of ATP require the presence of high density of receptors to accept the stimulation. Another possibility is the existence of large amount of inactive P_{2X}-purinoceptor or "acceptors" in human bladder, which do not mediate biological responses, but still have the ability to bind to the radioligand.

As has been observed in rat urinary bladder and vas deferens, the [^{3}H]α,β-MeATP binding sites in human bladder also showed two

affinity components. However, by using a radioligand binding assay it is not possible to discriminate whether they are two distinct binding sites or just one binding site with two affinity states. Further studies on the molecular structure properties of these binding sites may supply the final answer.

Competitive displacement experiments showed that the potency order of the cold ligands to displace the binding of [^{3}H]α,β-MeATP to human bladder membrane was α,β-MeATP > β,γ-MeATP > suramin > 2-MeSATP > ATP > ADP >> adenosine, which indicates that the binding sites are or are linked to P_{2X}-purinoceptors (Burnstock & Kennedy, 1985). In a pharmacological experiment on human bladder, it was observed that α,β-MeATP, A6PA, β,γ-MeATP, ADPβS and ATP elicited contraction concentration-dependently (Hoyle *et al.*, 1990; Palea *et al.*, 1992). α,β-MeATP and A6PA were far more potent than ATP, and their effects were significantly inhibited after desensitization of the preparation by prolonged exposure to α,β-MeATP (Hoyle *et al.*, 1990), which indicates that the contractile responses of human bladder to these purine compounds were mediated by P_{2X}-purinoceptors. In the sense of potency order, the results in the present study were in accord with those from *in vitro* recording experiments. However, a proper match between the potencies of the purine compounds in eliciting contraction and the affinities to [^{3}H]α,β-MeATP binding sites is unable to be performed at present due to the lack of detailed pharmacological experimental data. It is also difficult to obtain such data because several experiments showed that P_{2X}-purinoceptor-active ligands usually cannot elicit the maximum responses in human

urinary bladder (Hoyle *et al.*, 1990; Palea *et al.*, 1992). Furthermore, the existence of two affinity components of [^{3}H]α,β-MeATP binding would make such a match more difficult. Although all the cold ligands except adenosine could displace all the specific binding when a certain concentration was reached, they may also have different affinities to these two components. Non-linear regression analysis of the displacement experiment data showed that the cold ligands did have different K_i values to these two [^{3}H]α,β-MeATP binding components. However, due to significant variation between individual experiments, accurate estimates of the K_i values were not obtained.

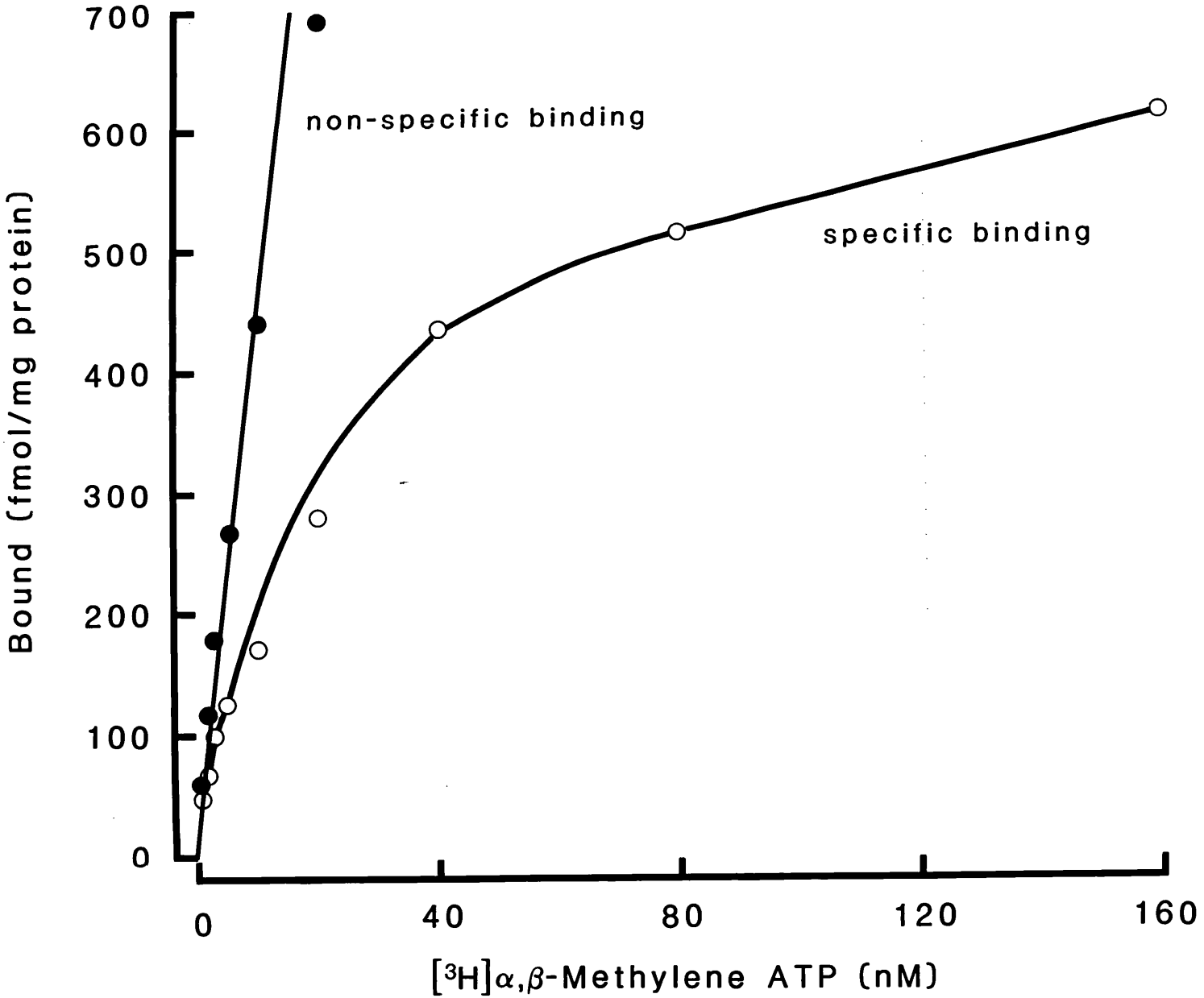
The rank order of potency of the cold ligands was similar to that obtained from the experiment on rat bladder membranes with the exception that 2-MeSATP appeared more potent than both ATP and ADP in this study. Another significant difference is that the IC_{50} values in this study were much lower than those from the experiments on rat bladder membranes. One possible explanation for this discrepancy is that, in this study, the incubation was carried out at 4°C in Mg^{2+}-free buffer, thus the degradation of the cold ligands including ATP, ADP, 2-MeSATP and β,γ-MeATP by ectonucleotidases would be greatly inhibited. The free cold ligand concentration would be constant throughout the incubation. The other reason may be that the interference of Mg^{2+} ions on the binding was abolished, and the affinities of the cold ligands might be changed. Differences in affinities of the binding sites to the ligands in the two tissues caused by the species

difference in molecular properties should also be considered.

In conclusion, the results from this study suggest the existence of P_{2X}-purinoceptors in human urinary bladder. The characteristics of the binding are similar to that in rat urinary bladder, although the densities of the receptors in human urinary bladder are lower.

Figure 5-1 **A representative of the saturation curves of [^{3}H]α,β-methylene ATP binding to the washed homogenate of human urinary bladder.**

The incubation was carried out at 30°C for 15 min. Non-specific binding was determined in the presence of 100 μM β,γ-methylene ATP.


700
600
500
400
300
200
100
0
Bound (fmol/mg protein)
non-specific binding
specific binding
0
40
80
120
160
[3H]α,β-Methylene ATP (nM)

Human bladder

9

Figure 5-2 **A representative of the Scatchard plots of the specific binding of [^{3}H]α,β-methylene ATP to the washed homogenate of human urinary bladder (data from Figure 5-1).**

The curve was fitted by the computer program EBDA-LIGAND, the two binding affinity state model was significantly better than that one binding affinity state model.

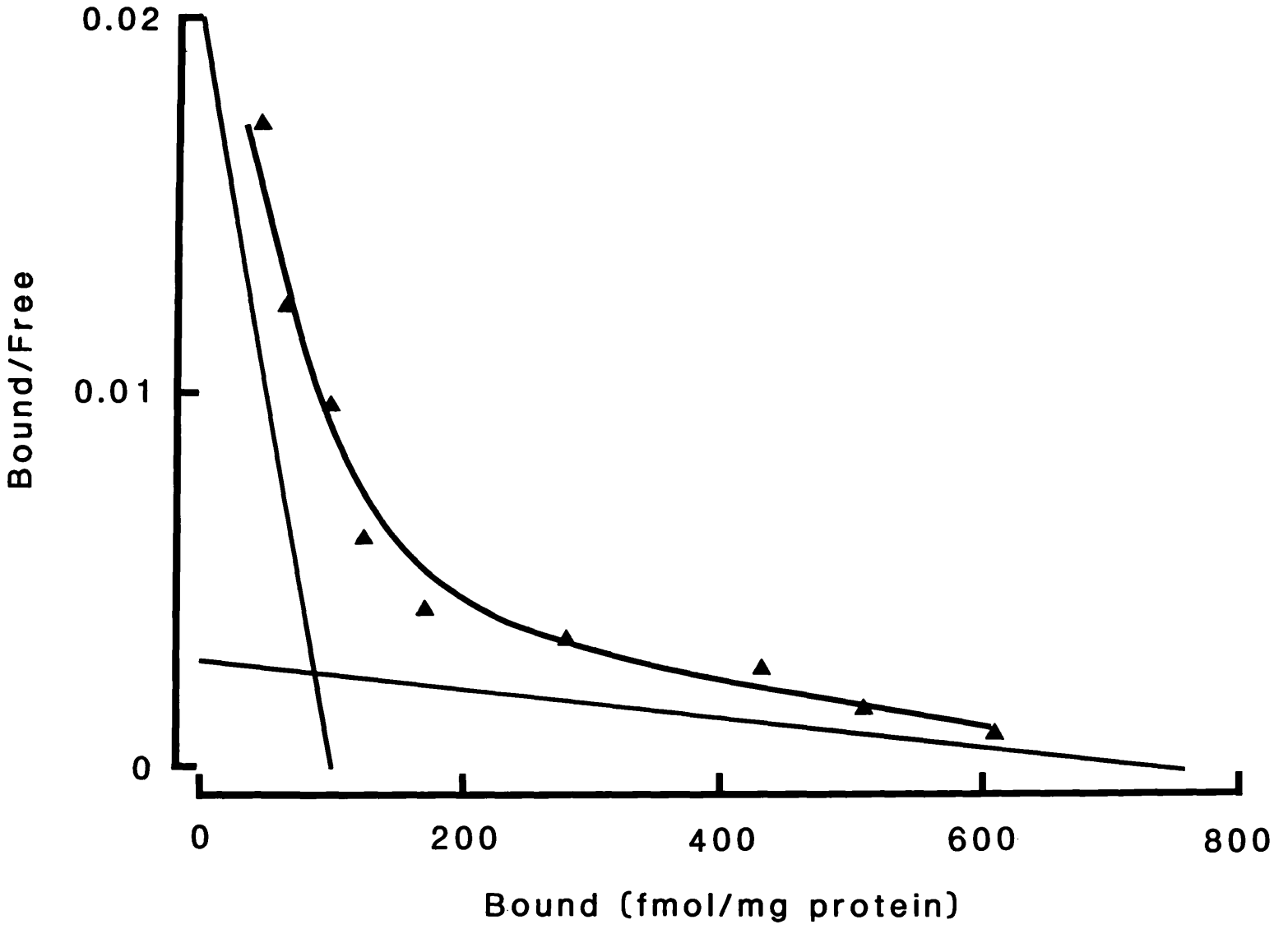

0.02
0.01
0
Bound/Free
0
200
400
600
800
Bound (fmol/mg protein)

Figure 5-3 **Displacement of [^{3}H]α,β-methylene ATP (10 nM) binding to human urinary bladder membrane preparations by unlabelled ligands:**

(○) α,β-methylene ATP; (●) β,γ-methylene ATP; (△) suramin; (▲) 2-methylthio ATP; (◇)ATP; (◆) ADP; (■) adenosine. Incubation was carried out at 4°C for 2 hr. Each point represents the mean of six duplicated experiments. Vertical bars represent the standard errors of the means where these exceeded the size of the symbol.

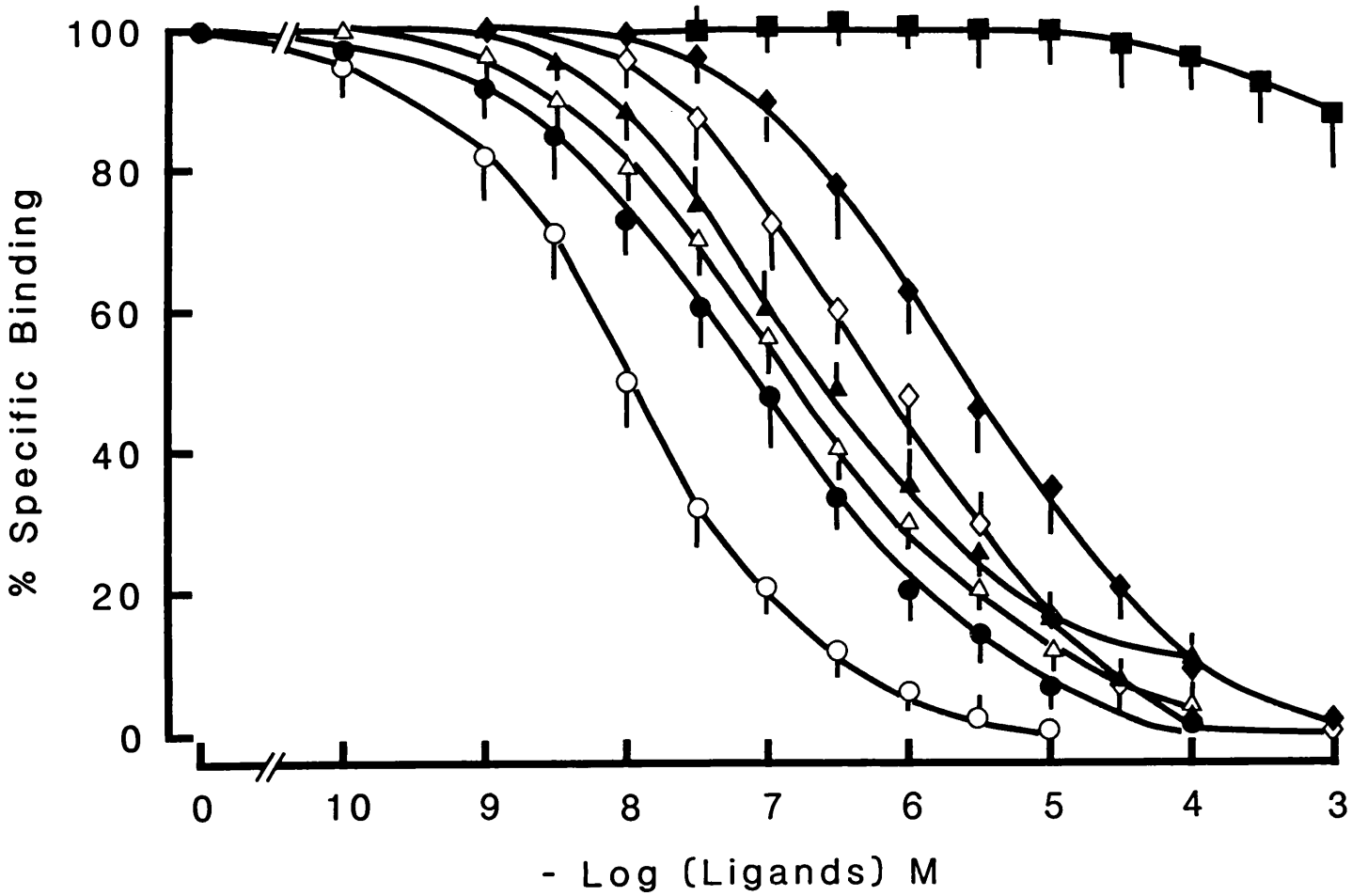
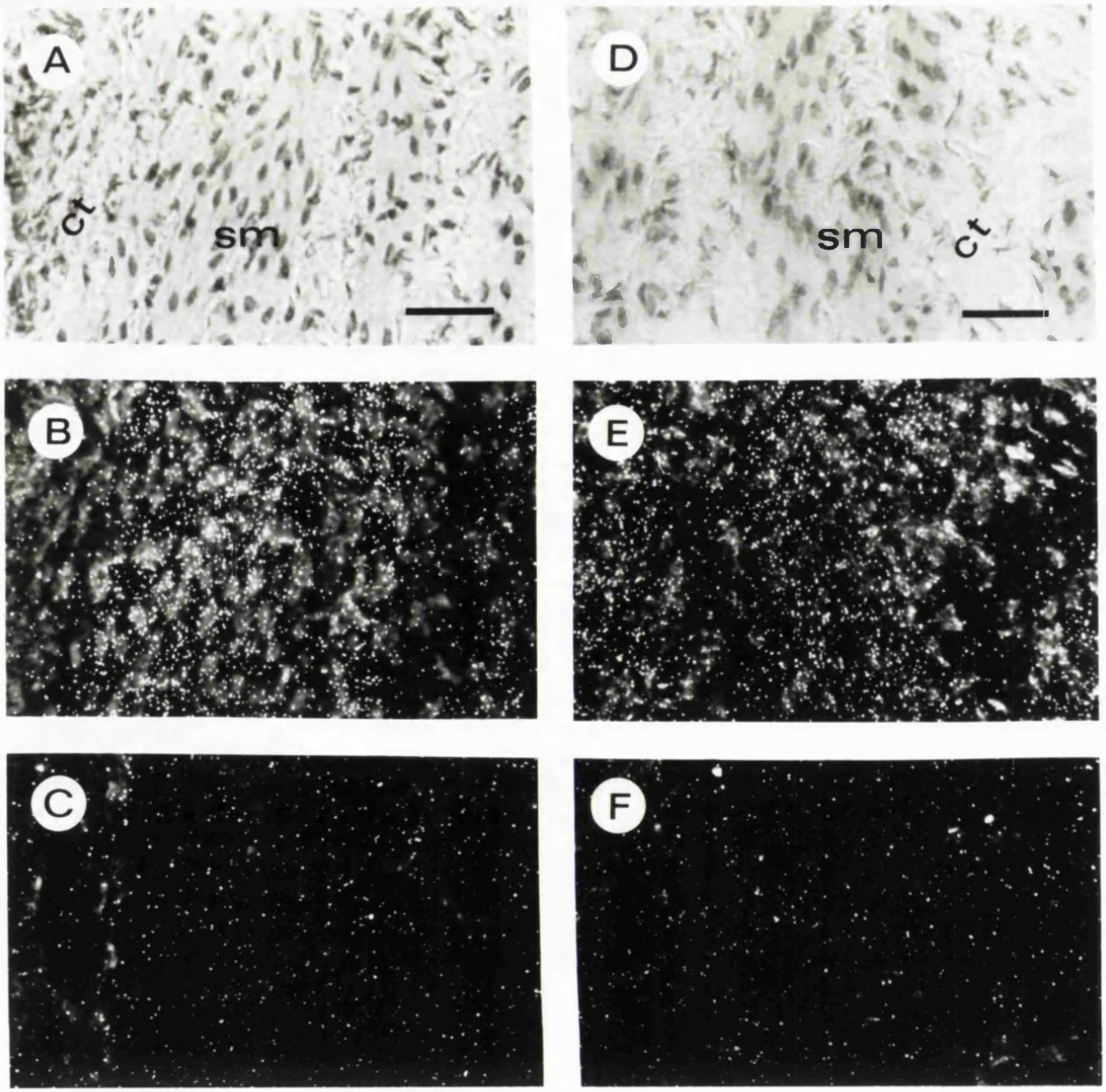

100
80
60
40
20
0
% Specific Binding
0
10
9
8
7
6
5
4
3
- Log (Ligands) M

Figure 5-4 **Autoradiographs of human urinary baldder.**

(A) and (D), bright-field views of the sections of human urinary bladder (15 μm thick, stained with 0.5% toluidine blue). (B) and (E), dark-field views of the same fields of (A) and (D). The grains represent the total binding of 10 nM [^{3}H]α,β-methylene ATP to the sections after exposure for 2 weeks. (C) and (F), dark-field views of the sections adjacent to (A) and (D). The grains represent the non-specific binding of 10 nM [^{3}H]α,β-methylene ATP to the sections in the presence of 100 μM β,γ-methylene ATP. (A), (B) and (C), sections were incubated in magnesium-free buffer; (D), (E) and (F), sections were incubated in the buffer containing 25 mM $MgCl_2$. sm, smooth muscle; ct, connective tissue. Calibration bar = 50 μm.


A
ct
sm
D
sm
ct
B
E
C
F

Chapter 6: Heterogeneous distribution of [^{3}H]α,β-methylene ATP binding sites in blood vessels

Summary

1. A radioligand binding assay and autoradiographic localization of P_{2X}-purinoceptors were carried out in several different blood vessels from rat, guinea-pig, and rabbit by using [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) as the radioligand.

2. The radioligand binding assay on rabbit ear artery showed that the binding process was saturable, and a high density of P_{2X}-purinoceptors was observed.

3. Autoradiographic localisation showed that the specific [^{3}H]α,β-MeATP binding sites were only associated with the smooth muscle of the blood vessels. Semi-quantitation of the autoradiographs revealed significant differences in the densities of P_{2X}-purinoceptors amongst the vessels studied. Generally, the medium- and small-sized arteries had higher densities of P_{2X}-purinoceptor than the elastic and large muscular arteries.

4. In some large muscular arteries, such as rabbit carotid, renal, hepatic, and mesenteric arteries, the outer region of vessel had a higher density of receptors than the inner region.

5. The veins in this study were sparsely labelled, except portal

veins where the longitudinal and circular layers of muscles were found to have different densities of P_{2X}-purinoceptors.

6. The results from this study provide direct evidence for the existence of P_{2X}-purinoceptors in blood vessels, futhermore, the distribution of the P_{2X}-purinoceptors is consistent with known pharmacological responses elicited by ATP in these vessels.

6-1 Introduction

The effects of extracellular purine nucleosides and nucleotides on the cardiovascular system were first observed in late 1920s (Drury & Szent-Györgyi, 1929). Since the proposal of the purinergic nerve hypothesis (Burnstock, 1972), much evidence has been presented which supports the existence of purinergic neurotransmission in organs such as urinary bladder, vas deferens, gut, and blood vessels (White, 1988; Dubyk & Fedan, 1990). In many vascular preparations such as the rat femoral artery (Kennedy *et al.*, 1985), pulmonary artery (Liu *et al.*, 1989; 1990), mesenteric artery (Ralevic & Burnstock, 1988), and rabbit ear artery (Kennedy & Burnstock, 1985a), activation of P_{2X}-purinoceptors on the smooth muscle in response to stimulation of the intramural nerve or by exogenous ligands, such as ATP and α,β-methylene ATP (α,β-MeATP), produces vasoconstriction. The endogenous ligand is likely to be ATP, which can be co-released from perivascular sympathetic nerves with noradrenaline (NA) following nerve stimulation (Su, 1975; 1978). Chemical denervation of sympathetic nerves with 6-hydroxydopamine or depletion of NA with reserpine have also demonstrated that ATP is a cotransmitter of NA in sympathetic nerves innervating some blood vessels (Warland & Burnstock, 1987; Saville & Burnstock, 1988; Brizzolara & Burnstock, 1990). Electrophysiological experiments have shown that excitatory junction potentials elicited in response to nerve stimulation can be mimicked by exogenously applied ATP and abolished following desensitisation of P_{2X}-purinoceptors by α,β-MeATP (Sneddon &

Burnstock, 1984; Miyahara & Suzuki, 1987; Inoue & Kannan *et al.*, 1988). P_{2Y}-Purinoceptors are generally located on endothelial cells where they mediate vasodilatation through the release of endothelium-derived relaxing factors (EDRF) (De May & Vanhoutte, 1981), although in some vessels P_{2Y}-purinoceptors are located directly on the smooth muscle (Kennedy & Burnstock, 1985b; Warland & Burnstock, 1987; Corr & Burnstock, 1992).

All the evidence accumulated so far indicates that purinergic cotransmission may play an important role in the regulation of blood flow in many systems, however, no direct evidence for the existence of P_{2X}-purinoceptors on blood vessels was available. In the present study the radioligand binding assay for [^{3}H]α,β-MeATP on the rabbit central ear artery was carried out. Autoradiographic localization of [^{3}H]α,β-MeATP binding sites was conducted in several blood vessels from rat, guinea-pig, and rabbit.

6-2 Materials and Methods

6-2-1 Radioligand binding assay

New Zealand white rabbits (male, 2.5 kg) were killed by overdose of sodium pentobarbital injection through the marginal ear vein. The central ear arteries were removed and dissected free of adventitia. They were minced and homogenised in 12 ml 50 mM Tris/HCl buffer (pH 7.4, 20°C, buffer A) with a Polytron PT3000 at 30,000 rpm for two 15 s bursts. The homogenate was centrifuged at 105,000 *g* for 50 min. The supernatant was discarded and the pellet was suspended in buffer A, which was refered to as the washed homogenate. Saturation assays were carried out as described in Chapter 2.

6-2-2 Autoradiography

Wistar rats (male, 200-250 g) were killed by asphyxia with CO_2. The femoral, saphenous, mesenteric and tail arteries, and the portal vein were removed. Guinea-pigs (male, 350-400 g) were killed in the same way as the rats: the femoral, saphenous and mesenteric arteries, and the portal vein were removed. New Zealand white rabbits (male, 2.5 kg) were killed as described above. The femoral, saphenous, mesenteric, central ear, renal, coeliac, hepatic, carotid, and coronary arteries, aorta, inferior vena cava, and mesenteric and portal vein were removed. All the vessels were placed in Krebs' solution and trimmed. Sections 14 μm thick

were cut in a Reichet-Jung Cryocut 1800 (Cambridge Instruments GmbH, Nussloch, Germany) cryostat at -22°C. Other procedures were the same as described in Chapter 3.

6-2-3 Chemicals

[^{3}H]α,β-MeATP was custom-synthesized by Amersham International Ltd. (Amersahm, U.K.). Specific activity was 27 Ci/mmol with the chemical purity of 98-99%. β,γ-MeATP was purchased from Sigma Chemicals (Poole, U.K.).

6-2-4 Data analysis

See Chapters 2 and 3.

6-3 Results

6-3-1 Radioligand binding assay

The binding of [^{3}H]α,β-MeATP to the washed homogenate of rabbit ear artery was saturable (Figure 6-1). Scatchard analysis revealed that only one affinity binding site existed in the preparations (Figure 6-2). The maximum binding site (B_{max}) was 1.3 ± 0.2 pmol/mg protein (n=6), and the dissociation constant (K_d) was 2.2 ± 0.6 nM (n=6).

6-3-2 Autoradiography

The specific [^{3}H]α,β-MeATP labelling was only observed over the smooth muscle cells of the vessels. Most of the vessels in this study were labelled, and significant differences in the densities of the specific binding sites amongst the vessels were observed (Figure 6-3; Table 6-1). The elastic arteries such as rabbit aortic trunks had almost no specific labelling (Figure 6-3-XIX), as did the rabbit coeliac artery (Figure 6-3-XXI). The rat femoral artery was moderately labelled (Figure 6-3-III), but most of the muscular arteries such as saphenous (Figure 6-3-IV, V, VI), mesenteric (Figure 6-3-VII, VIII, IX), rat tail (Figure 6-3-XII), and rabbit central ear (Figure 6-3-XI) were heavily labelled. Some striking changes of the densities of specific [^{3}H]α,β-MeATP binding sites along the artery were observed. For example, in the rabbit femoral artery, the proximal segment showed a very low

density of labelling (Figure 6-3-I), while the distal segment was heavily labelled (Figure 6-3-II); this difference was highly significant ($P < 0.001$) (Table 6-1).

In bigger muscular arteries, such as rabbit mesenteric (Figure 6-3-IX), hepatic (Figure 6-3-X), renal (Figure 6-3-XIII), and carotid arteries (Figure 6-3-XIV), the receptor densities were significantly higher ($P < 0.001$) in the outer region of the vessel wall than the inner region (Table 6-1).

The densities of [^{3}H]α,β-MeATP binding sites in veins were usually very low, such as in rabbit mesenteric vein (Figure 6-3-XVI), and in rabbit inferior vena cava (Figure 6-3-XX). However, the portal veins of rat, guinea-pig and rabbit showed high densities of binding sites. Significant differences of grain densities were observed between the two layers of smooth muscles, the longitudinal muscle of rat (Figure 6-3-XV) and guinea-pig portal veins (Figure 6-3-XVIII) had higher densities than the circular muscle, while the rabbit portal veins showed the opposite results (Figure 6-3-XVII).

6-4 Discussion

The importance of purinergic neurotransmission in the control of vascular tone is becoming increasingly evident from the results of numerous pharmacological and electrophysiological studies (see Burnstock, 1990b; Ralevic & Burnstock, 1991 for reviews). The results of both the radioligand binding assay and the autoradiography in this study demonstrate that many blood vessels from the rat, guinea-pig, and rabbit possess an abundance of P_{2X}-purinoceptors. The localization of the receptors is in agreement with results from pharmacological experiments, i.e., in vessels where potent P_{2X}-purinoceptor-mediated responses have been demonstrated, a dense specific labelling of [^{3}H]α,β-MeATP was observed (Burnstock, 1990b).

The rabbit central ear artery was chosen for the radioligand binding assay in this study because it is a vessel in which purinergic neurotransmission has been well studied (Kennedy & Burnstock, 1985; Saville & Burnstock, 1988). It has been suggested that both NA and ATP are co-stored in the perivascular sympathetic nerves, and are released upon nerve stimulation. These transmitters elicit vascular contraction via α-adrenoceptors and P_{2X}-purinoceptors, respectively. The results from both the radioligand binding assay and autoradiography confirmed that the ear artery has a high density of P_{2X}-purinoceptors with a high affinity for [^{3}H]α,β-MeATP. The high density of P_{2X}-purinoceptors in many vascular smooth muscles may be because released ATP is

rapidly degraded. The high content of the P_{2X}-purinoceptors should make it amenable to examine the changes in receptor densities and affinities in blood vessels in different physiological and pathological conditions by using radioligand binding assay.

All the autoradiographs showed that the grains, which represent specific [^{3}H]α,β-MeATP binding sites, were distributed over the smooth muscle cells, where pharmacological studies have proposed that P_{2X}-purinoceptors should be located. This is an important point because ATP and its binding proteins exist within every cell type, whereas P_{2X}-purinoceptors are only found extracellularly in relation to specific cell types.

Although ATP has been shown to be co-released with NA from rabbit aorta (Su, 1975) and P_{2X}-purinoceptor-mediated responses have been observed in rat aorta (White *et al.*, 1985), the density of [^{3}H]α,β-MeATP binding sites in rabbit aorta is low compared with that in muscular arteries. Medium-sized arteries such as the rat, guinea-pig, and rabbit saphenous and the rat tail arteries have denser binding sites than the large muscular arteries such as rabbit renal and carotid arteries. The receptor densities increased from the proximal segment of rabbit femoral artery to the distal segment, and from the rat femoral artery to the adjacent saphenous artery. All these observed phenomena may reflect differences in the relative importance of the purinergic control in these vessels. Few comparative studies have been performed on blood vessels using pharmacological techniques.

However, in some vessels great variations in the proportions of ATP and NA used as cotransmitters in sympathetic nerves have been observed (see Burnstock, 1988 for review). In rabbit small mesenteric arteries the purinergic component has been reported to account for 80% of the response to sympathetic nerve stimulation (Yamamoto *et al.*, 1988).

Another interesting phenomenon observed in this study is that in rabbit carotid, renal, hepatic, and large mesenteric arteries the densities of specific [^{3}H]α,β-MeATP binding sites in the outer region of the vascular wall were higher than those in the inner region. This may reflect the fact that the perivascular nerves are confined to the adventitial-medial border in the large arteries (see Burnstock, 1990b for review). The rapid degradation of ATP released from the sympathetic nerves prevents the diffusion of ATP to the inner region of the vascular wall. This is in accordance with the gradual reduction in density of P_{2X}-purinoceptors observed across the vascular wall.

Pharmacological and histochemical evidence shows that ATP is localised within different types of nerves in the portal vein in different species. In the rat portal vein, ATP and NA coexist in sympathetic neurons as shown by sympathectomy abolishing fluorescence staining due to quinacrine, which binds to high levels of ATP. In the rabbit portal vein, ATP is present in discrete purinergic nerves as well as existing as a cotransmitter with NA in adrenergic nerves, whereas in the guinea-pig portal vein only an inhibitory purinergic neurotransmission is observed

(Burnstock *et al.*, 1979; 1984). In this study it was found that the longitudinal muscle of guinea-pig and rat portal veins has much higher densities of P_{2X}-purinoceptor than does the circular muscle. P_{2X}-purinoceptor-mediated excitatory responses have been observed in longitudinal muscle of rat portal vein (Reilly & Burnstock, 1987). No comparable pharmacological results are available on the longitudinal muscle of guinea-pig portal vein. In rabbit portal vein the longitudinal muscle has a lower density of P_{2X}-purinoceptors, compared with the circular muscle. The results from pharmacological experiments indicate that both P_{2X}- and P_{2Y}-purinoceptors exist in the longitudinal muscle of the rabbit portal vein (Kennedy & Burnstock, 1985b).

With the exception of the portal vein, studies of purinergic neurotransmission in veins are scarce. One report shows that ATP can evoke contractile responses in canine cutaneous vein, which are augmented by cooling and abolished by α,β-methylene ATP (Flavhan & Vanhoutte, 1986). Very low densities of specific [^{3}H]α,β-MeATP binding sites were observed in rabbit mesenteric vein and inferior vena cava in this study, which may indicate that purinergic neurotransmission does not have much influence in the control of these veins. This also confirms that the distribution of P_{2X}-purinoceptor is tissue-dependent.

In conclusion, both radioligand binding assay and autoradiographic localization have demonstrated that P_{2X}-purinoceptors are present in many blood vessels from several

species. The distribution of the P_{2X}-purinoceptor is mostly consistent with the results from previous pharmacological experiments. It is also indicated that [^{3}H]α,β-MeATP may be used to examine the changes in P_{2X}-purinoceptors in blood vessels in physiological and pathological processes including ageing, hypertension, hypoxia, and migraine.

Figure 6-1 **A representative saturation curve of the [^{3}H]α,β-methylene ATP binding to the washed homogenate of rabbit central ear artery.**

Incubation was carried out at 30°C for 15 min. Non-specific binding was determined in the presence of 100 μM β,γ-methylene ATP.

Note: The last two points of the non-specific binding which were not shown in the figure fell on the line indicating the level of the non-specific binding.

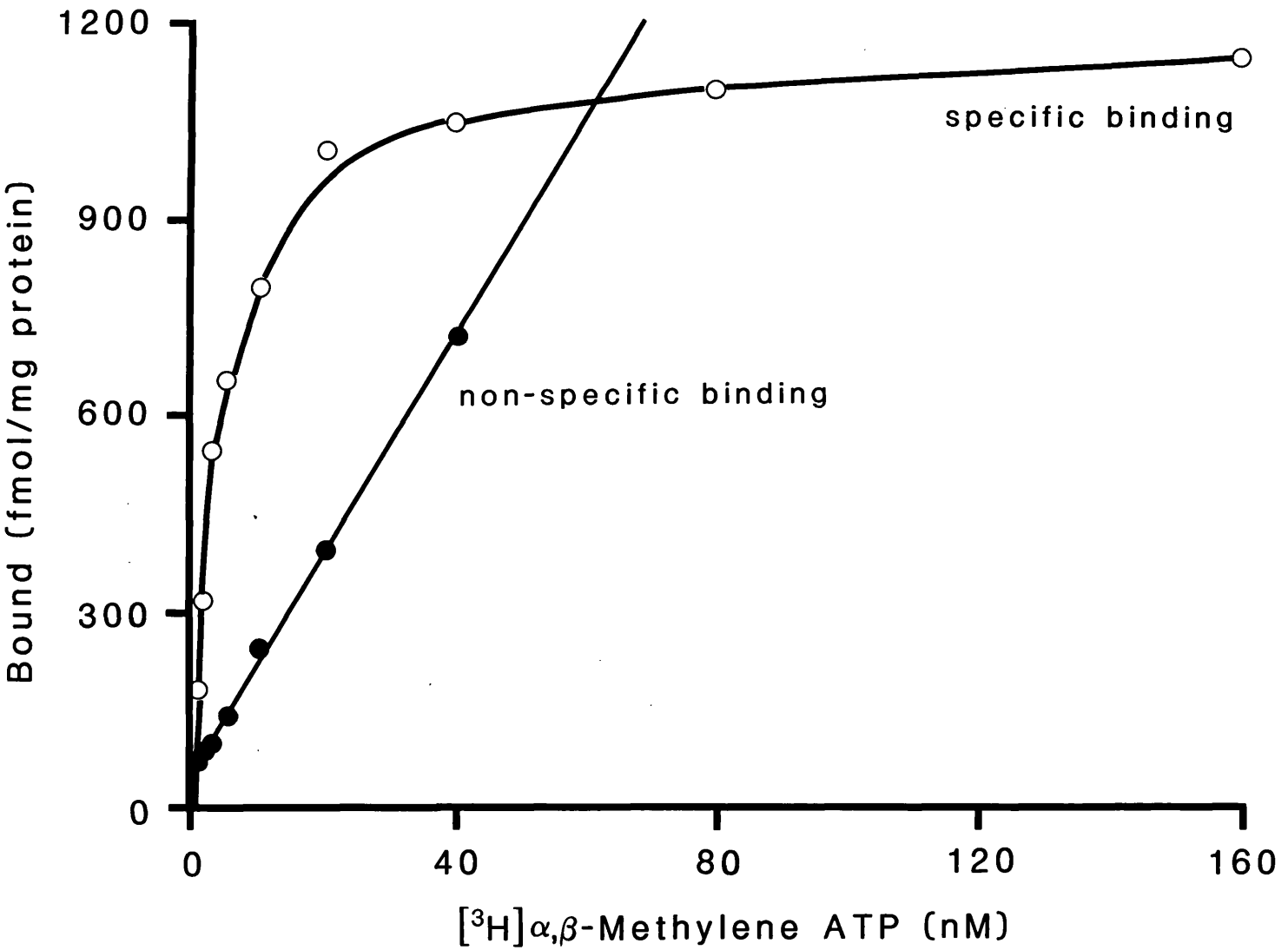

1200
900
600
300
0
Bound (fmol/mg protein)
specific binding
non-specific binding
0
40
80
120
160
[3H]α,β-Methylene ATP (nM)

Figure 6-2 **The Scatchard plot of the binding data in Figure 1.**

The curve-fitting was performed with the computer program EBDA-LIGAND. One binding site model was chosen based on the results of F-test of the weighted residual sum of squares.

Note: One point was rejected because it was too far away from the fitted line.

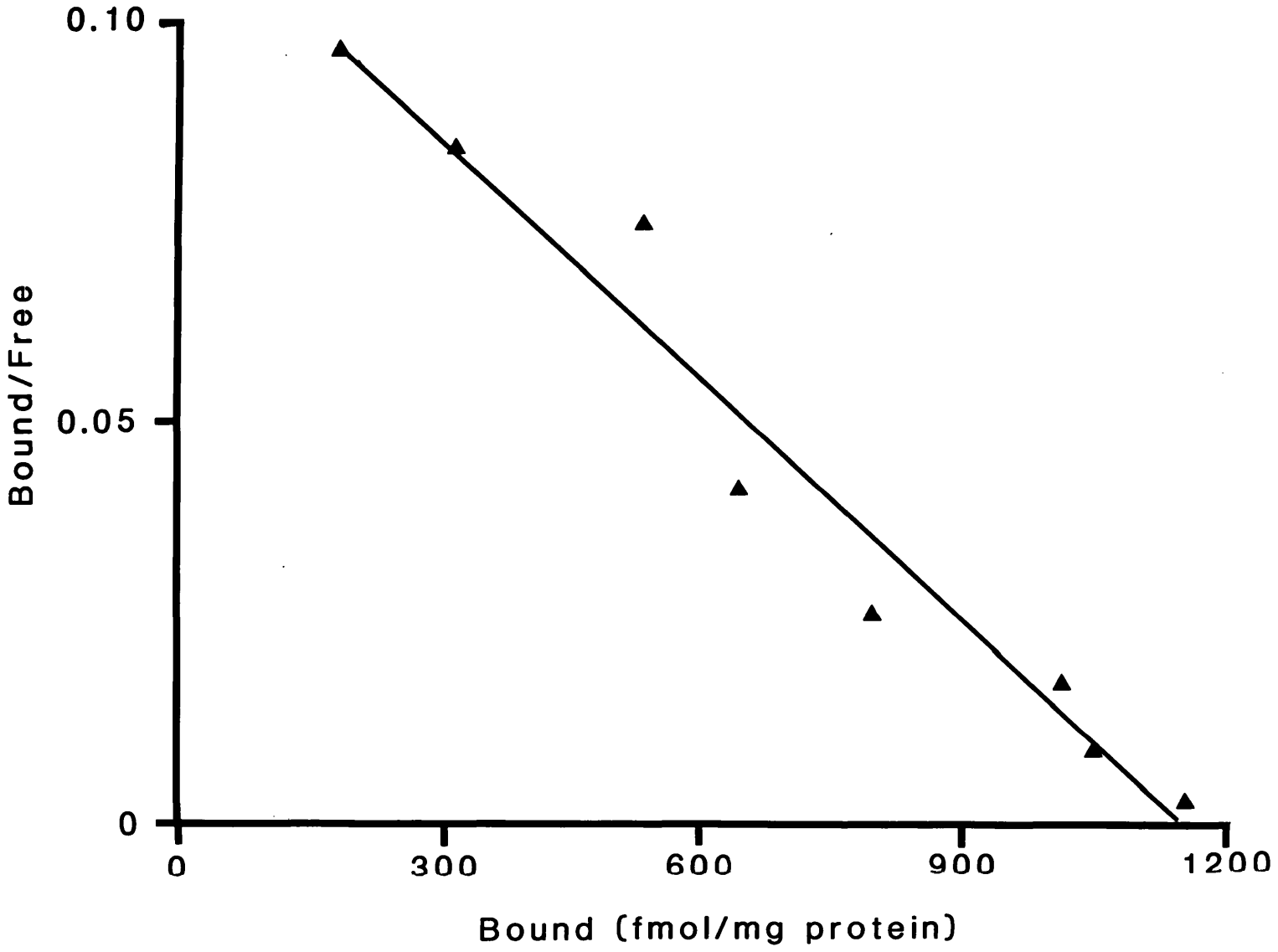
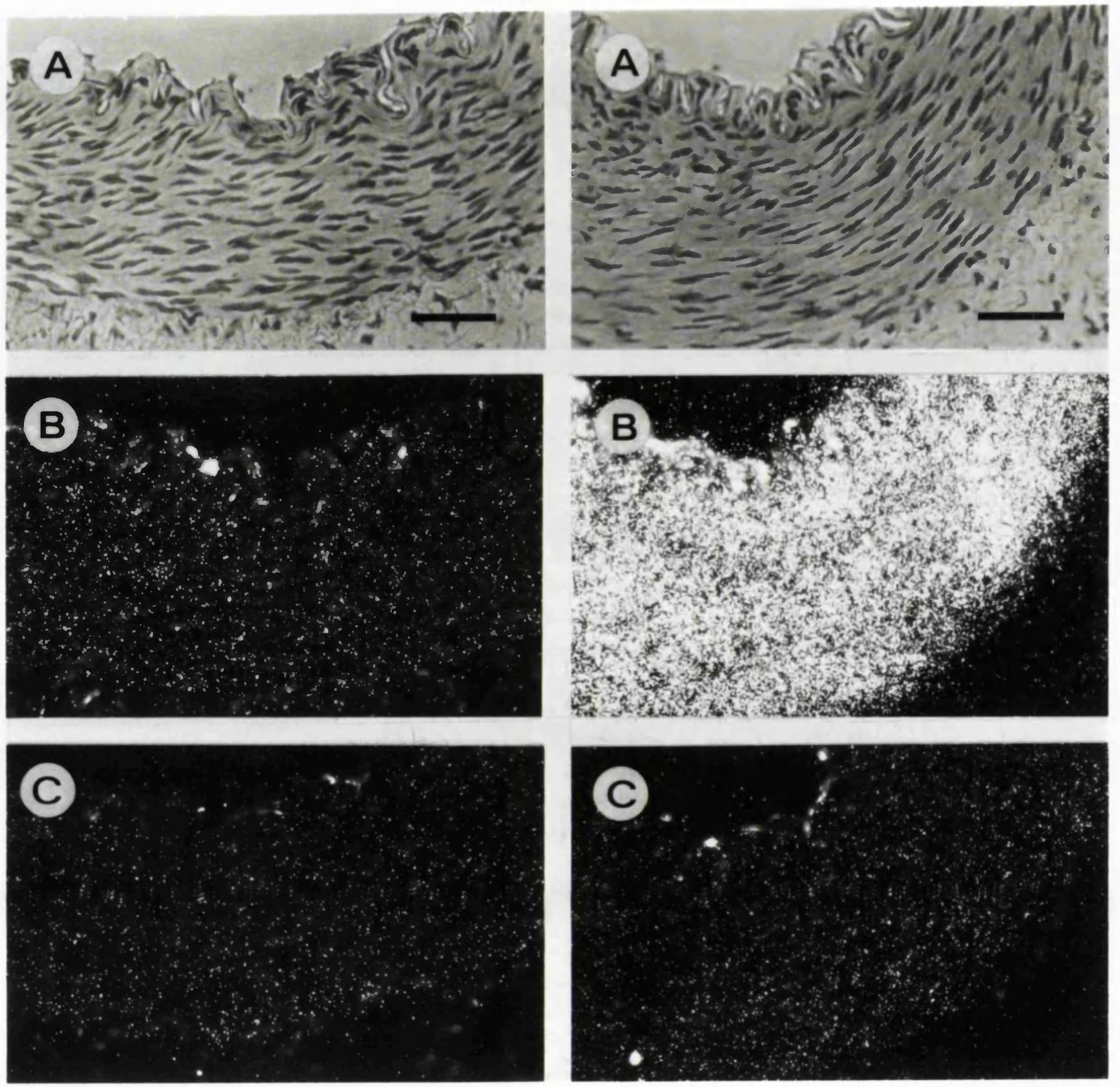

0.10
0.05
0
Bound/Free
0
300
600
900
1200
Bound (fmol/mg protein)

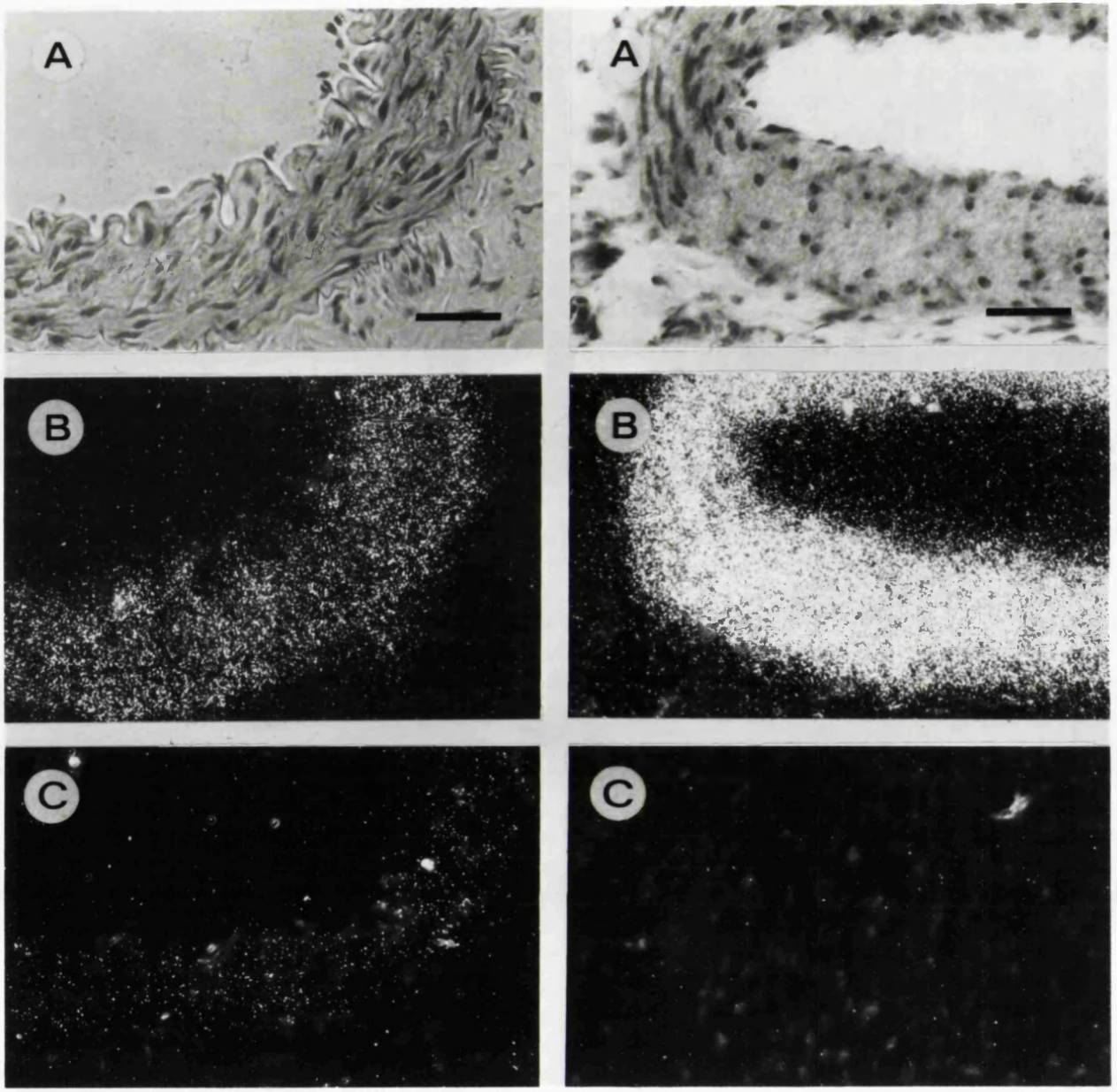
Figure 6-3 **Autoradiographs of blood vessels from rat, guinea-pig, and rabbit.**

A. Bright-field views of the vascular sections (15 μm thick, stained with 0.5% toluidine blue); B: Dark-field views of A, which show the overall distribution of [^{3}H]α,β-methylene ATP binding sites in the sections; C. Dark-field views of the adjacent sections of A, which show the non-specific binding sites of [^{3}H]α,β-methylene ATP in the sections. Ten nM [^{3}H]α,β-methylene ATP was used for the binding. Non-specific binding was determined in the presence of 100 μM β,γ-methylene ATP. Incubation was carried out at 30°C for 15 min. I, proximal segment of rabbit femoral artery; II, distal segment of rabbit femoral artery; III, rat femoral artery; IV, rat saphenous artery; V, rabbit saphenous artery; VI, guinea-pig saphenous artery; VII, rat mesenteric artery; VIII, guinea-pig mesenteric artery; IX, rabbit mesenteric artery; X, rabbit hepatic artery; XI, rabbit central ear artery; XII, rat tail artery; XIII, rabbit renal artery; XIV, rabbit carotid artery; XV, rat portal vein; XVI, rabbit mesenteric vein; XVII, rabbit portal vein; XVIII, guinea-pig portal vein; XIX, rabbit aorta; XX, rabbit inferior vena cava; XXI, rabbit coeliac artery. Calibration bars: 50 μm.



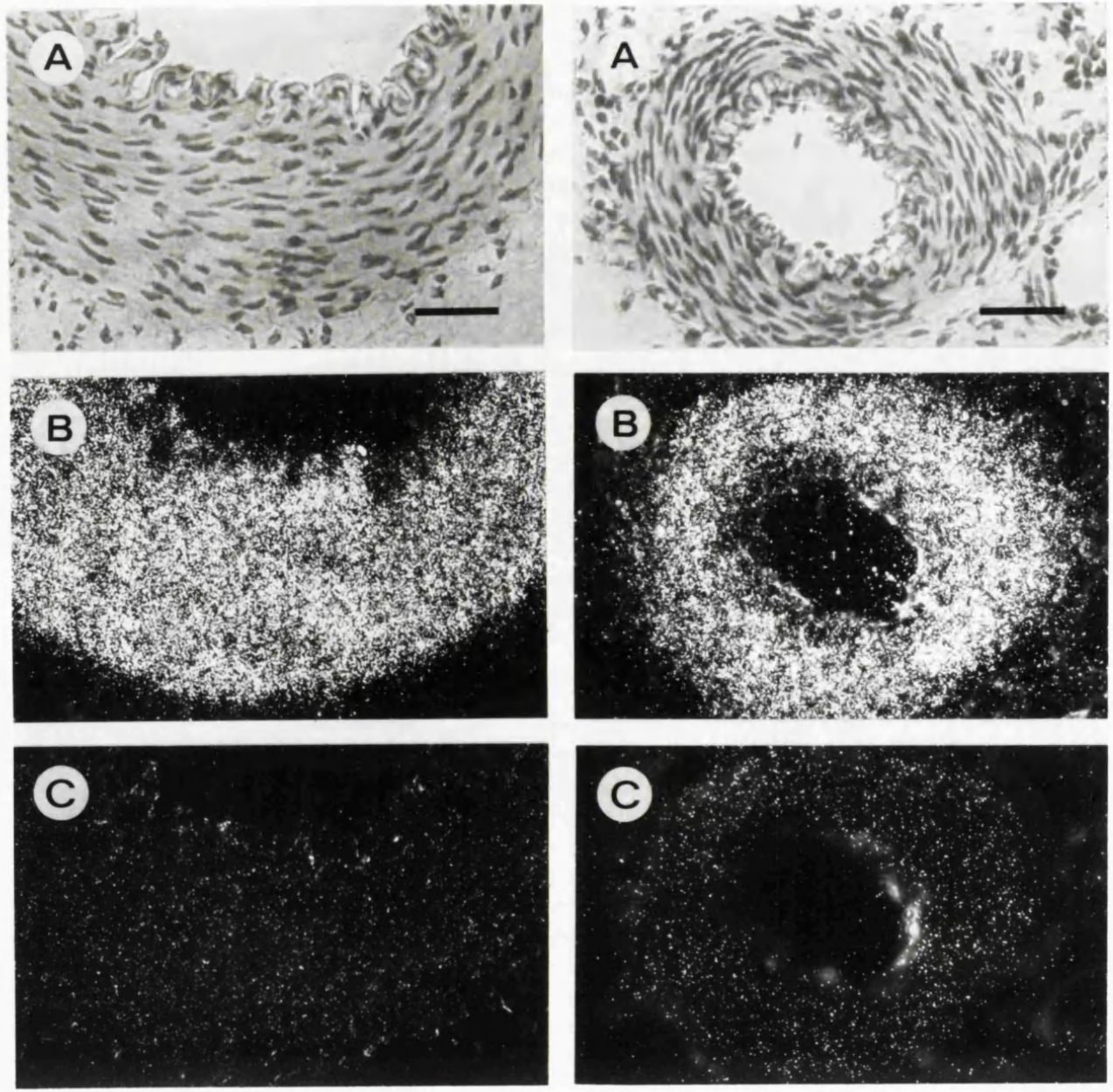
I Proximal segment II Distal segment

Rabbit femoral artery

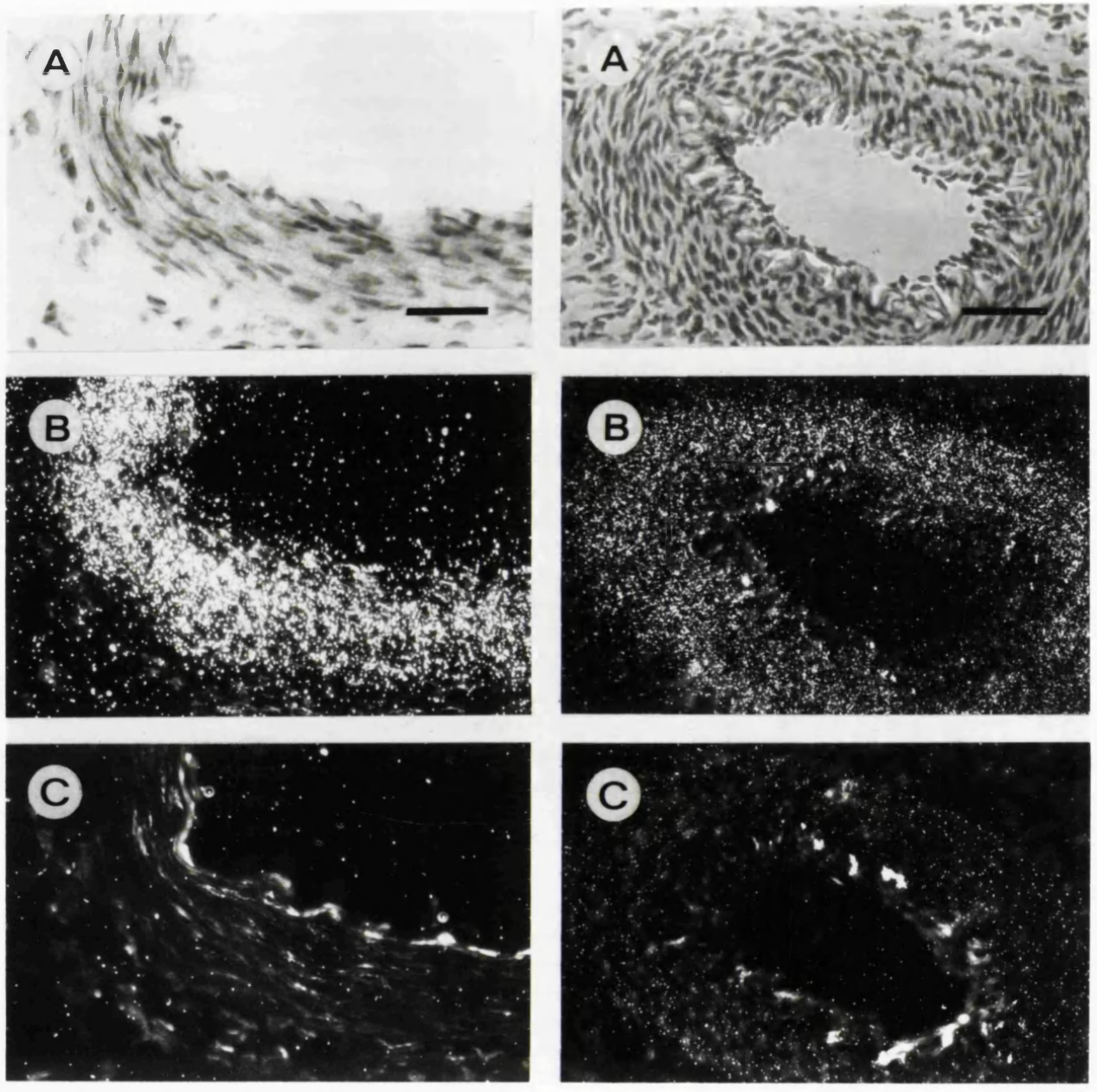


III Rat femoral artery

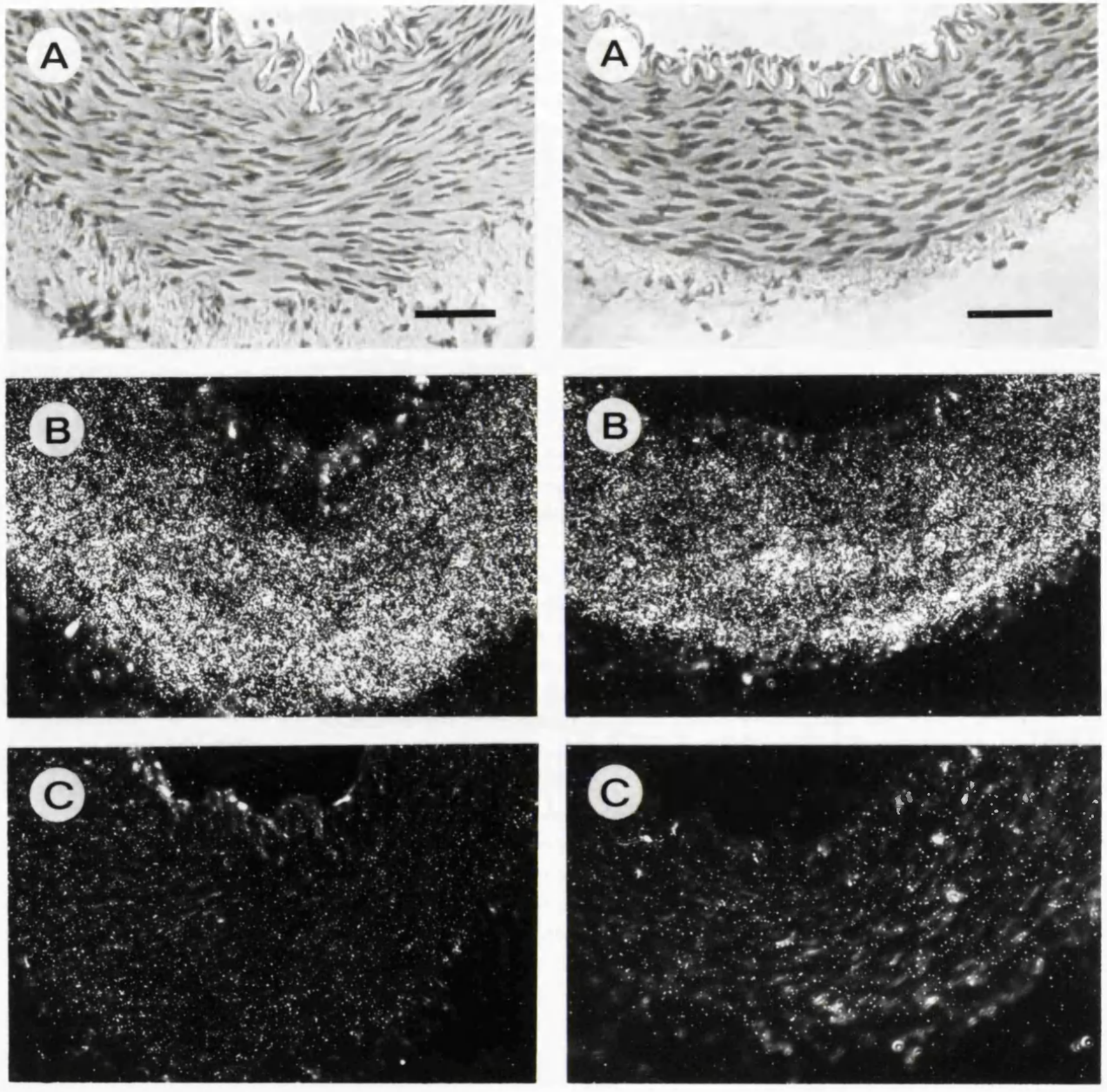
IV Rat saphenous artery



V Rabbit saphenous artery VI Guinea-pig saphenous artery

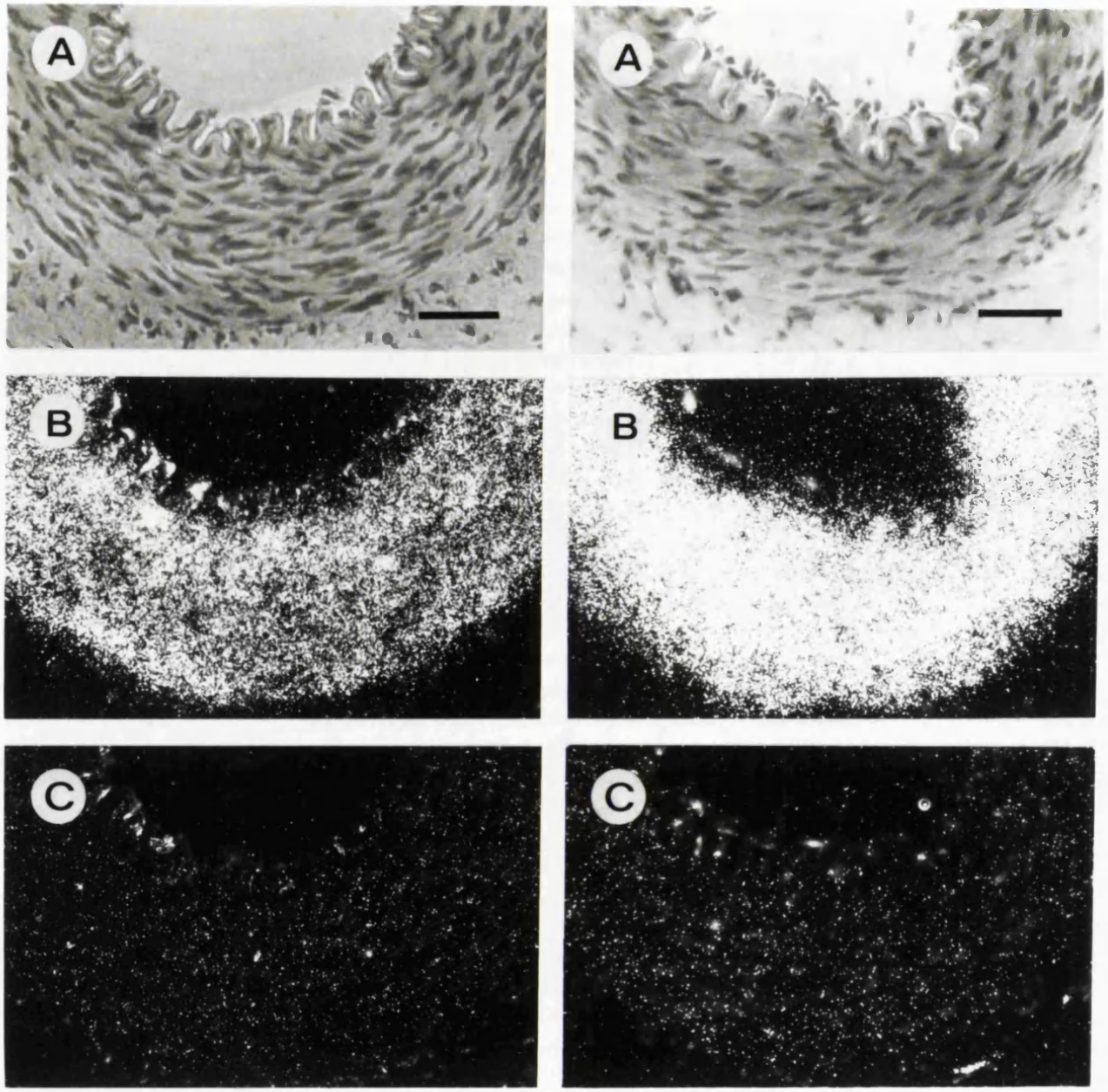


VII Rat mesenteric artery VIII Guinea-pig mesenteric artery



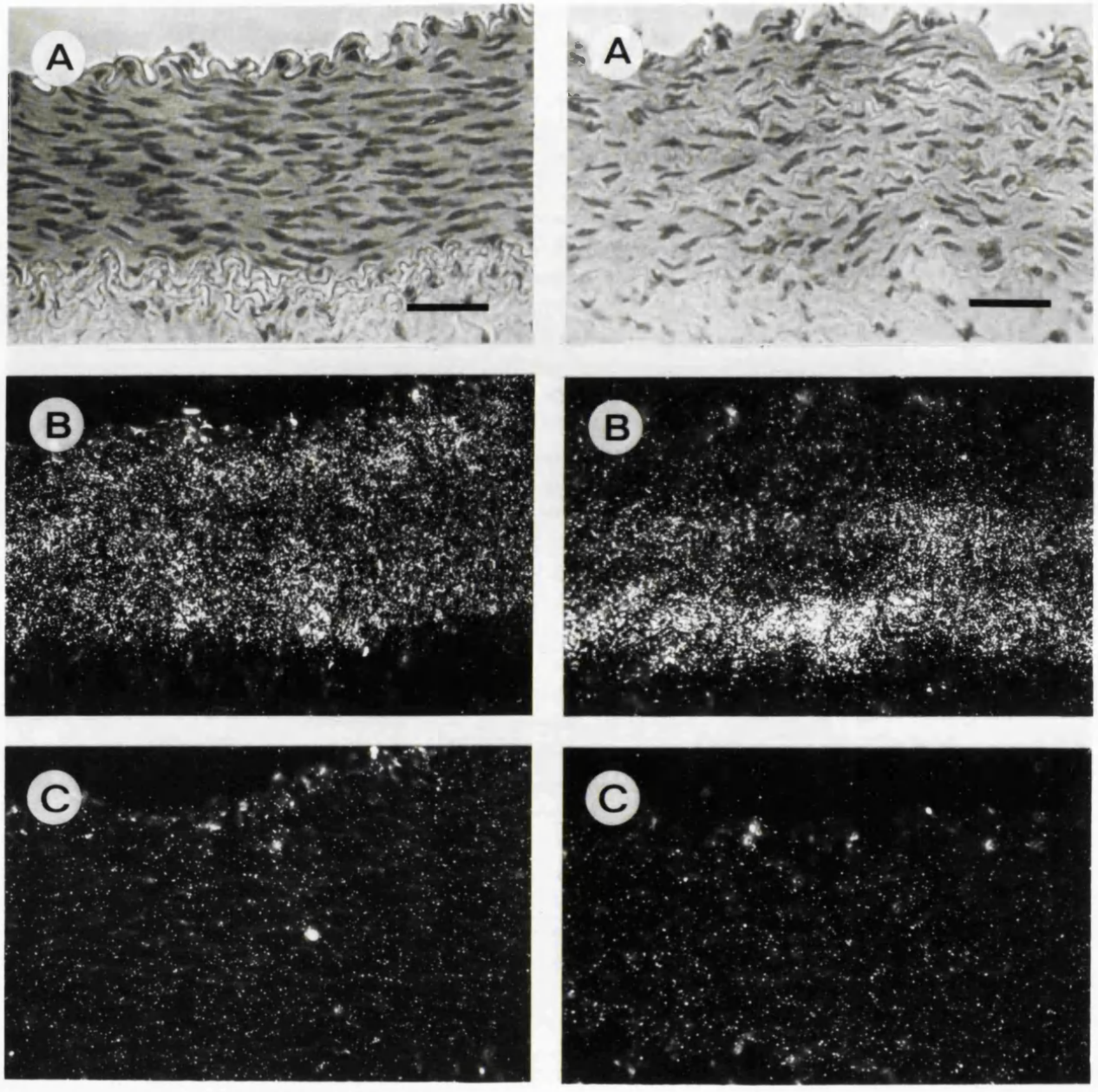
IX Rabbit mesenteric artery

X Rabbit hepatic artery



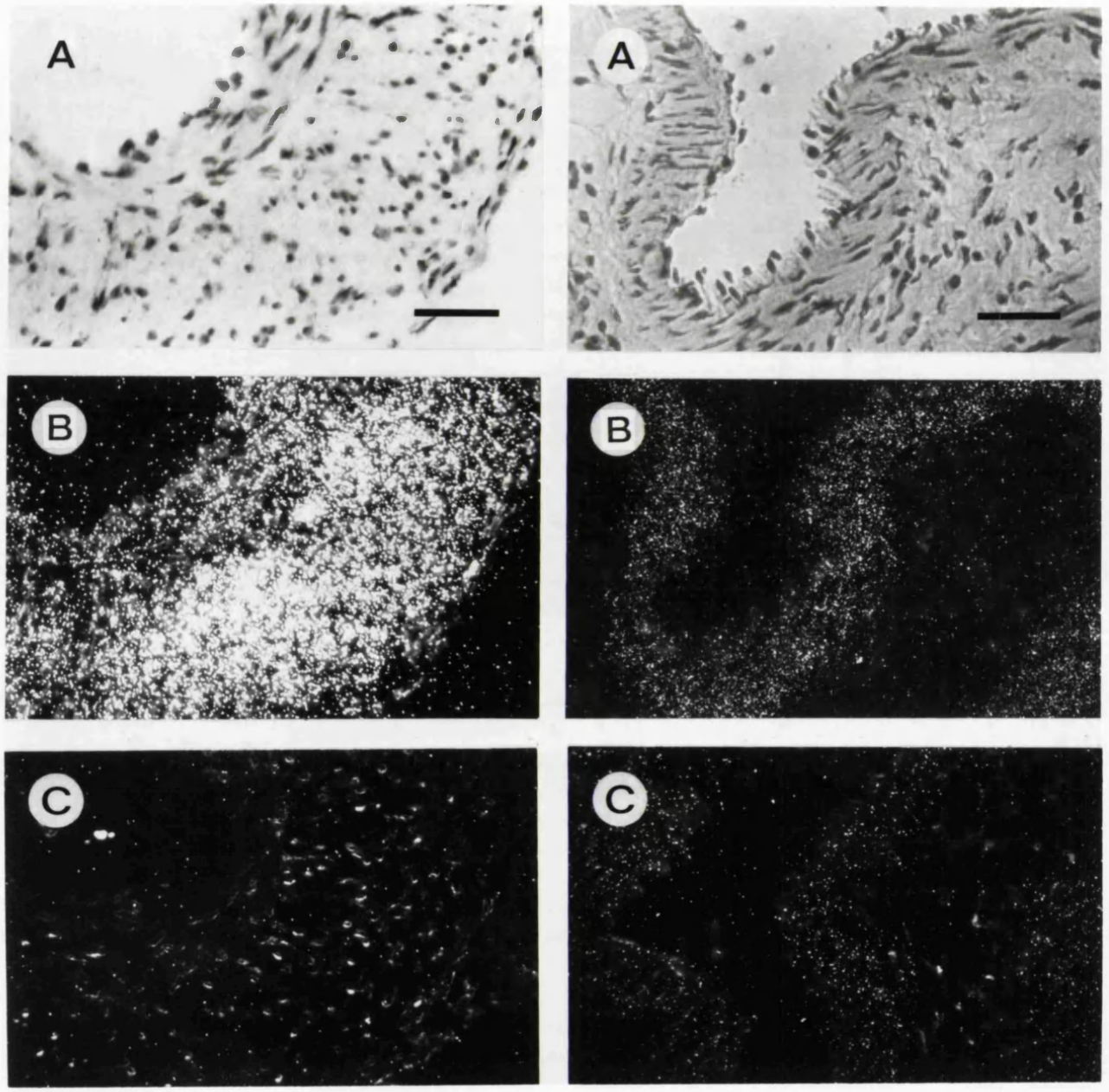
XI Rabbit ear artery

XII Rat tail artery



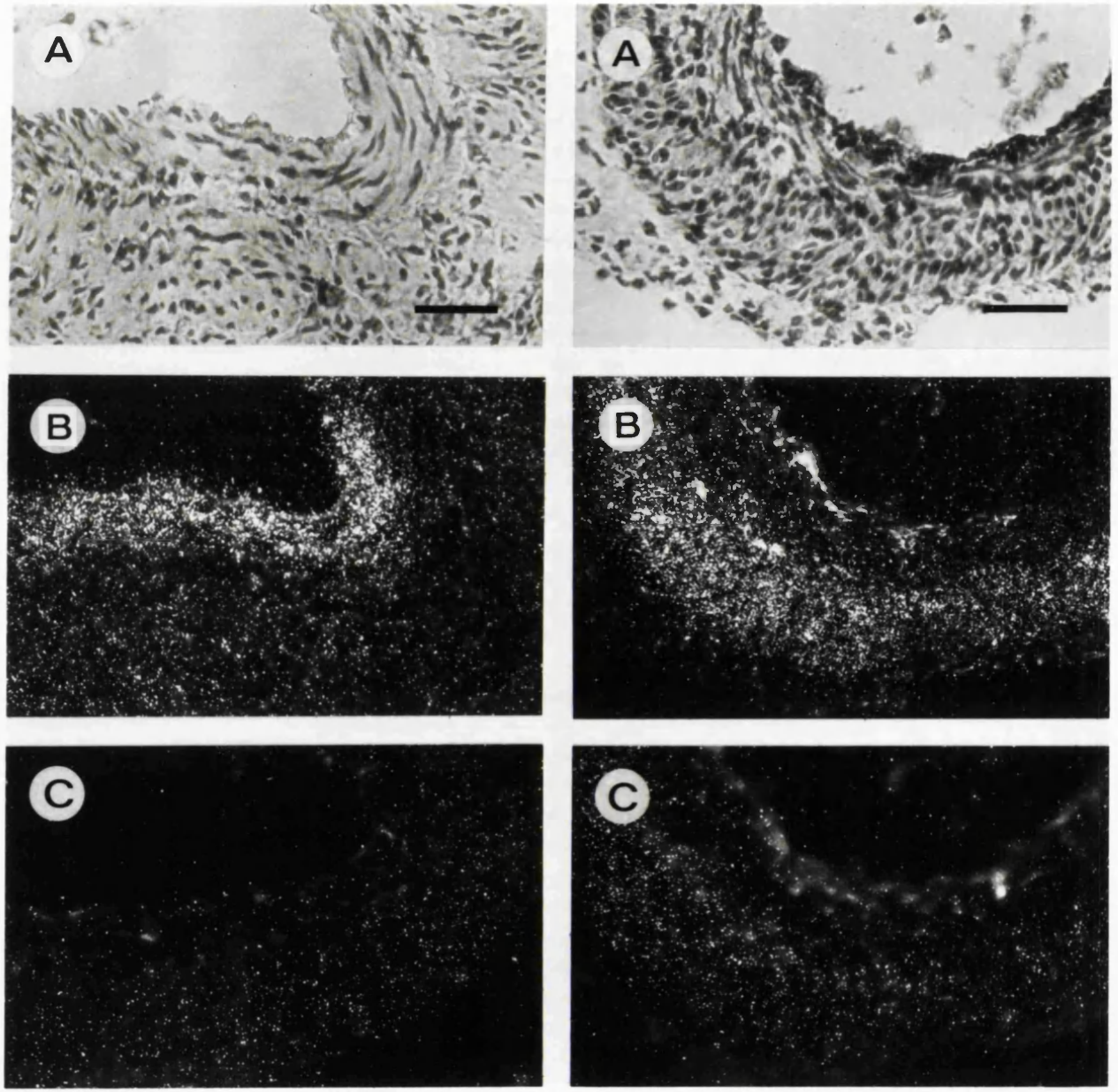
XIII Rabbit renal artery

XIV Rabbit carotid artery



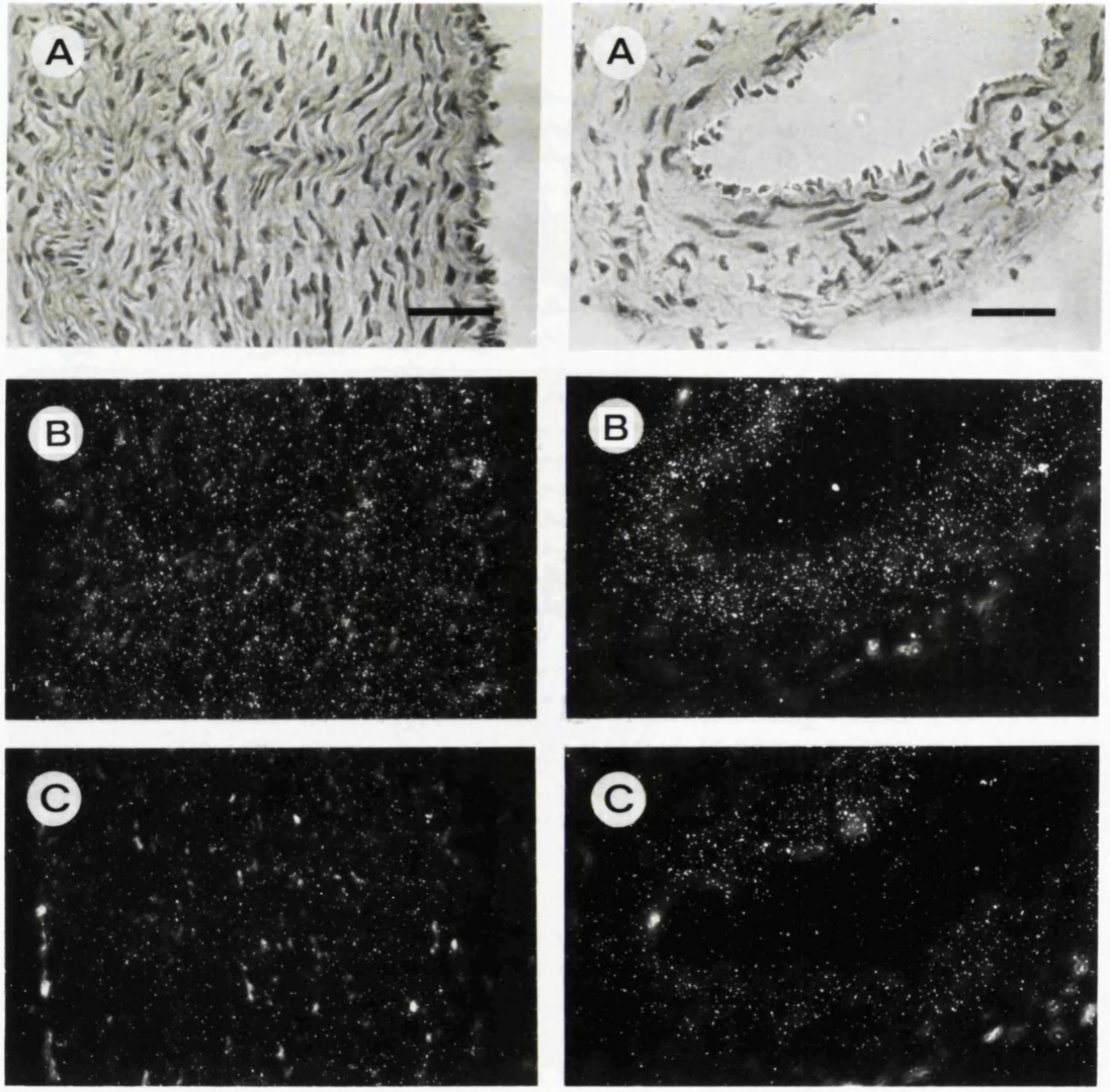
XV rat portal vein

XVI Rabbit mesenteric vein



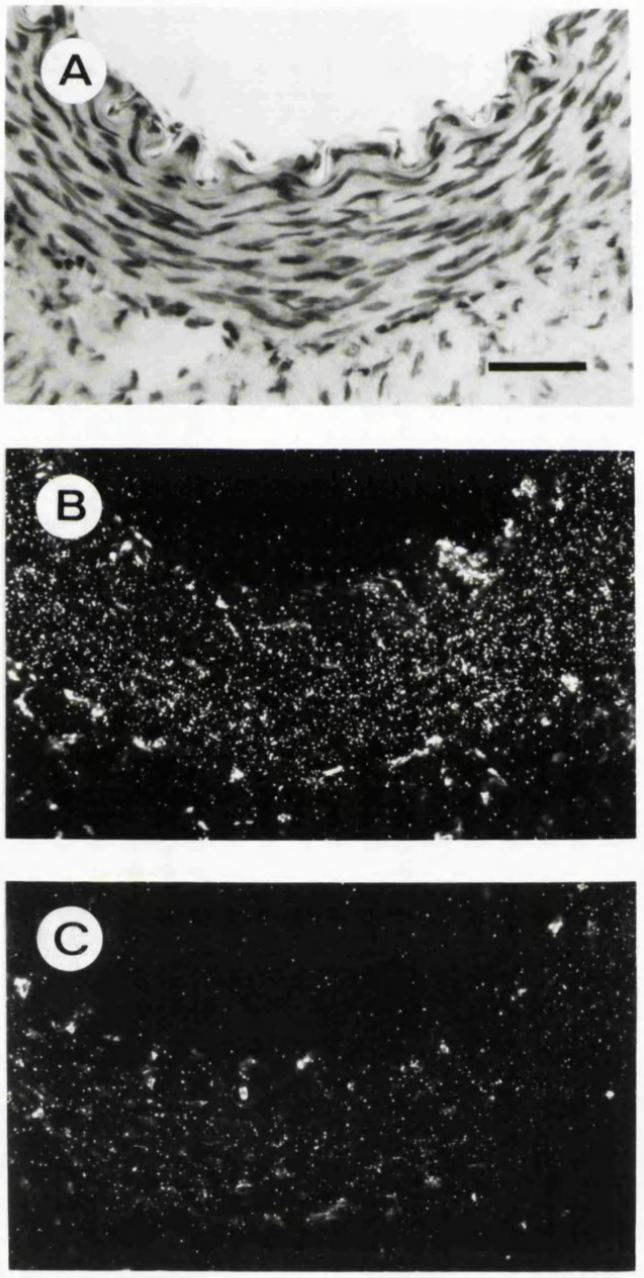
XVII Rabbit portal vein

XVIII Guinea-pig portal vein



XIX Rabbit aorta

XX Rabbit inferior vena cava



XXI rabbit coeliac artery

Table 6-1 **The relative densities of specific [^{3}H]α,β-methylene ATP binding sites in blood vessels from rat, guinea-pig, and rabbit.**

The grains were counted with an image-analysing system and expressed as grains per 1000 μm^2.

The results shown below are from three experiments and each experiment was carried out in triplicate (n=9).

Tissues	Animals	Specific binding sites (mean ± S.E.M)
Aorta	Rabbit	58 ± 4
Coeliac artery	Rabbit	63 ± 7
Carotid artery	Rabbit	
Outer region		289 ± 23***
Inner region		95 ± 13
Renal artery	Rabbit	
Outer region		388 ± 27***
Inner region		129 ± 17
Hepatic artery	Rabbit	
Outer region		377 ± 29***
Inner region		201 ± 19
Femoral artery	Rabbit	
Proximal segment		54 ± 7***
Distal segment		565 ± 31

(continue Table 1)

Tissues	Animals	Specific binding sites (mean ± S.E.M)
Saphenous artery	Rabbit	608 ± 4
	Guinea-pig	373 ± 32
	Rat	629 ± 55
Mesenteric artery	Rabbit	
Outer region		425 ± 47***
Inner region		245 ± 21
	Guinea-pig	271 ± 17
	Rat	226 ± 19
Central ear artery	Rabbit	529 ± 47
Tail artery	Rat	671 ± 51
Mesenteric vein	Rabbit	134 ± 13
Portal vein	Rabbit	
Longitudinal		180 ± 13***
Circular		402 ± 25
	Guinea-pig	
Longitudinal		262 ± 32***
Circular		99 ± 12
	Rat	
Longitudinal		402 ± 29***
Circular		180 ± 27
Inferior vena cava	Rabbit	91 ± 12

*** $P < 0.001$, significant difference in P_{2X}-purinoceptor densities between the outer and inner regions of rabbit carotid, heaptic, renal, and mesenteric arteries, between the proximal and distal segments of rabbit femoral artery, and between the longitudinal and circular muscle of rat, guinea-pig and rabbit portal veins.

Chapter 7: Solubilization and Molecular Size Determination of P_{2X}-Purinoceptors from Rat Vas Deferens

Summary

1. Membranes of the rat vas deferens were shown to contain a high density of [^{3}H]α,β-methylene ATP ([^{3}H]α,β-MeATP) binding sites. Analysis demonstrated two classes of high (K_d: 1.8 nM, B_{max}: 9.33 pmol/mg protein) and low (K_d: 33.7 nM, B_{max}: 29 pmol/mg protein) affinity. The rank order of potency of purinergic ligands in displacing [^{3}H]α,β-MeATP binding was in accordance with the pharmacological criteria for P_{2X}-purinoceptors.

2. The high affinity [^{3}H]α,β-MeATP binding sites were successfully solubilized with 2% digitonin, K_d was then 1.6 nM. Both the association and dissociation of the receptor-ligand complex were very rapid ($t_{½}$ = 6.5 min for association). The potency order of purinergic ligands in displacing the [^{3}H]α,β-MeATP binding to the solubilized preparation was similar to that for the high affinity sites in membranes.

3. The receptor-detergent complex was separated from ATPase enzymes, also present in the preparation, by sucrose gradient ultracentrifugation. The sedimentation coefficient of the receptor-detergent complex was 12.1 S.

4. It was shown that [^{3}H]α,β-MeATP can function as a photoaffinity labelling reagent upon exposure to ultraviolet light: it became cross-linked in a specific manner (in the rat vas deferens membrane) to a polypeptide with an apparent molecular weight of 62,000 daltons, proposed to be the ligand binding subunit of the functional P_{2X}-purinoceptor.

7-1 Introduction

As has been reviewed in Chapter 1, there is a massive pharmacological literature describing ATP induced functional responses. The signal transduction pathways activated by P_2-purinoceptors are also being studied (see Dubyak, 1991 for review). P_2-purinoceptors other than the P_{2X}-subtype are mainly linked to second messenger production and appear to be of the G-protein linked class (Okajima *et al.*, 1987; Morris *et al.*, 1990; Harden *et al.*, 1990; Jeffs *et al.*, 1991). The P_{2X} subtype, however, is distinct and has the properties of a transmitter-gated cation channel (Benham & Tsien, 1987). It would, therefore, constitute a novel member of the series of such receptor channels, which comprises of the nicotinic acetylcholine, $GABA_A$, glycine, $5\text{-}HT_3$ and ionotrophic glutamate receptors. Each of these contains two or more types of subunit of similar size in the receptor/channel oligomeric molecule (reviewed by Barnard, 1992). It would be of the greatest interest to see on what structural principles the P_{2X}-purinoceptor/channel is built.

Molecular analysis of the P_2-purinoceptor has been delayed by a lack of specific labelling tools. In the case of the P_{2Y}-purinoceptor, a photoaffinity label, [^{35}S]3'-O-(4-benzoyl)benzoyl ATP ([^{35}S]BzATP), has recently been synthesized and produced a specific incorporation of ^{35}S into a protein with an approximate molecular weight of 53,000 daltons in turkey erythrocyte membranes, which contain P_{2Y}- but not P_{2X}-purinoceptors (Boyer *et*

al., 1990). However, in a further study in the same system, an M_r of ≈70,000 was found (Jeffs *et al.*, 1991) and evidence was presented that this is present in a P_{2Y}-purinoceptor/G protein complex. An attempt to photoaffinity-label P_{2X}-purinoceptors has been made on guinea-pig vas deferens using [^{3}H]arylazido aminopropionyl ATP ([^{3}H]ANAPP$_3$) as the photoaffinity ligand. It was found that photolysis of [^{3}H]ANAPP$_3$ was incorporated into cellular components with apparent molecular weights of 54-66 and 43-57 kilodaltons (Fedan *et al.*, 1985).

Most neurotransmitter receptors discovered so far are membrane-bound proteins, therefore, in order to study further the molecular properties of the receptors, the first step is to solubilize the receptors from the membranes. In the present study, [^{3}H]α,β-MeATP is applied to a study of the P_{2X}-purinoceptors at molecular level, and evidence for successful solubilization and some biochemical characterization of P_{2X}-purinoceptors is provided.

The rat vas deferens was chosen as a receptor source in view of much physiological and pharmacological evidence that ATP is a co-transmitter in the sympathetic nerve ending and that only the P_{2X}-subclass can be detected in this tissue (see Hoyle & Burnstock, 1991 for review).

7-2 Materials and Methods

7-2-1 Preparation of membrane fraction

Male Wistar rats (230-250 g) were killed by asphyxiation with CO_2 and the vasa deferentia were removed immediately. They were immersed in cold Krebs solution, trimmed free from adventitia, minced, and homogenized in 10 ml 50 mM Tris/HCl buffer containing the following protease inhibitors: 1 mM EGTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01% bacitracin, and 0.002% soybean trypsin inhibitors (pH 7.4, buffer B) with a Polytron PT-3000 at 30,000 rpm for two 15 sec bursts. The homogenate was passed through double layers of nylon mesh and centrifuged at 620 *g* for 5 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 105,000 *g* for 50 min at 4°C. The ultracentrifugal pellets were suspended in buffer B to give a protein concentration of 5-6 mg/ml, kept on ice and used immediately.

7-2-2 Choice of detergents

Because neurotransmitter receptors are membrane-bound proteins, at least a portion of the receptor molecule is hydrophobic, thus, detergents are used for the solubilization of the receptors. Many types of detergents are available; it needs to be decided which type is the most appropriate for a particular receptor. The concentration of the detergents used and the influence of

detergents on the receptor binding also need to be determined.

In this study the effects of deoxycholate, digitonin, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) on the binding of [^{3}H]α,β-MeATP to rat vas deferens membranes were examined. Membrane preparations were incubated with 10 nM [^{3}H]α,β-MeATP in the presence of one of the detergents (0.1 and 0.05% deoxycholate and digitonin, 1 and 0.5 mM CHAPS) for 2 hrs at 4°C. Non-specific binding was determined by including 100 μM β,γ-MeATP in the incubation media. Reaction was terminated by vacuum filtration of the mixture through Whatman GF/B filters which had been soaked in 0.3% polyethyleneimine and 200 mM pyrophosphate.

The procedure to determine the efficiency of the detergents is described in 7-2-3.

7-2-3 Receptor solubilization

The membrane preparations of vas deferens were mixed with 10% (w/v) digitonin (room temperature) to a final detergent concentration of 2% and a detergent/protein ratio of 3:1. (Other detergents, at the stated concentration, were substituted in the noted case). After rotation (1 h) at room temperature, the suspension was centrifuged at 105,000 *g* for 1 h. The supernatant was the solubilized receptor preparation (2-3 mg protein/ml). Where noted, the ultracentrifugal pellets were also resuspended by vortexing in buffer B and kept on ice. The solubilized receptor

preparation was used immediately, because on storage at either 0°C or -25°C it showed a substantial loss of its specific binding.

7-2-4 Protein determination

Protein content was determined with a modified method of Lowry *et al.* which is routinely used in the MRC Molecular Neurobiology Unit (Cambridge, U.K.) (Peterson, 1977; 1979). Samples were mixed with an equal volume of 2% deoxycholate and the proteins were precipitated by adding two volumes of 24% trichloroacetic acid (TCA). The samples were left on ice for 20 min, and then centrifuged at 6000 rpm in an MSE desktop centrifuge for 10 min. The supernatant was discarded, 1 ml acetone was added and vortexed. They were centrifuged as above. The supernatant was discarded and the pellets were dried under vacuum. The pellets were dissolved in Solution 1 (2% Na_2CO_3, 0.4 NaOH, and 1% sodium dodecyl sulphate (SDS)). An aliquot of 200 µl was taken out and 800 µl of Reagent A (50 parts of Solution 1 with 0.16% sodium tartrate mixed with 1 portion of 4% $CuSO_4 \cdot 5H_2O$) was added. The reaction was carried out at room temperature for 30 min, then 60 µl Folin reagent (diluted 1:1 with ddH_2O) was added. Forty-five min later the optical density were measured at the wavelength of 750 nm. Bovine serum albumin was used as standard.

The marker proteins in the fractions collected from sucrose density gradient centrifugation were determined with the method of Bradford (1976).

7-2-5 Radioligand binding assays

Aliquots of membrane fraction containing 15-20 µg protein in buffer B were incubated with [^{3}H]α,β-MeATP (final concentration, 10 nM; 0.625 to 160 nM in saturation experiments) in the absence or presence of 100 µM unlabelled β,γ-MeATP for 2 hrs at 4°C (final volume 0.5 ml). The samples were vacuum-filtered through Whatman GF/B glass fibre filters presoaked in 20 mM disodium pyrophosphate and the filters were immediately washed twice with 5 ml ice-cold 50 mM buffer A.

For soluble receptor binding, the extract was diluted at least 20 times with buffer B in order to reduce the digitonin concentration below 0.1%. An aliquot containing 5-15 µg of solubilized protein was incubated with [^{3}H]α,β-MeATP as noted above (final volume of 1 ml). The reaction was terminated by total protein precipitation with polyethylene glycol (PEG, M.W. 8000) in the presence of 0.06% bovine γ-globulins (Simon *et al.*, 1984), and then followed by vacuum-filtration through Whatman GF/B filters which had been treated with 0.3% polyethyleneimine (Bruns *et al.*, 1983) and 200 mM tetrasodium pyrophosphate. The filters were washed twice with 5 ml of ice-cold buffer A containing 200 mM NaCl and 7% PEG.

In both cases the filters were dried under an infra-red lamp and their radioactivity was counted in OptiPhase 'HiSafe' II (LKB) scintillation cocktail in a Beckman LS 5000CE scintillation

spectrometer with an efficiency of about 56%.

7-2-6 Sucrose density gradient centrifugation

Linear sucrose gradients (10% to 30%) were prepared in buffer B, but omitting bacitracin and soybean trypsin inhibitors and including digitonin to 0.05% (buffer C). A 200 µl-aliquot of the solubilized receptor was layered on the top of each gradient and centrifuged at 200,000 *g* for 16 hr at 4°C in a Beckman SW40.1 rotor. Following the centrifugation, 23 drops per fraction (approx. 0.3-0.35 ml) were collected. In parallel experiments, a mixture of marker proteins (thyroglobulin, 19.3S, ferritin, 15.4S, catalase, 11.3S, aldolase, 6.9S, and haemoglobin, 4.2S) was also applied, and a linear calibration plot was obtained, with other details as in Mamalaki *et al.*(1989).

7-2-7 Determination of ATPase enzymic activity

The assay incubation was 100 mM Tris/HCl (pH 7.4) buffer containing 3 mM $MgCl_2$, 3 mM $CaCl_2$, 50 mM NaCl and 50 mM KCl. Aliquots of either the membrane suspension (vortexed), or of the digitonin solubilized preparation, or fractions from the sucrose gradient, in each case containing 5-20 µg of protein, were preincubated in the reaction mixture for 15 min at 37°C (final volume 1.0 ml). The reaction was initiated by the addition of 3 mM ATP (vanadium-free, Sigma). After 10 min at 37°C, the reaction was terminated by adding 100 µl of 50% TCA to the samples. Inorganic phosphate (Pi) released by the enzyme reaction was measured

according to the method of Baginski *et al.* (1967).

7-2-8 UV mediated cross-linking of [^{3}H]α,β-MeATP and SDS/polyacrylamide gel electrophoresis

Aliquots of membrane preparation (approx. 100 µg protein) were equilibrated with 50 nM [^{3}H]α,β-MeATP in buffer B (saturated with N_2) containing 1 mM reduced glutathione in the absence or presence of 1 mM β,γ-MeATP for 1 hr on ice in siliconized glass vials. UV irradiation was then carried out by direct exposure to ultraviolet light (254 nm) at a distance of 8 cm for 15 min using CAMAG Universal UV lamp (Switzerland). During the UV illumination the reaction mixtures were kept at 4°C in a flat dish and continuously magnetically stirred. Control experiments without the ligand showed that there was no destruction of receptor sites by the irradiation. Finally the samples were precipitated by chloroform/methanol (Wessel and Flüge, 1984). Each pellet was solubilized in 15 µl of SDS sample buffer at 80°C, containing 62.5 mM Tris/HCl (pH 6.8), 6% SDS, 8 M urea, and 100 mM dithiothreitol (DTT). The samples were boiled for 3 min and subjected to electrophoresis through 10% polyacrylamide gel at 200V, essentially as described by Laemmli (1970). Parallel lanes contained a mixture of prestained standard proteins (Bio-Rad: phosphorylase b, 106,000 Da; bovine serum albumin, 80,000 Da; ovalbumin, 49,500 Da, carbonic anhydrase, 32,500 Da; soybean trypsin inhibitor, 27,500 Da, and lysozyme, 18,500 Da). Subsequently the gels were sliced (1 mm) and solubilized with 400

µl H_2O_2, overnight at 75°C in tightly capped vials. Finally, 500 µl Of 1% SDS solution containing 4 M urea was also added, followed by 14 ml OptiPhase 'HiSafe' II cocktail and counting as above.

7-2-9 Chemicals

[^{3}H]α,β-MeATP was custom-synthesised by Amersham International (Amersham, U.K.) with a specific activity of 19.2 Ci/mmol and chemical purity of 98 - 99%. α,β-Methylene ATP (α,β-MeATP), β,γ-methylene ATP (β,γ-MeATP), ATP, ADP, adenosine, digitonin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and sodium deoxychalate were from Sigma Chemicals (Poole, U.K.). 2-Methylthio ATP (2-MeSATP) was from Research Biochemical Inc. (Natick, U.S.A.). L-β,γ-Methylene ATP (L-β,γ-MeATP) was a gift from Fisons Pharmaceuticals (Loughborough, U.K.). Suramin was a gift from Bayer Pharmaceuticals, (West Sussex, U.K.). The calibration kit containing standard proteins for sucrose gradient ultracentrifugation was from Pharmacia, (Central Milton Keynes, U.K.). Prestained low molecular weight standards for SDS polyacrylamide gel electrophoresis, acrylamide and N,N'-methylene bisacrylamide were purchased from Bio-Rad (Hemel Hempstead, U.K.). All other chemicals were from BDH (Poole, U.K.).

7-2-10 Data analysis

See Chapter 2.

7-3 Results

7-3-1 [^{3}H]α,β-MeATP binding to the rat vas deferens membranes

The binding of [^{3}H]α,β-MeATP to rat vas deferens membranes was saturable (Figure 7-1A). The data were best fitted (see Methods) to two binding sites, the heterogeneity being clearly illustrated in the Scatchard plot (Figure 7-1B). The analysis gave a K_d value of 1.8 ± 0.5 nM for the high affinity sites and a K_d value of 33.7 ± 10.0 nM for the more numerous, low affinity binding sites (Table 7-1). The calculated Hill coefficient (n_H) for each site was very close to 1 (Table 7-1), suggesting the non-cooperative nature of the binding process.

[^{3}H]α,β-MeATP binding to the membrane could be displaced by a range of compounds active pharmacologically on purinoceptors (Figure 7-2). Computer fitting of the data revealed that this displacement was biphasic with all but one of the P_2-purinergic ligands tested, again denoting the existence of two binding sites. The one exception seen was with the highest-affinity displacer, α,β-MeATP, where binding at any second site was too weak to measure in the conditions used. The computed Ki values for the ligands are listed in Table 7-2. The rank order of potency of the purinergic ligands tested, in displacing [^{3}H]α,β-MeATP binding from vas deferens membranes, was α,β-MeATP > β,γ-MeATP > L-β,γ-MeATP > 2-MeSATP > ATP > Suramin > ADP for the high-affinity binding sites. The rank order was different for the low affinity sites, being there β,γ-MeATP > ATP > ADP > 2-MeSATP > L-β,γ-MeATP

= Suramin > α,β-MeATP. Adenosine was of very low potency, only about 13% of specific binding of [^{3}H]α,β-MeATP being displaced at the concentration of 1 mM (Table 7-2).

7-3-2 Efficiency of detergents in solubilization of [^{3}H]α,β-MeATP binding sites

Three types of detergents were applied to solubilize P_{2X}-purinoceptors from rat vas deferens membranes. The anionic detergent sodium deoxycholate (1%) and the zwitterionic detergent CHAPS (10 mM), were equally effective in solubilization of membrane proteins, but only a small percentage of α,β-MeATP specific binding was then recovered in the centrifugal supernatant. Most of the [^{3}H]α,β-MeATP binding sites remained bound in the pellet. In contrast, the nonionic detergent, digitonin, at a final concentration of 2% (w/v) showed reasonably good recovery of [^{3}H]α,β-MeATP binding sites (45%) in the supernatant (Table 7-3). Lower detergent concentrations were less effective; the optimal digitonin/protein ratio was found to be ≈ 3:1, and this was used in all further experiments.

The binding in solution decreased (data not shown) with concentration of digitonin present in the assay tube (and the same was true of the other two detergents). This is an effect generally found with other receptors, but there was no change with detergent concentration in the range below 0.1% (w/v) digitonin (Figure 7-3). Therefore, all assays of the solubilized receptor were

performed with final digitonin concentration below 0.1%.

7-2-3 [^{3}H]α,β-MeATP binding to the solubilized preparations

The equilibrium binding of [^{3}H]α,β-MeATP to digitonin extract of rat vas deferens membranes was also saturable (Figure 7-4A). However, the Scatchard plot derived for this (Figure 7-4B), as well as direct analysis of the binding data, gave evidence for the existence of only one binding site there (up to 160 nM ligand). The calculated K_d: 1.6 ± 0.5 nM, was in close agreement with the K_d for the high-affinity binding sites found in the membranes (Table 7-1).

The ultracentrifugal pellet of the digitonin-extracted membranes was finely redispersed and assayed for [^{3}H]α,β-MeATP binding, to perform saturation binding analysis on this, also. It contained 27% of the original total binding sites in the membranes. These insoluble sites completely resisted a repeat extraction; they were only of the high-affinity type, as detailed in Table 7-3. In the digitonin extraction, 28% of the initial high-affinity sites and all of the low-affinity sites disappeared.

Kinetic studies were performed on digitonin-solubilized preparation to confirm that the incubation conditions used were adequate to measure steady-state binding of [^{3}H]α,β-MeATP to it. In association experiments, even if performed at 4°C, the binding to the digitonin extract reached a steady state within 15 min. The binding equilibrium remained stable for up to 2 hr of incubation

period (Figure 7-5A). In dissociation experiment, the solubilized preparation was fully equilibrated with 10 nM [^{3}H]α,β-MeATP over a 2 hrs incubation at 4°C and the dissociation was then initiated by addition of unlabelled β,γ-MeATP to 100 μM final concentration. The dissociation was rapid (half-time 6.5 min) and was complete within 60 min (Figure 7-5B). Computer fitting of the association and dissociation data revealed that both processes were monophasic throughout, supporting the deduction that there is only one class of binding site in the solubilized receptors. The kinetic parameters are shown in Table 7-4. The kinetically determined K_d value of 1.3 nM is (within the error limits) identical to the equilibrium K_d value (Table 7-1).

Competition studies with purinergic ligands performed on solubilized preparation also showed the existence of high-affinity binding sites (Figure 7-6). The displacement curves for α,β-MeATP, β,γ-MeATP, L-β,γMeATP and 2-MeSATP could be fitted by a single competitive, non-cooperative high-affinity binding constant, K_i, but high-affinity interaction there and at a second site of lower affinity was discerned in the cases of ATP and suramin (Figure 7-6). The high-affinity K_i values for the ligands are mostly in good agreement with their K_i values at the high-affinity [^{3}H]α,β-MeATP binding sites in the membrane preparations (Table 7-2), but for 2-MeSATP and suramin the interaction is much weaker in the solubilized material. Taking the solubilized sites and their high-affinity K_i values, the rank order of potency of the ligands tested was α,β-MeATP = β,γ-MeATP > L-β,γ-MeATP > ATP > 2-MeSATP >

ADP > suramin >> adenosine. This potency order is in excellent agreement with the pharmacological definition of P_{2X}-purinoceptor subtype (Burnstock and Kennedy, 1985; Cusack and Hourani, 1990).

7-3-4 Molecular size of the binding component and separation from ATPase

Sucrose density gradient ultracentrifugation of the solubilized material revealed a single peak of [^{3}H]α,β-MeATP specific binding, with a sedimentation coefficient ($s_{20,\ w}$) for the digitonin-protein complex of 12.1 S (Figure 7-7). Considerable ATPase activity was present in the soluble preparation, when all types of this enzyme were measured in the combined presence of Na^+, K^+, Ca^{2+} and Mg^{2+}, but this could largely be separated on the gradient from the [^{3}H]α,β-MeATP binding sites (Figure 7-7). The ATPase peak was homogeneous, and had a median sedimentation coefficient of 9.3 S.

In the solubilized preparation, and also in the gradient separation, cations other than Tris were not added and 1 mM EGTA was present, the above-mentioned inorganic cations being added only to the aliquots taken for ATPase assays. When the ATPase activity in the membranes was measured with the inorganic cations present (see Methods) the hydrolysis of ATP was 0.570 μmole Pi/mg protein, but when those cations were omitted and 1 mM EGTA/ Tris was added (with or without a pre-extraction of the membranes with 1 mM EDTA/Tris (pH 7.4) to remove membrane-bound Ca^{2+}), this activity dropped to a negligible level (<0.009 μmole Pi/mg

protein). Further, when the assay of ATPase was conducted (with the inorganic cations present) in the presence of 0.6 mM α,β-MeATP (and ATP at 3 mM), there was no measurable decrease in the initial enzymic velocity. Hence, in the solubilized preparation in the conditions normally used here, there was no evidence for significant binding of [^{3}H]α,β-MeATP to any ATPase.

To detect the polypeptides carrying the P_{2X}-purinergic binding site when separated in gel electrophoresis, photo-affinity cross-linking was employed on the original vas deferens membranes. Photoactivation of [^{3}H]α,β-MeATP (at 50 nM concentration) was achieved with a low-intensity UV energy flux, with a maximum irreversible labelling occurring within 15 min. This incorporation was equivalent to 80% of the high-affinity binding sites for [^{3}H]α,β-MeATP present in the membrane sample.

SDS/polyacrylamide gel electrophoresis of the irradiated samples revealed that the radioligand was irreversibly incorporated into protein species with a molecular weight of 62,000 Da (Figure 7-8). This radioactivity peak completely disappeared when cross-linking of [^{3}H]α,β-MeATP was performed in the presence of 1 mM unlabelled β,γ-MeATP. Minor additional labelled components in the range of 47,000 - 70,000 Da might be present, but the relatively low specific activity of the ligand and the low energy of tritium disintegration prevented higher resolution being attainable, since the receptor is at an extremely low density in the membranes.

7-4 Discussion

In known classes of purinoceptors (Burnstock, 1991b; 1991c) detailed molecular information is available so far only on the A_1 and A_2 subtypes of the P_1 type, for which the encoding cDNA's have been cloned (Maenhaut *et al.*, 1990; Libert *et al.*, 1989; 1991). These, as expected, are single polypeptides of ≈ 50,000 Da of the G-protein-coupled superfamily. For the P_2 types, the P_{2Y}-purinoceptors would be expected to be of the same general type, being linked to inositol phospholipid turnover or the production of nitric oxide (see Burnstock, 1991b; 1991c; Ralevic & Burnstock, 1991 for review). A solubilization from avian erythrocyte membranes of a complex containing P_{2Y}-purinoceptors, to a B_{max} of 4.5 pmol/mg protein (measured with [^{35}S]ADPβS), has recently been reported (Jeffs *et al.*, 1991), but no other information on P_2-purinoceptor proteins is available and no solubilization of P_{2X}-purinoceptors has been described hitherto.

In the present study we have solubilized and characterized P_{2X}-purinoceptors from rat vas deferens using [^{3}H]α,β-MeATP as the selective probe for this subtype. Digitonin (2%), but not sodium deoxycholate or CHAPS, solubilized a considerable proportion of the [^{3}H]α,β-MeATP binding sites; if the binding assay was carried out at a concentration of digitonin below 0.1%, an inhibitory effect of the detergent was avoided.

The rat vas deferens membranes were shown to contain a high-

affinity and a lower-affinity receptor populations labelled by [^{3}H]α,β-MeATP, although both K_d values are below 100 nanomolar (Figure 7-1). The competition studies performed here confirmed the heterogeneity of [^{3}H]α,β-MeATP binding sites in the rat vas deferens membranes: all of the ligands except the most tightly bound (α,β-MeATP) acted biphasically in the displacement of the radioligand.

In contrast, in the digitonin-solubilised preparation only one binding site for [^{3}H]α,β-MeATP was detectable in direct binding isotherms (Figure 7-4). The K_d value for this site is identical (within the experimental error of the determination) to the K_d for the high-affinity binding sites in the membrane (Table 7-1). This was confirmed by independent kinetic estimation of K_d (Table 7-4). However, the more sensitive test for heterogeneity that is provided by displacement over a very wide concentration range of a competing ligand showed that [^{3}H]α,β-MeATP does interact very weakly at additional sites in the soluble preparation (Figure 7-6, curves of ATP and suramin). This interaction occurs in an affinity range more than 1,000-fold weaker than at the first site and was too weak and of too small an amount to be measured accurately. The presence of these very low affinity sites, however, could give for the weaker ligands some deviation of the estimated K_i values from their true affinity for the P_{2X}-purinoceptor site.

The situation found, therefore, from the data of Table I and

III, is that upon extraction of the vas deferens membranes with digitonin at the optimum level, 45% of the high-affinity sites for [^{3}H]α,β-MeATP are recovered in the solution, while 27% of them remained unextracted. All of the more numerous low-affinity sites (K_d ≈ 30 nM) in the membranes also disappeared, but there was an indication from competition data of a far weaker set, impossible to quantitate, also being present in the solution. Since the high-affinity sites in the solution, and also those unextracted, exhibit the original high-affinity K_d values (Table 7-1), we deduce that there is an overall loss of about 28% of the P_{2X}-purinoceptor binding sites during the extraction procedure, which is a reasonably low value. The sites of lower affinity in the membrane were shown to have a quite different pharmacological profile to the high-affinity sites and we can note that α,β-MeATP has been found pharmacologically to interact weakly with a P_2-purinoceptor other than the P_{2X}-subtype (Burnstock, 1991b; 1991c), so we attribute those low-affinity sites to other P_2-purinoceptor as well as to ATPase and other ATP-binding enzymes. The set of ATPases present do not bind [^{3}H]α,β-MeATP in its high affinity range (Figure 7-7, and other data reported above) in the conditions used, in which the levels of Mg^{2+}, Ca^{2+}, Na^+, or K^+ needed to activate various ATPases were excluded. We conclude, therefore, that these low-affinity components in the membrane, when extracted in digitonin in the absence of appreciable levels of inorganic cations, become disrupted, or are complexed with the detergent, in such a manner as to transform them to yet lower affinity, or perhaps to selectively inactivate some of these components.

The high-affinity sites, in contrast, are mostly recovered as such. The total set of high-affinity binding sites for [^{3}H]α,β-MeATP is attributed to P_{2X}-purinoceptors because (a) the rat vas deferens contains essentially only this type among the purinoceptor, as assayed physiologically (Burnstock & Kennedy, 1985; Hoyle & Burnstock, 1991); (b) the rank order of ligands competing at the solubilized high-affinity binding sites (Table 7-2) matches completely that predicted from the pharmacological behaviour of the P_{2X}-purinoceptor in tissue studies (Cusack and Hourani, 1990; Burnstock, 1991b; 1991c); (c) for the P_{2Y}-purinoceptors, in contrast, the known rank order of potency is very different from that which we find from the binding in the solution, since for P_{2Y}-purinoceptor 2-MeSATP is the most potent of the ligands which were tested here, whereas α,β-MeATP, used as our probe, has much lower affinity for the P_{2Y}-subclass (Burnstock, 1991b; 1991c); (d) a subtype-specific P_{2X} agonist is recognised by these sites, in that they show a high affinity (K_d 17 nM) for L-β,γ-MeATP, which has a very high physiological potency on the P_{2X} subtype in a variety of tissues but no activity on any other purinoceptors (Hourani *et al.*, 1985; 1986; Cusack and Hourani, 1990).

There are few differences in the ligand affinities between the membrane-bound and the solubilized sites (Table 7-4). We presume that these arise because [^{3}H]α,β-MeATP is, in the membranes, more weakly labelling some additional sites (Table 7-

1), whereas in the soluble preparation these have mostly disappeared, as noted above. Suramin, a trypanocidal drug which was found to act as a P_{2X}-purinoceptor antagonist (Dunn and Blakeley, 1988), shows the largest difference seen between the two sites (Table 7-2). However, its lower potency relative to the methylene ATPs in the soluble preparation is more like that relationship in the physiological responses (Hoyle *et al.*, 1990). Moreover, its effect on the latter is not selective for P_{2X}-purinoceptors because it can also antagonize P_{2Y}-purinoceptor responses (Hoyle, *et al.*, 1990) and it inhibits various ATPases (Fortes *et al.*, 1973; Hourani & Chown, 1989). Some of those sites may be involved in its greater effect on the membrane than on the soluble preparation.

We observed (Figure 7-7) that an abundant ATPase activity could be evoked in our soluble preparation, but for several reasons we can discard the twin possibilities that this destroys some of the [^{3}H]α,β-MeATP in the binding assays or that any significant part of the high-affinity binding of that ligand occurs to any ATPase. The latter case might have been considered to be a possibility for it acts as an inhibitor even when there is no hydrolysis. Firstly, the ATPases could be separated physically from the [^{3}H]α,β-MeATP binding sites (Figure 7-7). However, there was a small amount of overlap there, so this question was considered further. Secondly, then, the methylene analogues of ATP can be only very slowly hydrolysed by smooth muscle ATPases in their normal assay conditions, as was shown by Ruggieri *et al.* (1990) by chromatographic separation of the products. Thirdly, in

autoradiographic studies of the rat vas deferens and other peripheral tissues the distribution of [^{3}H]α,β-MeATP binding sites was found to be extremely tissue- and region- specific (see Chapters 4, 5, 6, and 7), whereas the ATPase enzymes are universally found in all cell types. Finally, the evidence noted on the ATPase activity demonstrated that in the conditions of our soluble extract (absence of inorganic cations) there is no significant activity of the ATPases present, in any case, and that when they are active α,β-MeATP is not an enzymic inhibitor, i.e., it does not bind to the ATPases present.

For affinity labelling of the receptor, we applied UV excitation of the adenine group in [^{3}H]α,β-MeATP. This reaction has been shown to occur in a specific manner in ATP when bound strongly to some ATP-dependent enzymes, e.g. terminal nucleotidyl transferase, *E. coli* DNA polymerase, etc. (Modak & Gillerman-Cox, 1982; Basu & Modak, 1987). We show here that it can be utilized for labelling a membrane receptor which binds ATP. The irreversibly cross-linked product has an apparent M_r of 62,000 Da, although it may contain several polypeptides in similar sizes. By analogy with the other known transmitter-gated ion channels (reviewed by Barnard, 1992), the P_{2X}-purinoceptor is expected to contain between two and four different types of subunit, but with the high-affinity transmitter-binding site likely to be on only one of these. On the other hand, a multiplicity of subtypes of such receptors is common, so that several isoforms of the transmitter-binding subunit may well exist in a given tissue.

Hence several labelled subunits of quite similar size in SDS gel electrophoresis could indeed arise from a specific receptor-labelling reaction. Such subunits would be expected to occur in the membrane in oligomers, which in other such receptors, wherever the stoichiometry is known, are pentamers (Barnard, 1992): those would readily account for the size of the labelled components which migrate in the complex with digitonin, of $s_{20,w} \approx 12$ S (Figure 7-7). By subjecting that peak to a repeat of the gradient separation the binding sites could be significantly purified relative to the total extract and could be freed of ATPases. The solubilization and that separation constitute the necessary first stage for the affinity purification of the P_{2X}-purinoceptor, which is now in progress.

Figure 7-1 Saturation isotherm of [^{3}H]α,β-MeATP specific binding to rat vas deferens membranes

Aliquots of the membrane fractions were incubated with the indicated concentration of [^{3}H]α,β-MeATP for 2 hrs at 4°C. Non-specific binding was defined as the binding in the presence of 100 μM unlabelled β,γ-MeATP. Panel A shows the saturation isotherm, Panel B shows the Scatchard plot of the specific binding data. The data are representative of results obtained from five different membrane preparations and they were analysed using the EBDA-LIGAND program. The best fit was achieved by the two binding site model.

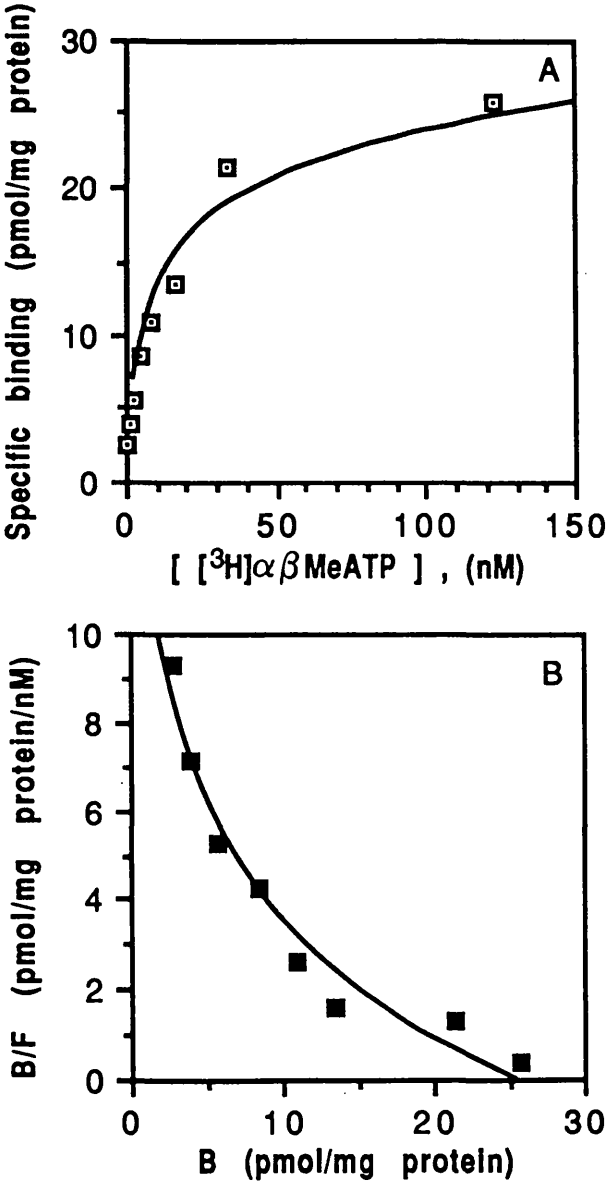

A
Specific binding (pmol/mg protein)
30
20
10
0
0
50
100
150
[[3H]αβMeATP] , (nM)
B
B/F (pmol/mg protein/nM)
10
8
6
4
2
0
0
10
20
30
B (pmol/mg protein)

Figure 7-2 **Competition of purinergic ligands for the binding of [^{3}H]α,β-MeATP to rat vas deferens membranes**

Aliquots of the membranes were incubated with 10 nM [^{3}H]α,β-MeATP in the absence or presence of various type of purinergic ligands under the conditions as described in the *Materials and Methods*. Non-specific binding was defined with 100 μM β,γ-MeATP. Data represent the mean ± SEM of at least three experiments and they were analysed by non-linear regression.

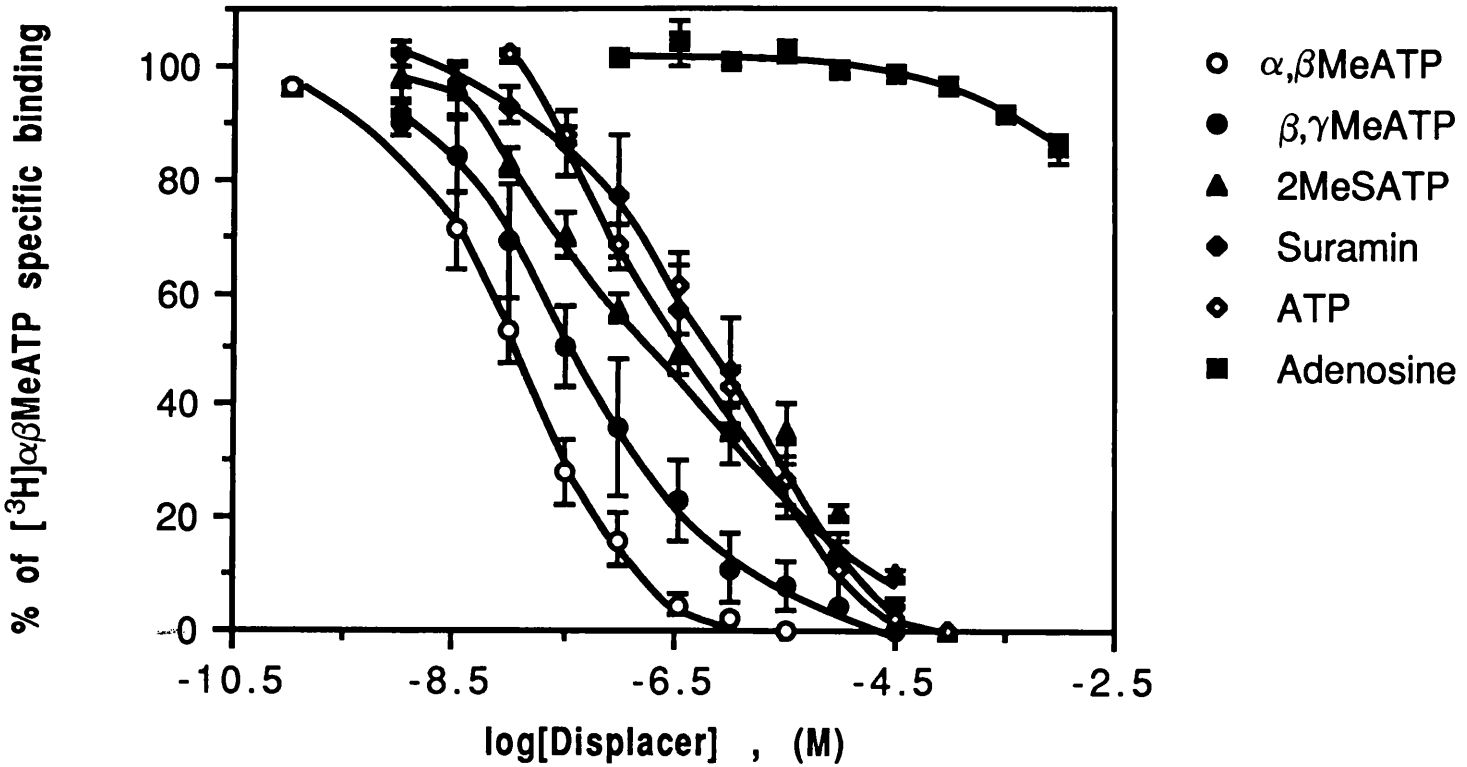

% of [3H]αβMeATP specific binding
100
80
60
40
20
0
-10.5
-8.5
-6.5
-4.5
-2.5
log[Displacer] , (M)
α,βMeATP
β,γMeATP
2MeSATP
Suramin
ATP
Adenosine

Figure 7-3 **The effects of detergents on the specific [^{3}H]α,β-MeATP binding to rat vas deferens membranes**

Aliquots of rat vas deferens membranes were incubated with 10 nM [^{3}H]α,β-MeATP for 2 hrs at 4°C in the presences of 0.1% or 0.05% of digitonin (panel D), disodium deoxycholate (panel C), and 1 or 0.5 mM CHAPS (panel B). Non-specific binding was determined in the presence of 100 μM β,γ-MeATP. CON: control; DOC: deoxycholate; DIGI: digitonin.

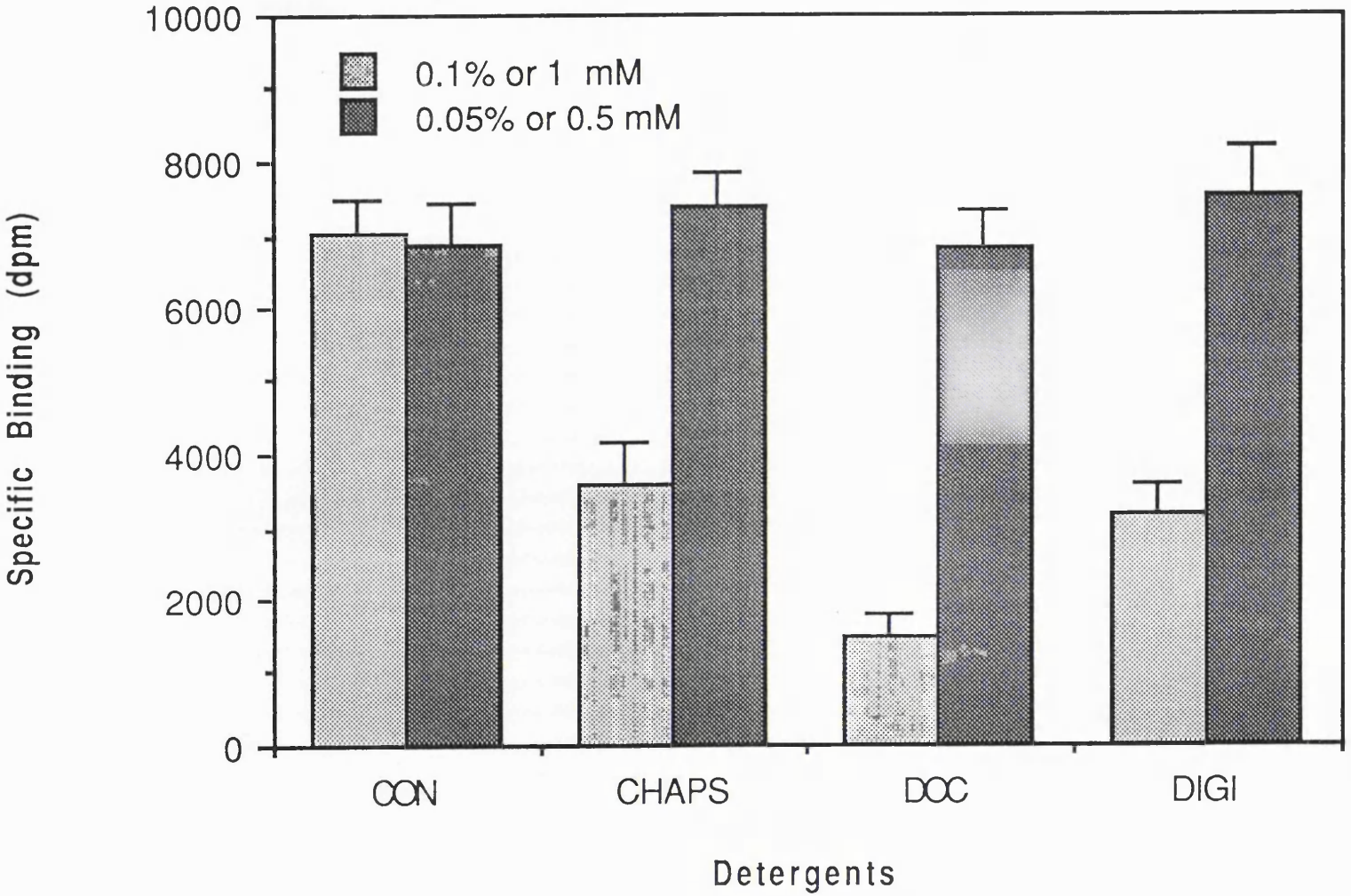

10000
8000
6000
4000
2000
0
0.1% or 1 mM
0.05% or 0.5 mM
Specific Binding (dpm)
CON
CHAPS
DOC
DIGI
Detergents

Figure 7-4 **Saturation isotherm of [^{3}H]α,β-MeATP specific binding to digitonin solubilised preparation**

Digitonin extract of rat vas deferens membranes was incubated with the indicated concentration of [^{3}H]α,β-MeATP for 2 hrs at 4°C. Non-specific binding was defined as the binding in the presence of 100 μM unlabelled β,γ-MeATP. Panel A shows the saturation isotherm, Panel B shows the Scatchard plot of the specific binding data, while Panel C shows the Hill plot.
The data are representative of results obtained from five different preparations and they were analysed using the EBDA-LIGAND program. The best fit fulfils the single population of binding site model.

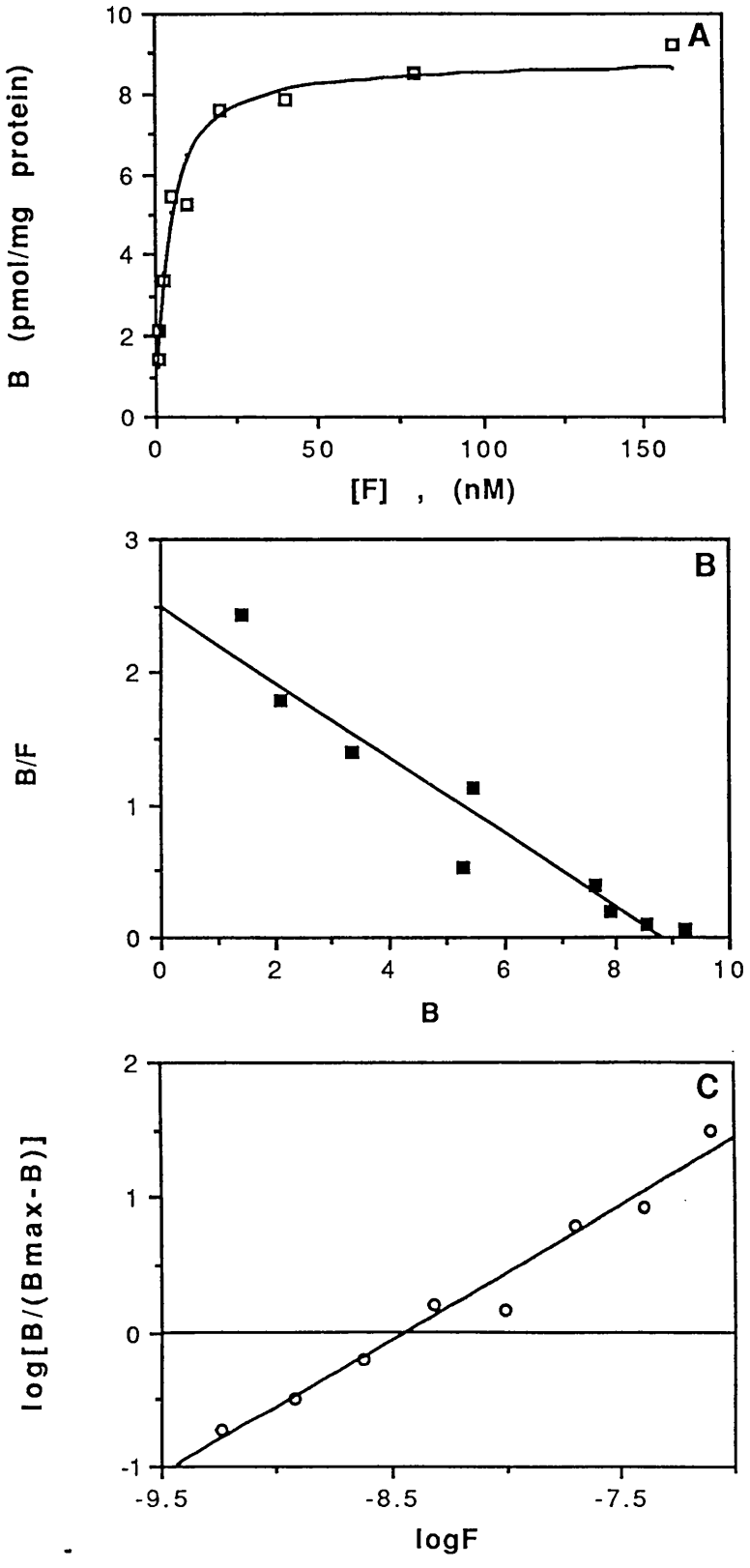
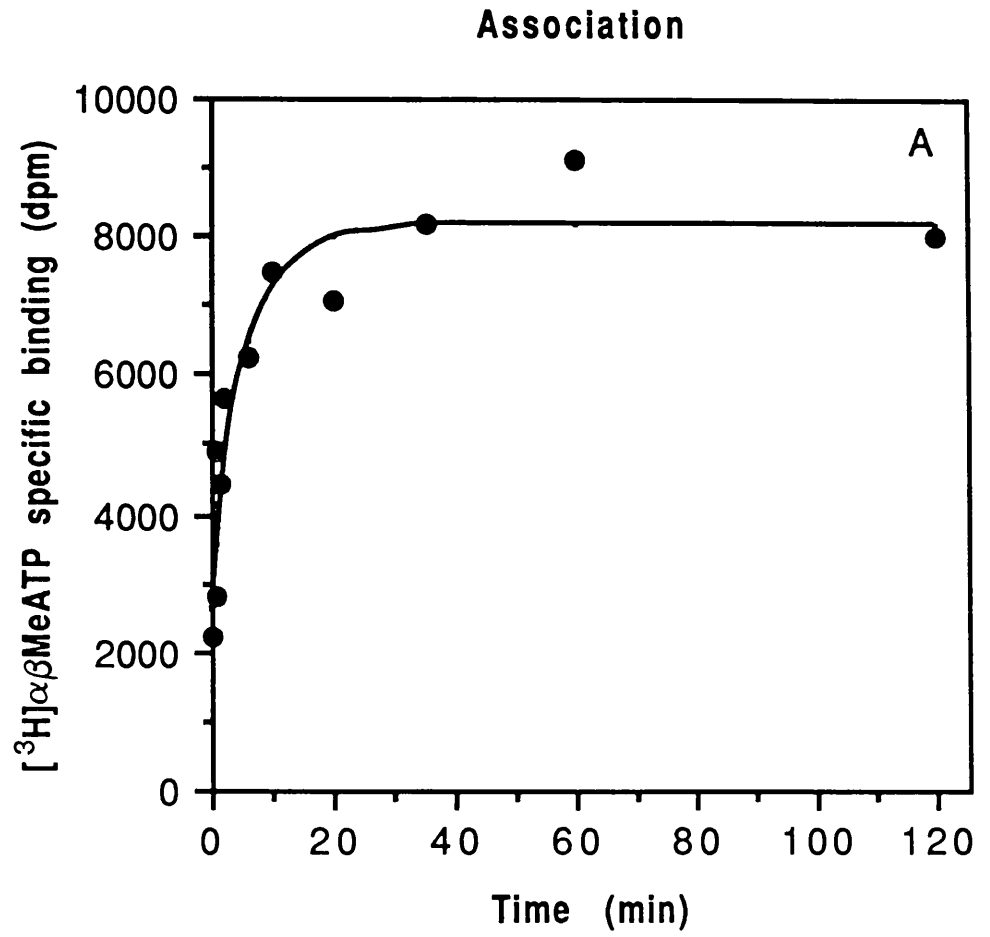

A
B (pmol/mg protein)
[F] , (nM)
B
B/F
B
C
log[B/(Bmax-B)]
logF

Figure 7-5 **Kinetics of [^{3}H]α,β-MeATP binding to the digitonin-solubilised preparation**

Aliquots of the digitonin-solubilised preparation were incubated with 10 nM [^{3}H]α,β-MeATP at 4°C. At various time intervals (0 - 120 min) the reaction was terminated by rapid filtration of the incubation mixture as described in *Materials and Methods*. Dissociation of the radioligand was achieved by adding 100 μM β,γ-MeATP to the incubation mixture at equilibrium, and then the reaction was terminated at different time intervals (0 - 70 min). The binding data were analysed with a computer program KINETICS included in the program EBDA-LIGAND. The best fit in each case was given by a mono exponential form (curves, computed). The curve shown is representative of five similar experiments.


Association
A
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
120
[3H]αβMeATP specific binding (dpm)
Time (min)

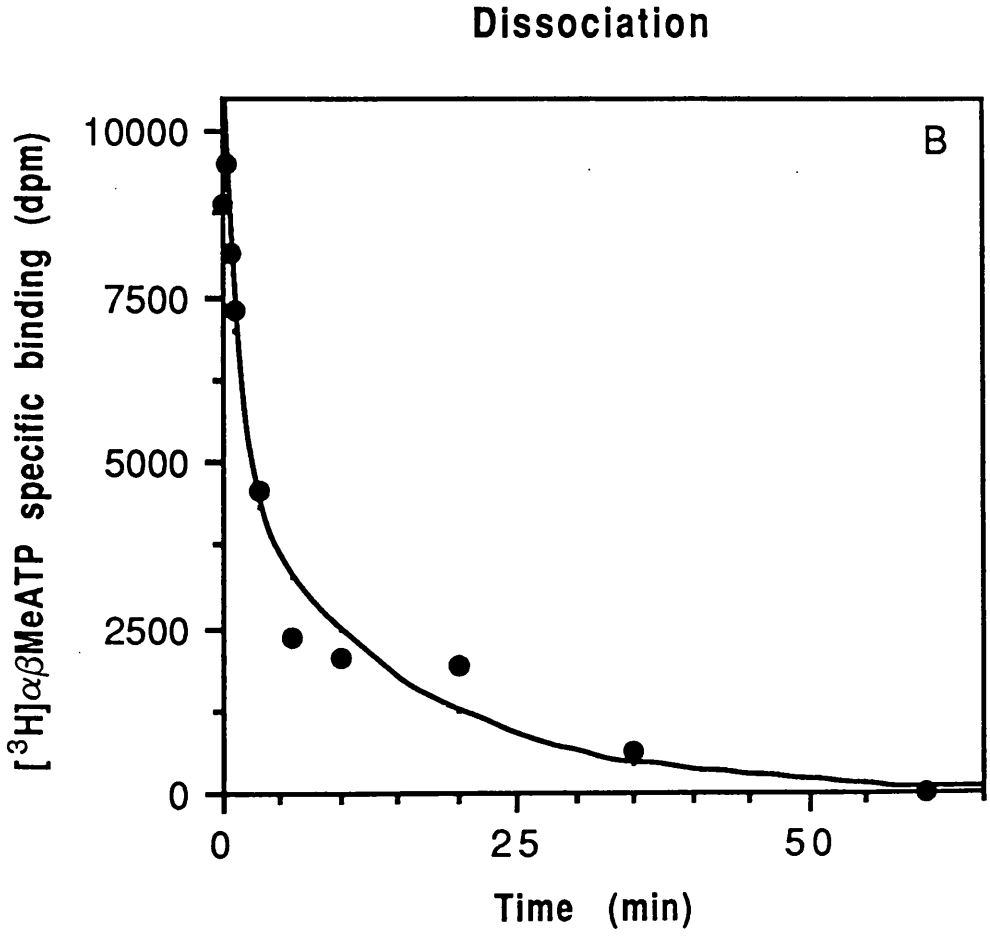

Dissociation
B
10000
7500
5000
2500
0
0
25
50
[3H]αβMeATP specific binding (dpm)
Time (min)

Figure 7-6 **Competition of purinergic ligands for the specific binding of [^{3}H]α,β-MeATP to solubilised rat vas deferens membranes**

Aliquots of the digitonin-solubilised preparation were incubated with 10 nM [^{3}H]α,β-MeATP in the absence or presence of various types of purinergic ligands under the conditions as described in the *Materials and Methods*. Non-specific binding was defined with 100 μM β,γ-MeATP.

Data represent the mean ± SEM of at least three experiments and they were analysed by non-linear regression. Lines are computer-generated fits to the data.

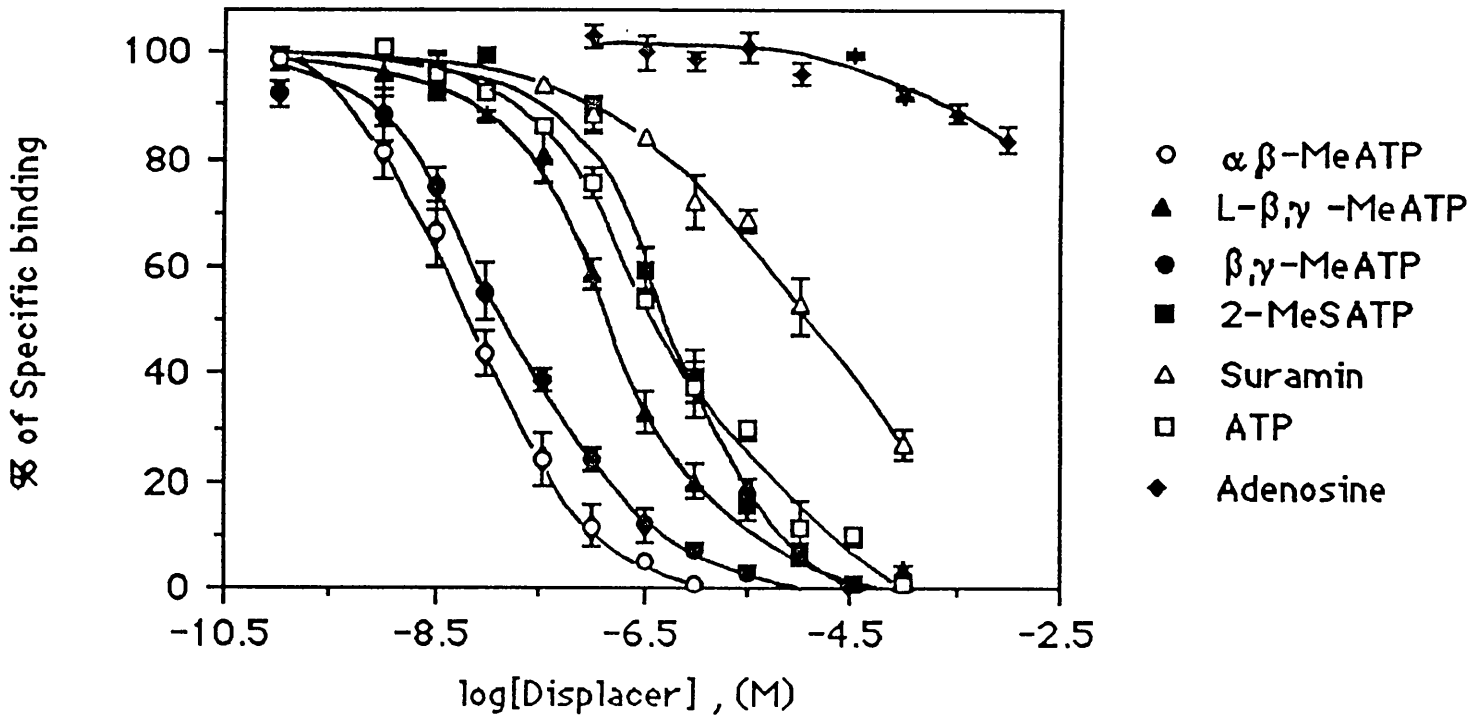

% of Specific binding
100
80
60
40
20
0
-10.5
-8.5
-6.5
-4.5
-2.5
log[Displacer] , (M)
αβ-MeATP
L-β,γ -MeATP
β,γ-MeATP
2-MeSATP
Suramin
ATP
Adenosine

Figure 7-7 **Separation by sucrose density gradient centrifugation of the [^{3}H]α,β-MeATP binding sites and ATPases**

Aliquots of 200 µl of the digitonin-solubilised preparation were layered onto 10-30% sucrose linear gradients and centrifuged at 200,000 *g* for 16 hrs. at 4°C. Twenty-three drops of each fraction were collected and assayed for [^{3}H]α,β-MeATP binding (●) and for ATPase enzyme activity (⊡) as described in the *Materials and Methods*. The figure also shows the relative positions and sedimentation coefficients of the marker proteins, centrifuged on parallel gradients in order to estimate the sedimentation coefficients and the sizes of the detergent-protein complexes. Data is representative of results obtained from three different preparations. Two gradients were assayed in duplicate each time.

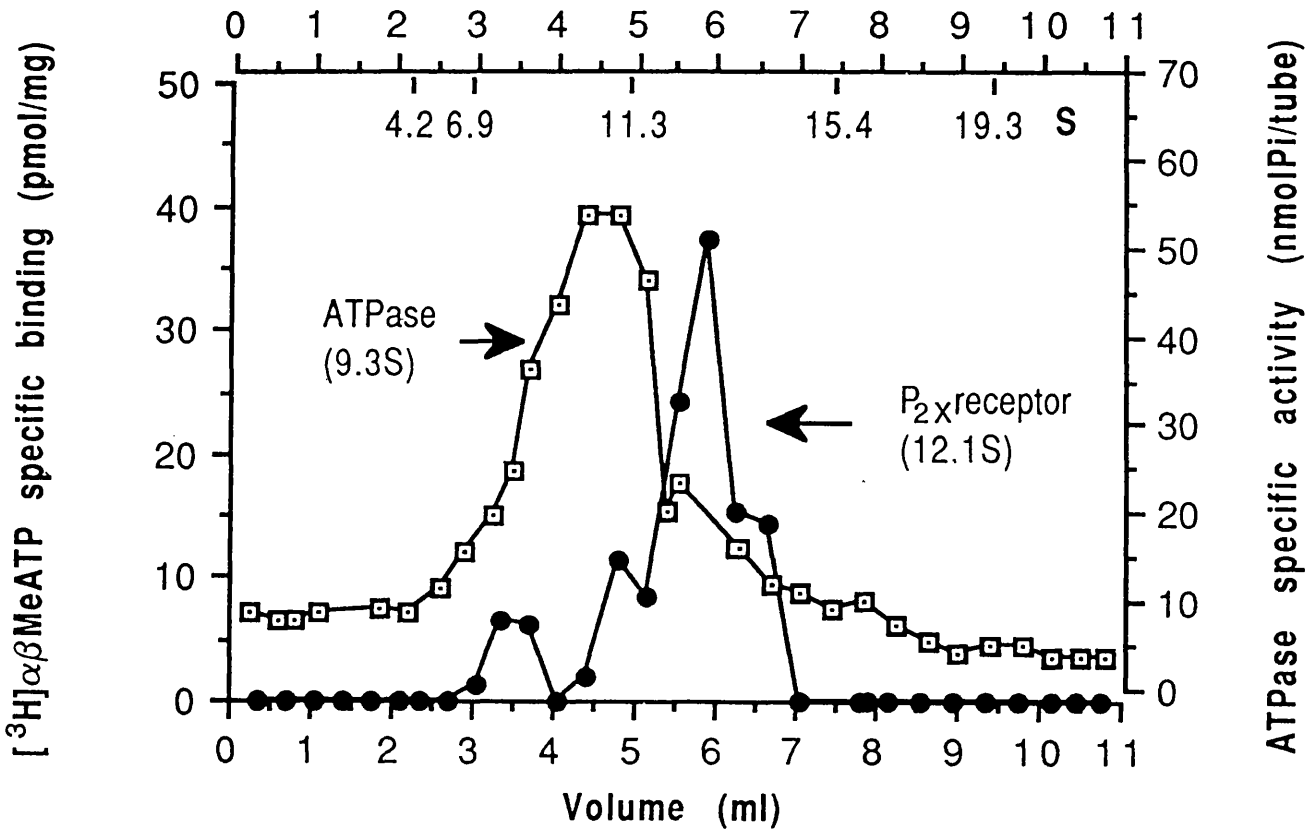
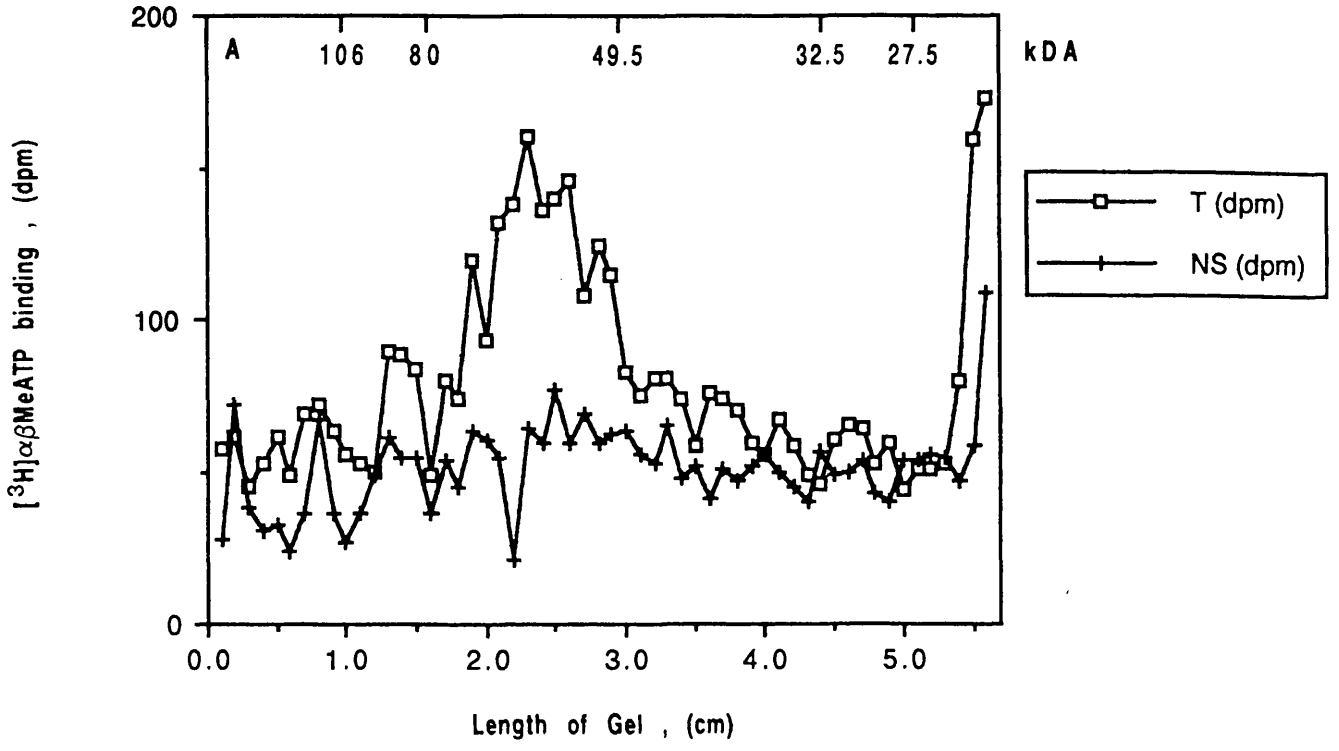
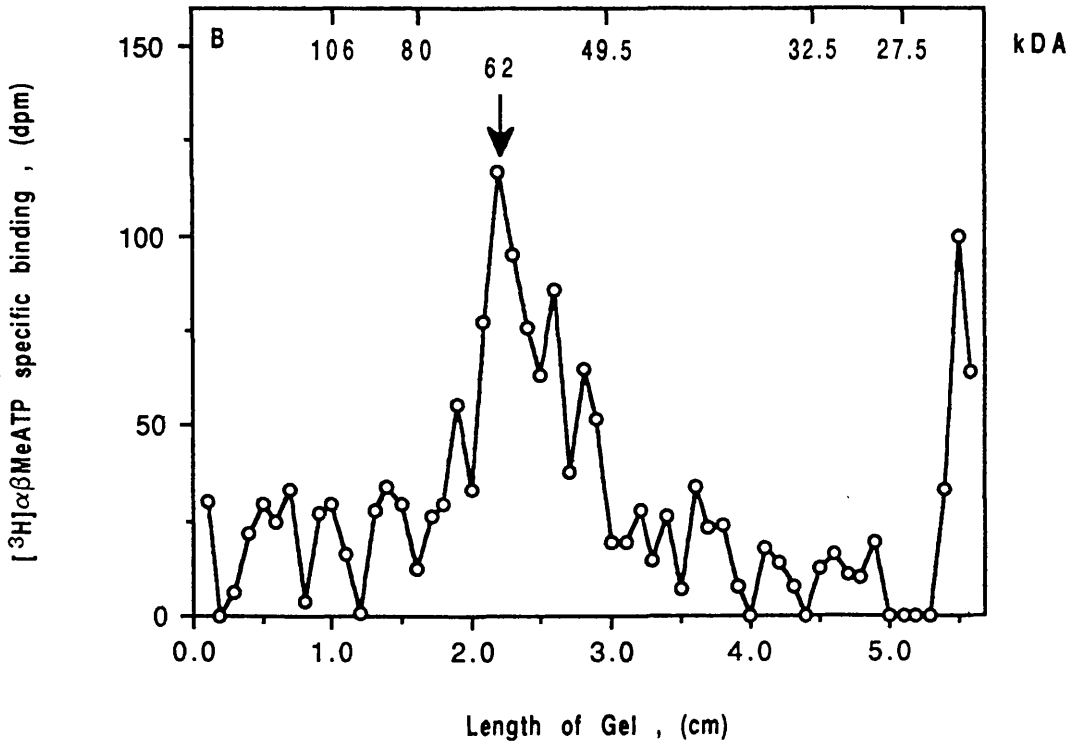

0 1 2 3 4 5 6 7 8 9 10 11
4.2 6.9 11.3 15.4 19.3 S
50 40 30 20 10 0
70 60 50 40 30 20 10 0
ATPase
(9.3S)
P2X receptor
(12.1S)
[3H]αβMeATP specific binding (pmol/mg)
ATPase specific activity (nmolPi/tube)
Volume (ml)

Figure 7-8 **Cross-linking of [^{3}H]α,β-MeATP to P_{2X}-purinoceptors in rat vas deferens membranes**

Aliquots of membrane were incubated with 50 nM [^{3}H]α,β-MeATP in the absence and presence of 1 mM unlabelled β,γ-MeATP and UV irradiated as described in the *Materials and Methods*. The samples were then subjected to SDS polyacrylamide gel electrophoresis, the gels were cut into 1 mm slices and their radioactivity was measured. Panel A shows the incorporation of [^{3}H]α,β-MeATP into membrane species in the absence (□), or presence (+) of β,γ-MeATP. The specific incorporation (o), shown on Panel B, was calculated as a difference between the total (□) and non-specific (+) incorporation. Relative positions and molecular weights of prestained protein standards electrophoresed together with the irradiated samples are also shown on both panels.
The figure is a representative of the experiments performed on three different preparations.


A
106
80
49.5
32.5
27.5
kDA
200
100
0
0.0
1.0
2.0
3.0
4.0
5.0
T (dpm)
NS (dpm)
[3H]αβMeATP binding , (dpm)
Length of Gel , (cm)


B
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49.5
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0.0
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3.0
4.0
5.0
[3H]αβMeATP specific binding , (dpm)
Length of Gel , (cm)

Table 7-1 **Equilibrium parameters of [^{3}H]α,β-MeATP binding to membrane-bound and digitonin-solubilized P_{2X}-purinoceptors**

Each value represents the mean ± SEM of triplicate determinations on five independent preparations (three for the residue preparation) n.s., not significantly present, as detected with up to 160 nM radioligand.

Preparation	B_{max} (pmol/mg protein)		K_d (nM)		n_H	
	High[a]	Low[a]	High	Low	High	Low
Membrane-Bound	9.3±4.1	28.8±8.6	1.8±0.5	33.7±10.0	0.95±0.04	1.06±0.04
Digitonin-extract	8.2±1.3	n.s.	1.6±0.5	n.s.	0.92±0.04	n.s.
Insoluble residue	4.9±0.4	n.s.	2.3±0.5	n.s.	1.03±0.07	n.s.

[a] High and low affinity binding.

Table 7-2 **Potencies of purinoceptor-active ligands to displace [^{3}H]α,β-MeATP binding to membrane and digitonin-solubilized preparations**

The inhibition constant (K_i) for each ligand was computed from the data of the competition curves using the high-affinity K_d values for the radioligand of Table I. The concentration of the radioligand used was 10 nM. K_i values (computed only for the high affinity site) are expressed as the mean ± SEM of three independent determinations.

Ligands	Membrane K_i (nM)	Digitonin extract K_i (nM)
α,β-MeATP	1.1±0.5	2.2±0.8
β,γ-MeATP	1.5±0.1	1.8±0.6
L-β,γ-MeATP	8.0±2.0	17±2
2-MeSATP	27±3.0	99±12
ATP	70±3.0	73±26
Suramin	123±22	1,359±256
ADP	151±13	98±14
Adenosine	> 5000	> 5000

Table 7-3 **Efficiency of detergents in the solubilization of P_{2X}-purinoceptors**

Membranes from rat vas deferens were solubilized with 2% digitonin, or 1% sodium deoxycholate, or 10 mM CHAPS, as described in *Materials and Methods*. All the data in this table are from one preparation that is representative of three similar experiments.

Detergent	Protein solubilized (%)	Binding sites in supernatant (pmol/mg)	Binding sites solubilized	
			% Hi-Affi*	% Total
Digitonin	51	5.22[a]	43[b]	12[b]
Deoxycholate	64	0.21	2[a]	
CHAPS	61	1.17	15[a]	

[a] This binding was measured (in both the solution and the membranes)only at a fixed concentration of [^{3}H]α,β-MeATP, 40 nM.

[b] (Binding sites (B_{max}) in the supernatant/binding sites (B_{max}) in the membranes) x 100. This percentage is shown using as the reference *either* the B_{max} value for the high-affinity sites in the membranes (giving 45%), *or* the B_{max} value for the total of the high and low affinity sites in the membranes (12%).

* Hi-Affi, High-affinity binding sites

Table 7-4 **Kinetic parameters of [^{3}H]α,β-MeATP binding to digitonin-solubilised P_{2X}-purinoceptors**

k_{obs} (min^{-1})	k_1 (x $10^8 M^{-1}.min^{-1}$)	k_{-1} (min^{-1})	K_d (nM)	$t_{\frac{1}{2}}$ (min)
0.156±0.020	2.57±0.01	0.087±0.009	1.3±0.3	6.5±1.0

The apparent association rate constant (k_{obs}) and the dissociation rate constant (k_{-1}) were derived from computer fits to the data using EBDA-LIGAND program. k_1 is the first-order association rate constant, $t_{\frac{1}{2}}$ is the half-time of the dissociation. The values represent the mean ± SEM of five independent experiments.

Chapter 8 General Discussion

The main achievements in this study can be summarized as follows: (1) [^{3}H]α,β-MeATP has been shown to fulfil the basic criteria for a radioligand binding to P_{2X}-purinoceptors; (2) the biochemical characteristics of P_{2X}-purinoceptors have been systematically studied by using radioligand binding assay; (3) the distribution of P_{2X}-purinoceptors has been displayed in various tissues with the autoradiographic localization technique; (4) this is the first report of successful solubilization of P_{2X}-purinoceptors, and of the biochemical characterization of the soluble P_{2X}-purinoceptors. Therefore, the principle aims outlined in the Preface of the thesis have been achieved. In this Chapter the advantages and disadvantages of the radioligand and of the techniques used are discussed. Also, some observations are presented that were not included in the main text of the thesis, and some ideas presented for future work in this field.

8-1 [^{3}H]α,β-MeATP

α,β-MeATP is an ATP analogue with the esoteric oxygen atom between the α and β positions of the polyphosphate chain being replaced with a methylenic carbon atom. Such a modification makes it a more potent activator of the P_{2X}-purinoceptors than ATP, and relatively resistant to dephosphorylation (Welford *et al.*, 1987). Another distinct feature is that α,β-MeATP can desensitize P_{2X}-purinoceptors after repeated or prolonged exposure (Kasakov &

Burnstock, 1983; Meldrum & Burnstock, 1983, Kennedy & Burnstock, 1985a and b; Kennedy *et al.*, 1985; Houston *et al.*, 1987; Reilly & Burnstock, 1987). Based on these properties α,β-MeATP was considered a promising label for P_{2X}-purinoceptors. [^{3}H]α,β-MeATP was custom-synthesized by Amersham Int. by a catalyzed exchange reaction using tritium gas and purified by high performance liquid ion exchange chromatography. The experiments carried out in this study proved that tritium-labelled α,β-MeATP possesses the basic criteria for a radioligand for receptor binding assays: (a) the specific activity (first batch 27 Ci/mmol, second batch 19.2 Ci/mmol) is sufficiently high to allow accurate measurement at low concentration; (b) high radiochemical purity to ensure the bound radioligand is at the receptor sites (98 - 99% checked by using thin-layer chromatography on PEI cellulose); (c) affinity to the receptor sites is high enough to make the filtration method for the separation of bound and free ligand feasible; (e) relatively stable when exposed to the tissue preparations; (f) relatively selective, it has no affinity for P_1-purinoceptors and ATPases, however, it is not a pure P_{2X}-purinoceptor agonist and may have a weak affinity to P_{2Y}-purinoceptors. In autoradiographic studies specific [^{3}H]α,β-MeATP binding sites were also observed in P_{2Y}-purinoceptor-dominant tissues such as the guinea-pig taenia coli; such a property will make it difficult to estimate the proportions of P_{2X}-purinoceptor and P_{2Y}-purinoceptor if the tissue contains both subtypes (such as in some blood vessels) because of the lack of pure agonist and antagonist for both the receptors. Nevertheless, in the tissues dominated by P_{2X}-purinoceptor such as

urinary bladder and vas deferens it is still a powerful tool to study the biochemical properties and localization of P_{2X}-purinoceptor.

β,γ-MeATP is considered to have better selectivity at P_{2X}-purinoceptors, but this ligand is more susceptible to dephosphorylation. It has been tritium-labelled by Du-Pont NEN for use as a radioligand to characterize P_{2X}-purinoceptors in urinary bladder (Ruggieri *et al.*, 1990). However, the specific activity is low (4.53 Ci/mmol), thus its use could be limited. L-β,γ-MeATP was claimed to be a potent and extremely selective P_{2X}-purinoceptor agonist (Cusack & Hourani, 1984; Hourani, 1986; Hourani *et al.*, 1985; 1986), which is devoid of any activity on other P_2-purinoceptor subtypes, and was completely resistant to dephosphorylation. In this study it was shown that L-β,γ-MeATP could competitively displace the [^{3}H]α,β-MeATP binding to rat vas deferens membranes and solubilized receptors, although its affinity was lower than both α,β-MeATP and β,γ-MeATP. Nevertheless, with advantage of its high selectivity and high resistance to enzymic degradation it would be worthwhile to have this ligand radiolabelled for P_{2X}-purinoceptor studies in the future.

8-2 [^{3}H]α,β-MeATP binding sites

The [^{3}H]α,β-MeATP binding sites in both urinary bladder and vas deferens fulfilled the criteria for the recognition of a transmitter receptor: (a) the binding is saturable and has

relatively high affinities; (b) both the association and dissociation processes of the binding were very rapid; (c) the binding could be displaced competitively by the unlabelled ligands active on P_{2X}-purinoceptors; (d) in most cases the specific binding sites appeared only in the tissues where P_{2X}-purinoceptor-mediated responses were observed. Other features include temperature sensitivity and cation regulation of the binding. Therefore, we have enough evidence to believe that [^{3}H]α,β-MeATP binding sites in urinary bladder, vas deferens and some blood vessels are P_{2X}-purinoceptor, or directly related to them, although this cannot be regarded as fully established until the receptor has been successfully purified and reconstituted.

One question that might be raised is why the K_d values for both the high- and low-affinity sites of [^{3}H]α,β-MeATP binding are in the nanomolar range, while the EC_{50} values of α,β-MeATP in eliciting contractile responses in smooth muscle are in the micromolar range? Ideally, the pharmacology of the binding should correlate quantitatively with the pharmacology of receptor-mediated effects. In practice, such quantitative correlation is difficult to achieve, especially when agonists are used as radioligands (Burt, 1985; Ehlert *et al.*, 1984), because of some unknown sequence of events between agonist receptor occupation and measured pharmacological responses. For α,β-MeATP, the situation is more complicated because an accurate EC_{50} value is difficult to obtain due to its ability to desensitize the receptor. A proportion of P_{2X}-purinoceptors would be turned into an inactive

state after exposure to α,β-MeATP, and while the pharmacological responses can only be displayed by the receptors in their active state, [^{3}H]α,β-MeATP may bind to both the active and the inactive states. Furthermore, there is even some evidence indicating that agonists preferentially bind to desensitized receptors (Birdsall & Hulme, 1976) and that the low-affinity sites are the pharmacologically relevant receptors (Birdsall *et al.*, 1977). The influence of environmental factors also have important implications in the interpretation of agonist binding data. Pharmacological experiments on the P_{2X}-purinoceptor-mediated responses were carried out on intact tissues or cells, while the binding assays were performed on homogenates or separated membranes. A pharmacological response depends on the link of transmitter-receptor-effector coupling, while the binding assay only deals with transmitter-receptor interaction. On the other hand, the binding of antagonists is not as sensitive to the coupling constraints of the receptor, since antagonists do not isomerize the receptor into active conformation.

8-3 Quantitative autoradiography

As has been pointed out in Chapter 3, autoradiography has two advantages over radioligand binding assay: high sensitivity and accurate localization. Recently developed quantitative methods have extended the information that can be obtained from autoradiography. This technique provides the advantages of: (a) measuring radioligand binding in small, anatomically distinct, and relatively intact tissue sections; (b) doing so in a single

section, thus increasing the reliability of between-region comparisons; and (c) avoiding potential artifacts in inter-regional comparisons caused by differences in tissue concentration or composition (Marien & Altar, 1988). So far, most of the work with this technique has been carried out to map various receptors in the brain, which supplied a more accurate estimation of receptor densities in different structures in the brain (Boast *et al.*, 1986). However, this technique is far from perfect. First, the grain densities in nuclear emulsion and the optical densities on film are not linearly related to the radioactive content of the tissues, thus the quantitative interpretation of autoradiograms will require the simultaneous use of autoradiographic standards along with the experimental tissues. Another problem is encountered when one uses tritiated ligands. Because of the relatively low energy of β-particle emission, there is a significant degree of tissue absorption of the β-rays. Moreover, the magnitude of the absorption will vary depending on the density of the tissue. Thus, if two tissues with different densities contain the same quantity of radioactivity, the autoradiographic signals over the two tissues would be different. Comprehensive binding studies often involve redundant and labour-intensive steps, and in some cases, rigorous quantitation is difficult to achieve.

Because [^{3}H]α,β-MeATP is the first radioligand which could be used for autoradiography, in the present study, a screening experiment has been performed. In addition to the results which

were described in Chapters 4, 5, and 6, autoradiographic localization of [^{3}H]α,β-MeATP binding sites has been carried out on many other tissues from rat, guinea-pig, rabbit, cat, human, frog, starfish, and snail. With the assistance of an image analysis system, the grain densities could be efficiently and accurately counted, thus, a semi-quantitative study was conducted to compare the densities of [^{3}H]α,β-MeATP binding sites in different tissues. Because only one concentration of radioligand was used and the receptor densities were not converted into such a unit as fmol/mg protein, we do not claim that we had revealed the real difference in the densities of [^{3}H]α,β-MeATP binding sites in different tissues. However, these results provide important information for further studies in the these tissues. If necessary, more intensive quantitative autoradiographic studies will be carried out.

8-4 Summary of the autoradiographic results on various tissues

8-4-1 Genito-urinary system: the ureters of rat, guinea-pig and rabbit were moderately labelled by [^{3}H]α,β-MeATP, and the binding could be displaced by β,γ-MeATP; no obvious labelling was observed in rat kidney; the smooth muscle in rat prostate gland was moderately labelled and most of the labelling could be displaced by β,γ-MeATP. The vas deferens of rat, guinea-pig and rabbit was heavily labelled and the specific binding was completely displaced by β,γ-MeATP.

8-4-2 Digestive system: the smooth muscle of rat stomach,

duodenum, ileum, gall-bladder, and biliary duct showed a low density of [^{3}H]α,β-MeATP binding sites and the binding was only partially displaced by β,γ-MeATP; guinea-pig colon and taenia coli were heavily labelled and only a small portion of the binding was displaced by β,γ-MeATP; rat anococcygeus and rabbit rectococcygeus were heavily labelled and most of the binding could be displaced by β,γ-MeATP. Parotid gland, liver and the pancreatic A cells were not labelled while the pancreatic islets showed a low density of labelling. The gastric ligament of star-fish was sparsely labelled, and the oesophagus and rectum of snail were not labelled.

8-4-3 Pulmonary system: In rat and cat lung, the smooth muscle of the bronchi and the alveoli were moderately labelled with [^{3}H]α,β-MeATP, which was partially displaced by β,γ-MeATP. The labelling on vessels will be described later.

8-4-4 Cardiovascular system: The cardiac atrium and ventricle of rat, guinea-pig and rabbit, and the ventricle of frog and snail were not labelled, while the frog atrium showed a low density of specific labelling. The rabbit coronary artery of rabbit showed a low density of specific labelling, which was not observed in the rat coronary artery. The large pulmonary arteries of rat and cat were moderately labelled, while the medium and small arteries showed a higher density of specific labelling. The pulmonary veins also showed a low density of specific labelling. The displacement of the [^{3}H]α,β-MeATP binding by β,γ-MeATP was not complete. Human

pulmonary arteries were also labelled, but the densities were lower than those in rat and cat pulmonary arteries. Human umbilical artery and vein were moderately labelled by [^{3}H]α,β-MeATP and the labelling was partially displaced by β,γ-MeATP and totally displaced by 2-MeSATP. The phenomenon was also seen on the medium-sized cerebral arteries. The penile artery, vein and spongiosium of rat and guinea-pig were heavily labelled and only partially displaced by β,γ-MeATP.

8-4-5 Nervous system: No specific binding was observed in rat brain, spinal cord, dorsal root ganglion, and guinea-pig intermuscular plexus of ileum. A study carried out on cultured neurons from intracardiac ganglia also showed a negative result for P_{2X}-purinoceptor.

8-4-6 Other tissues: Rat skeletal muscle, adipose tissue, lymph node, and adrenal and thymus glands of newborn rat showed no specific binding.

Thus, many tissues have been screened for specific binding sites of [^{3}H]α,β-MeATP, some of them have not been studied for their pharmacological responses to purinergic ligands, such as ureter, prostagland, penile vessels and spongiosium, which may give a hint to the researchers in this area. In most tissues specific binding was found where the P_{2X}-purinoceptor-mediated responses were observed, and the labelling was completely displaced by β,γ-MeATP, including urinary bladder, vas deferens, and arteries from rat, guinea-pig, rabbit and human, and frog

atrium (Burnstock & Meghji, 1981; Hoyle & Burnstock, 1986). In the tissues where only P_{2Y}-purinoceptor-mediated responses or no P_2-purinoceptor-mediated responses were observed, no [^{3}H]α,β-MeATP specific labelling was shown, such as, liver (Keppens & De Wulff, 1985), mammalian heart, rat and guinea-pig urethra, snail heart and rectum (Knight *et al.*, 1992), and guinea-pig intracardiac neurons (Allen & Burnstock, 1990). In the gastrointestinal tract, gall-bladder and biliary duct, the responses to ATP are usually mediated by P_{2Y}-purinoceptors, however, a low density of [^{3}H]α,β-MeATP binding sites was observed, although the binding was only partially displaced by β,γ-MeATP, which indicate that [^{3}H]α,β-MeATP still has some affinity to the P_{2Y}-purinoceptors in some tissues. Such a phenomenon was also seen in bronchial smooth muscle (Welford & Anderson, 1988), and starfish gastric ligament (Knight *et al.*, 1990). In tissues where both P_{2X}-and P_{2Y}-purinoceptors exist the situation is more complicated, such as in pulmonary arteries (Liu *et al.*, 1989a and b), human umbilical vessels (Sexton, personal communication), and human cerebral arteries, the binding can be partially displaced by β,γ-MeATP and completely displaced by the combination of β,γ-MeATP and 2-MeSATP. Because their selectivity are not so high for the P_{2X}- and P_{2Y}-subtypes, it is difficult to assess the proportions of these two subtypes by binding assays. It should be pointed out that many tissues have not been as intensively studied for purinergic responses as the urinary bladder, vas deferens, and blood vessels. Usually only the ATP-elicited responses were measured, it is, therefore, difficult to say which subtype of P_2-purinoceptor

mediates the responses.

In the present autoradiographic localization study, no specific binding site of [^{3}H]α,β-MeATP was observed in the rat brain, spinal cord and dorsal root ganglia. However, there are many reports indicating the existence of ATP-mediated neurotransmission in the CNS, especially in the primary sensory nervous pathways (Krishtal *et al.*, 1983; Jahr & Jessel, 1983; Pyffe & Perl, 1984; Salt & Hill, 1983; Salter & Henry, 1985; Krishtal *et al.*, 1985b). Release of ATP from synaptosomes from different regions of rat brain has been reported (Potter & White, 1980) and stimulation-depedent release of ATP from rat hippocampal slices have also been reported (Wieraszko *et al.*, 1989). Extracellular ATP was found to potentiate the population spike in rat hippocampal slices (Wieraszko & Seyfried, 1989) and evoke inward currents in cultured hippocampal neurons (Inoue *et al.*, 1992). In cultured PC12 cells a large and fast ATP-activated inward current was obseved, followed by a slowly rising outward current (Neuhaus *et al.*, 1991). All these evidence suggests that ATP or its analogues may play an important role in the signal transmission in the CNS, which may be mediated by some other types of P_2-purinoceptors than the P_{2X}-subtype.

8-5 Future work on the molecular characteristics of P_{2X}-purinoceptor

The structure studies on cell-surface receptors have made great strides in recent years. Owing to the application of the powerful

and universal techniques of DNA cloning to these scarce and difficult molecules, the amino acid sequences of a large and increasing number of cell-surface receptors have been, and are being determined. An important outcome is that the evolutionary and structure similarities that exist at the sequence level with the various classes of cell-surface hormone, neurotransmitter, and growth factor receptors.

Based on the structures and functions the transmembrane receptors have been classified into three groups. (a) Receptors with a single transmembrane segment (group 1). The endogenous ligands for group 1 receptors are polypeptide hormones, whose action is related to metabolic changes, or cell proliferation and survival. Most of the receptors in this group characterized to date have an intracellular domain which has tyrosine kinase catalytic activity. (b) Oligomeric receptors incorporating both ligand-binding sites and an ion channel (group 2). The endogenous ligands for group 2 receptors are typical neurotransmitters. The functional distinction is that the response of group 2 receptors are fast. A representative receptor in this group is nicotinic cholinoceptors (nAChRs), which activate a cation (Na^+/K^+) channel. The nAChRs from electric organs and skeletal muscle consists of five subunits with $\alpha_2\beta\gamma\delta$ stoichiometry. The oligomer forms a rosette structure with approximate pentagonal symmetry, which incorporates the ion channel, selectivity filter, and gating mechanism, as well as at least two acetylcholine binding sites. (c) Receptors which exert their effects by coupling to GTP-binding

proteins (G-protein) (group 3). The endogenous ligands for group 3 receptors include both hormones and neurotransmitters, and also external stimulants such as light and adourants.

According to the pharmacological and electrophysiological studies the P_{2X}-purinoceptor-mediated responses in smooth muscle cells are very fast, which lead to the opening of cation channels, and subsequently the changes of membrane potentials. Thus, it is possible that P_{2X}-purinoceptors belong to group 2 receptors. To understand fully the molecular properties of the receptors, the following steps are to be taken:

(a) solubilization of the receptor polypeptide in a form in which the ligand binding activity is preserved;

(b) isolation and purification of the polypeptides carrying the binding sites;

(c) determination of the structure of the receptor polypeptide(s);

(d) reconstitution, identification and study of the function of the receptor polypeptide.

In this study the P_{2X}-purinoceptors with the [^{3}H]α,β-MeATP binding sites have been successfully solubilized and characterized. The purification of P_{2X}-purinoceptors has already been started in cooperation with the MRC Molecular Neurobiology Unit (MRC Centre, Cambridge). The first technique employed was affinity chromatography. α,β-MeATP was linked to gel matrix with a spacer arm between them. Several affinity gels with different lengths of spacer arms linked to different positions on α,β-MeATP molecules (on ribose and adenine base, but not on the

polytriphosphate chain) were synthesized. A digitonin-extract of rat vas deferens membrane preparation was incubated with the affinity gel. Following the elution of bulk proteins, the gel was circulated with a high concentration of ATP and eluted. After the removal of ATP in the eluate, the sample was subjected to radioligand binding assay and electrophoresis. So far, we have not succeeded in detecting the purified receptors with radioligand binding assay. Upon SDS-PAGE electrophoresis of the ATP-eluate, three bands were observed. However, whether these bands are the subunits of P_{2X}-purinoceptor needs to be confirmed. It seemed that the α,β-MeATP gel synthesized had a affinity high enough to detain most of the binding sites in the digitonin-extract (only about 15 to 20% of the binding sites appeared in the breakthrough), which raised three possibilities responsible for the failure to detect the purified receptors with radioligand binding assay. Firstly, the binding of the receptors to the affinity gel was so tight that only a very small amount of receptors was eluted by ATP; secondly, ATP in the eluate was not efficiently removed; thirdly, the receptors lost their binding activity during the purification processes. Now we are modifying the techniques to achieve a higher recovery of the receptors. Recently, we have turned to use another non-ionic detergent, dodecyl maltoside, for the solubilization of the P_{2X}-purinoceptors from the vas deferens membranes. This detergent is also very efficient to extract P_{2X}-purinoceptors and has no obvious inhibitory effect on the [^{3}H]α,β-MeATP binding at its critical micellization concentration (CMC). This detergent has a low CMC (about 0.12 mM) and a pure composition (unlike

digitonin), thus, it works more steadily and is more friendly for the following purification procedures.

Several methods are being tested for the removal of the ATP in the eluate, including dialysis, molecular sieve chromatography, absorption with hydroxylapatite gel, lectin affinity chromatography, or the combination of the methods. An experiment on the reconstitution of the purified receptors into liposomes has been carried out. This technique makes the removal of the eluting ligand simpler and more efficient. In the first attempt, 7% of the purified receptors has been recovered in the liposomes.

For the purification of P_{2X}-purinoceptors there exist two more restrictions, one is that no specific antagonist for the P_{2X}-purinoceptor is available at present, the other is that so far P_{2X}-purinoceptors are richly found in rodent urinary bladder, vas deferens, and blood vessels. Thus, the receptor sources are small, and if a large amount of purified receptors is needed for further studies, such as analysis of amino acid sequences of the receptors, the cost would be too high. Therefore, another task in the near future is to find a receptor source in big animals. Pig vas deferens was once tested, it was found that the receptor density was much lower than that in rat vas deferens.

Owing to the advances in recombinant DNA techniques, it is now possible to determine the amino acid sequence of receptors without using purified receptors. Different receptors in the same group have been shown to have common sequences, and therefore, the

complementary DNA (cDNA) or genomic clones for another receptor in the same group can be isolated by using oligonucleotides corresponding to the common sequences. Two different genomic clones were isolated by this strategy using partial sequences of a subtype of the mAChRs and were shown to be genes for the previously unidentified subtypes of mAChRs (Bonner *et al.*, 1988). The genes for canine A_1 and A_{2a} adenosine receptors have been discovered by using homology-screening protocol whereby the oligonucleotides were designed on the basis of the similarities among genes encoding the superfamily of proteins with seven transmembrane helixes (Libert *et al.*, 1989; 1991; Maeuhant *et al.*, 1990; Linden *et al.*, 1991).

The activities of the expressed receptors can be monitored by measuring the change in membrane potentials induced in *Xenopus* oocytes, into which mRNAs transcribed from successively fractioned cDNA clones have been injected. It is essential in this strategy that the cDNA clone contains the full length that is necessary for expression of the receptor activity, and therefore the length of the mRNA may be a limiting factor. This strategy is especially useful for the receptors composed of a single homogeneous protein, while for those composed of hetero-oligomers it would become more complicated (Haga *et al.*, 1990).

Another technique which will be used for the study of the P_{2X}-purinoceptor functions is the translation of P_{2X}-purinoceptors from crude mRNA extracted from rodent urinary bladder or vas

deferens. The expression of P_{2X}-purinoceptors could be achieved using *Xenopus* oocytes. Such a technique has been employed for the study of P_{2Y}-purinoceptors by using crude mRNA extracted from guinea-pig brain (Honoré *et al.*, 1991). In normal *Xenopus* oocytes it was found that ATP could evoke a depolarizing current response, which was carried out by Cl^- (Lotan *et al.*, 1986), while the activation of P_{2X}-purinoceptors would lead to influx of cations through receptor-operated cation channels. Therefore, the expression of P_{2X}-purinoceptors in *Xenopus* oocytes after injection of mRNA could be monitored by measuring the membrane potentials elicited by P_{2X}-purinoceptor selective ligands and by radioligand binding assays and autoradiography. However, it should be predicted that the yields of P_{2X}-purinoceptors would be very low because the mRNAs for P_{2X}-purinoceptors may account for only 0.01% of the total mRNAs. However, the yields might be increased by mRNA enrichment techniques. Consecutive fractionation may finally lead to the discovery of the mRNA encoding the P_{2X}-purinoceptor, from which the cDNA can be synthesized by reverse transcription and amplified by polymerase chain reaction technique. The cloned cDNA can be used to express large quantity of P_{2X}-purinoceptors.

One of the approaches for the purification of P_{2X}-purinoceptors is to use the monoclonal antibodies against the receptors. Animals can be immunized with membranes prepared from P_{2X}-purinoceptor-rich organs (rodent urinary bladder or vas deferens). The activated spleen cells are fused with myeloma cells. The hybridoma cells are cultured and then cloned. A screening assay will be carried out to identify the cells which

produce the antibodies against P_{2X}-purinoceptors. The clones of the hybridoma cells can be cryopreserved or kept alive by producing hybridoma tumours in mouse. For large scale production of monoclonal antibodies, the hybridoma cells can be cultured *in vitro* or injected intraperitoneally in rats or mouse and then the ascites fluid and serum of the tumour bearing animals are collected.

The monoclonal antibodies are then purified which would become a powerful tool for the study of molecular properties of P_{2X}-purinoceptors. The first important application would be the immunoaffinity chromatography for the purification of the receptors. Another important usage is to identify cDNA clones for receptor subunits. Other applications include: (a) to identify receptor subunits and localize substructures within the receptor subunit; (b) to test the role of structures to which they bind in receptor function; (c) to compare the structures of receptors from different tissues and species; (d) to localize subunits within the quaternary structure of the receptor molecule; (e) to study the synthesis, conformational maturation, and assembly of receptor subunits.

The future of the study on P_{2X}-purinoceptors using the molecular biology technologies is certainly exciting. However, many obstacles on the road should be expected because of the complexity of these techniques and low content of the receptors.

References

ABRAHAM, K.I. & MODAK, M.J. (1984). Affinity labeling of *Escherichia coli* DNA polymerase I with thymidine 5'-triphosphate and 8-azidoadenosine 5'-triphosphate: conditions for optimum labeling, specificity and identification of the labeling site. *Biochem.*, **23**, 1176-1182.

ACEVEDO, C.G. & CONTRERAS, E. (1985). Possible involvement of adenine nucleotides in the neurotransmission of the mouse urinary bladder. *Comp. Biochem. Physiol.*, **82C**, 357-361.

ACEVEDO, C.G. & CONTRERAS, E. (1989). Effect of extracellular calcium and calcium channel antagonists on ATP and field stimulation induced contractions of the mouse urinary bladder. *Gen. Pharmacol.*, **20**, 811-815.

ALLEN, T.G.J. & BURNSTOCK, G. (1990). The actions of adenosine 5'triphosphate on guinea-pig intracardiac neurones in culture. *Br. J. Pharmacol.*, **100**, 753-756.

ANDERSSON, K-E., MATTIASSON, A. & SJÖGREN, C. (1983). Electrically induced relaxation of the noradrenaline contracted isolated urethra from rabbit and man. *J. Urol.*, **129**, 210-214.

ANDREWS, T.M. & HOLTON, P. (1958). The substance P and adenosinetriphosphate (ATP) contents of sensory nerve on

degeneration. *J. Physiol. (London)*, 143, 45-46P.

AREND, L.J., HANDLER, J.S., RHIM, J.S., GUSOVSKY, F. & SPIELMAN, W.S. (1989). Adenosine-sensitive phosphoinositide turnover in a newly established renal cell line. *Am. J. Physiol.*, 256, F1067-F1074.

ASMAWI, M.Z., IRVING, G. & McCURRIE, J.R. (1980). The effect of magnesium ions on the responses of arterial smooth muscle to noradrenaline, histamine and adenosine 5'triphosphate (ATP). *Proc. Br. Pharmacol. Soc.*, 129, P39.

BAGINSKI, E.S., FOA, P.P. & ZAK, B. (1967). Microdetermination of phosphate: study of labile organic phosphate interference. *Clin. Chim. Acta*, 15, 155-158.

BARNARD, E.A. (1992). in *Receptor subunits and Complexes*, eds. Burgen, A.V. and Barnard, E.A. (in press) (Cambridge:Cambridge University Press).

BASU, A. & MODAK, M.J. (1987). An affinity labeling of *ras* p21 protein and its use in the identification of *ras* p21 in cellular and tissue extracts. *J. Biol. Chem.* 262, 2369-2373.

BATRA, S. (1980). Discussion summary: cell fractionation. in *Vascular Neuroeffector Mechanisms*. ed. Bevan, J.A., Godfraind, T., Maxwell, R.A. & Vanhoutte, P.M. pp. 9-12. (New York:Raven Press).

BATRA, S. (1986a). Identification and characterization of muscarinic cholinergic receptors in the isolated plasma membranes and intact tissue of the urinary bladder. *J. Recep. Res.*, **6**, 227-246.

BATRA, S. (1986b). Use of ^{3}H-QNB in the isolation of plasma membrane from smooth muscle of the urinary bladder: effect of oxalate on calcium uptake by the membrane fractions. *Experi-entia*, **42**, 608-611.

BEAN, B.P. & FRIEL, D.D. (1990). ATP-activated channels in excitable cells. in *Ion Channels*, vol. 2, ed. Narahashi, T., pp. 169-203 (New York:Revan Press).

BENHAM, C.D. (1989). ATP-activated channels gate calcium entry in single smooth muscle cells dissociated from rabbit ear artery. *J. Physiol. (London)*, **419**,689-701.

BENHAM, C.D. (1990). ATP-gated channels in vascular smooth muscle cells. in *Biological Actions of Extracellular ATP*, Ann-als of the New York Academy of Sciences, vol. 603, eds. Dubyak, G.R. & Fedan, J.S. pp. 275-299 (New York:The New York Academy of Sciences).

BENHAM, C.D. & TSIEN, R.W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature*, **328**,275-278.

BENHAM, C.D., BOLTON, T.B., BYRNE, N.G. & LARGE, W.A. (1987). Action of externally applied adenosine triphosphate on single smooth muscle cells from rabbit ear artery. *J. Physiol. (London)*, 387, 473-488.

BENNETT, J.P., COCKROFT, S. & GOMPERTS, B.D. (1981). Rat mast cells permeabilized with ATP secrete histamine in response to calcium ions buffered in the micromolar range. *J. Physiol. (London)*, 317, 335-345.

BENNETT, J.P. & YAMAMURA, H.I. (1985). Neurotransmitter, hormone, or drug receptor binding methods. in *Neurotransmitter Receptor Binding*, 2nd ed., eds. YAMAMURA, H.I. & Enna, S.J. pp. 61-89 (New York:Revan Press).

BERRIE, C.P., HAWKINS, P.T., STEPHENS, L.R., HARDEN, T.K. & DOWNES, C.P. (1989). Phosphatidylinositol 4,5-biphosphate hydrolysis in turkey erythrocytes is regulated by P_{2Y}-purinoceptors. *Mol. Pharmacol.*, 35, 526-532.

BERTRAND, G., CHAPAL, J., LOUBATIERES-MARIANI, M.M. & ROYE, M. (1987). Evidence for two different P_2-purinoceptors on β cell and pancreatic vascular bed. *Br. J. Pharmacol.*, 91, 783-787.

BIEGON, A., RAINBOW, T.C. & McEWEN, B.S. (1982). Quantitative autoradiography of serotonin receptors in the rat brain. *Brain Res.*, 242, 197-204.

BIRDSALL, N.J.M. & HULME, E.C. (1976). Biochemical studies on muscarinic acetylcholine receptors. *J. Neurochem*, **27**, 7-16.

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1977). in *Cholinergic Mechanisms and Psychopharmacology*, ed. Jenden, D.J. pp. 25-33 (New York:Plenum).

BJÖRNSSON, O.G., MONCK, J.R. & WILLIAMSON, J.R. (1989). Identification of P_{2Y} purinoceptor associated with voltage-activated cation channels in cardiac ventricular myocytes of the rat. *J. Biochem.*, **186**, 395-404.

BO, X. & BURNSTOCK, G., (1990), The effects of Bay K 8644 and nifedipine on the responses of rat urinary bladder to electrical field stimulation, β,γ-methylene ATP and acetylcholine, *Br. J. Pharmacol.*, 101:494-498.

BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science*, **237**, 527-532.

BOAST, C.A., SNOWHILL, E.W. & ALTAR, C.A. (eds.) (1986) *Quantitative Receptor Autoradiography*, (New York:Alan R. Liss, Inc.).

BOUVIER, M.M., EVANS, M.L. & BENHAM, C.D. (1991). Calcium influx induced by stimulation of ATP receptors on neurons cultured from

rat dorsal root ganglia. *Eur. J. Neurosci.*, 3, 285-291.

BOYER, J.L., DOWNES, C.P. & HARDEN, T.K. (1989). Kinetics of activation of phospholipase C by P_{2Y} purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.*, 264, 884-890.

BOYER, J.L., COOPER, C.L. & HARDEN, T.K. (1990). [^{35}S]3'-O-(4-Benzoyl)benzoyl ATP as a photoaffinity label for a phospholipase C-coupled P_{2Y}-purinergic receptor. *J. Biol. Chem.* 265, 13515-13520.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254

BRADING, A.F. (1990). in *Neurobiology of Incontinence, Ciba Foundation Symposium* 151, p.19 (Wiley:Chichester).

BRADING, A.F. & MOSTWIN, J.L. (1989). Electrical and mechanical responses of guinea-pig bladder muscle to nerve stimulation. *Br. J. Pharmacol.*, 98, 1083-1090.

BRADING, A.F. & WILLIAMS, J.H. (1990). Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and α,β-methylene ATP. *Br. J. Pharmacol.*, 99, 493-498.

BRINDLEY, G.S. & CRAGGS, M.D. (1975). The effect of atropine in the urinary bladder of the baboon and man. *J. Physiol. (London)*, 256, 55P.

BRIZZOLARA, A.L. & BURNSTOCK, G. (1990). Evidence for noradrenergic-purinergic cotransmission in the hepatic artery of the rabbit. *Br. J Pharmacol.*, 99, 835-839.

BRUNS, R.F. (1990). Adenosine receptors: Roles and pharmacology. in *Biological Actions of Extracellular ATP, The Annuals of the New York Academy of Sciences. Vol. 603*, eds. Dubyak, G.R. & Fedan, J.S., pp. 211-226 (New York:The New York Academy of Sciences).

BRUNS, R.F., DALY, J.W. & SNYDER, S.H. (1980). Adenosine receptors in brain membranes: binding of N^6-cyclohexyl[^{3}H]adenosine and 1,3-diethyl-8-[^{3}H]phenylxanthine. *Proc. Nat. Acad. Sci. USA.*, 77, 5547-5551.

BRUNS, R.F., LAWSON-WENDLING, K. & DUGSLEY, T.A. (1983). A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.*, 132, 74-81.

BUCKLEY, N. & BURNSTOCK, G. (1983). Autoradiographic demonstration of peripheral adenosine binding sites using [^{3}H]NECA. *Brain Res.*, 269, 374-377.

BURNSTOCK, G. (1969). Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. *Pharmacol.*

Rev., **21**, 247-324.

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmacol. Rev.*, **24**, 509-581.

BURNSTOCK, G. (1976). Do some nerve cells release more than one transmitter? *Neuroscience*, **1**, 239-248.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. in *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach, eds.* Straub, R.W. & Bolis, L. pp. 107-118 (New York:Raven Press).

BURNSTOCK, G. (1986). The changing face of autonomic neurotransmission. *Acta Physiol. Scand.*, **126**, 67-91.

BURNSTOCK G. (1988). Sympathetic purinergic transmission in small blood vessels. *Trends Pharmacol. Sci.* 9, 116-117

BURNSTOCK, G. (1990a). Purinergic mechanism, in *Biological Actions of Extracellular ATP, The Annuals of the New York Academy of Sciences.* Vol. 603, eds. Dubyak, G.R. & Fedan, J.S., pp. 1-17 (New York:The New York Academy of Sciences).

BURNSTOCK, G. (1990b). Dual control of local blood flow by purines. in *Biological Actions of Extracellular ATP. Annals of the New York Academy of Sciences*, vol. 603, eds. Dubyak, G.R. & Fedan,

J.S., pp. 31-45 (The New York: New York Academy of Sciences).

BURNSTOCK, G. (1990c). Classification and characterization of purinoceptors. in *Purines in Cellular Signalling: Targets for New Drugs*. eds. JACOBSON, K., DALY, J. & MANGANIELLO, V., pp. 241-253. (New York: Springer).

BUNRSTOCK, G. (1991a). Plasticity in expression of co-transmitters and autonomic nerves in aging and disease. in *Plasticity and Regeneration of the Nervous System*, eds. TIMIRAS, P.S. *et al.*, pp. 291-301. (New York:Plenum Press)

BURNSTOCK, G. (1991b). Distribution and roles of Purinoceptor subtypes, *Nucleosides and Nucleotides*, **10**, 917-930.

BURNSTOCK, G. (1991c). Overview (purinergic receptors), in *Role of Adenosine and Adenine Nucleotides in the Biological System*, eds. IMAI, NAKAZAWA, M., pp. 3-16. (Elsevier Science Publisher BV).

BURNSTOCK, G. & CAMPBELL, G. (1963). Comparative physiology of the vertebrate autonomic nervous system II. Innervation of the urinary bladder of the ringtail possum (*Pseudocheirus peregrinus*). *J. Exp. Biol.*, 40, 421-436.

BURNSTOCK, G. & MEGHJI, P. (1981). Distribution of P_1- and P_2-purinoceptors in the guinea-pig and frog heart. *Br. J. Pharmacol.*, 73, 879-885.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, 5, 433-440.

BURNSTOCK, G., DUMSDAY, B. & SMYTHE, A. (1972). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br. J. Pharmacol.*, 44,451-461.

BURNSTOCK, G, CROWE, R, WONG, H.K. (1979). Comparative pharmacological and histochemical evidence for purinergic inhibitory innervation of the portal vein of the rabbit, but not guinea-pig. *Br. J. Pharmacol.*, 65, 377-388

BURNSTOCK, G., CAMPBELL, G., BENNETT, M. & HOLMAN, M.E. (1964). Innervation of the guinea-pig taenia coli: are there intrinsic inhibitory nerves which are distinct from sympathetic nerves? *Int. J. Neuropharmacol.*, 3, 163-166.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, 63, 125-138.

BURNSTOCK, G., CROWE, R., KENNEDY, C. & TÖRÖK, J. (1984). Indirect evidence that purinergic modulation of perivascular adrenergic neurotransmission in the portal vein is a physiological proess. *Br. J. Pharmacol.*, 82, 359-368.

BURNSTOCK, G., STEWART-LEE, A., BRIZZOLARA, A., TOMLIINSON, A. & CORR, L. (1991). Dual control by nerves and endothelial cells of arterial blood flow in atherosclerosis. in *Atherosclerotic Plaques*, eds. WISSLER, R.W. *et al.*, pp. 285-292. (New York: Plenum Press).

BURT, D.R. (1985). Criteria for receptor identification. in *Neurotransmitter receptor Binding*. ed. Yamamura, H.I., Enna, S.J. & Kuhar, M.J. pp. 41-60 (New York:Revan Press).

CALLAHAN, S.M. & CREED, K.E., (1981). Electrical and mechanical activity of the isolated lower urinary tract of the guinea-pig. *Br. J. Pharmacol.*, 74, 353-358.

CASADO, V. CANTI, C., MALLOL, J, CANELA, E.I., LLUIS, C. & FRANCO, R. (1990). Solubilization of A_1 adenosine receptor from pig brain: characterization and evidence of the role of the cell membrane on the coexistence of high- and low-affinity states. *J. Neurosci. Res.*, 26, 461-473.

CHANG, R.S.L. & SNYDER, S.H. (1980). Histamine H_1 receptor binding sites in guinea pig brain membranes: regulation of agonist interaction by guanine nucleotides and cations. *J. Neurochem.*, 34, 916-922.

CHAUDRY, I.H. (1990). Use of ATP following shock and ischemia. in

Biological Actions of Extracellular ATP, Annals of the New York Academy of Sciences, vol. 603, eds. Dubyak, G.R. and Fedan, J.S. pp. 130-141 (New York:New York Academy of Sciences).

CHEN, H-I., (1990). Evidence for the presynaptic actions of 5-hydroxytryptamine and the involvement of purinergic innervation in the rabbit lower urinary tract. *Br. J. Pharmacol.*, **101**, 212-216.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099-3108.

CHEUNG, D.W. & FUJIOKA, M. (1986). Inhibition of the excitatory junction potential in the guinea-pig saphenous artery by $ANAPP_3$. *Br. J. Pharmacol.*, **89**, 3-5.

CHRISTIE, A., SHARMA, V.K. & SHEU, S.-S. (1992). Mechanism of extracellular ATP-induced increase of cytosolic Ca^{2+} concentration in isolated rat ventricular myocytes. *J. Physiol. (London)* , **445**, 369-388.

COCKROFT, S. & GOMPERTS, B.D. (1979a). Activation and inhibition of calcium-dependent histamine secretion by ATP ions applied to rat mast cells. *J. Physiol. (London)*, **296**, 229-243.

COCKROFT, S. & GOMPERTS, B.D. (1979b). ATP induces nucleotide permeability in rat mast cells. *Nature*, **279**, 541-542.

COCKROFT, S. & GOMPERTS, B.D. (1980). The ATP^{4-} receptor of rat mast cells. *Biochem. J.*, 188, 789-798.

COHN, M. (1990). Structure and chemical properties of ATP and its metal complexes in solution. in *Biological Actions of Extracellular ATP. Ann-als of the New York Academy of Sciences*, Vol. 603, ed. Dubyak, G.R. & Fedan, J.S. pp. 151-164 (New York:The New York Academy of Sciences).

COOPER, C.L., MORRIS, A.J. & HARDEN, T.K. (1989). Guanine nucleotide-sensitive interaction of a radiolabeled agonist with a phospholipase C-linked P_{2Y}-purinergic receptor. *J. Biol. Chem.*, 264, 6202-6206.

COOPER, D.M.F., YEUNG, S-M.H., PEREZ-REYES, E. & FOSSOM, L.H. (1984). A central role for magnesium in the regulation of inhibitory adenosine receptors. In *Neurotransmitter Receptors, Mechanisms of Action and Regulation*. eds. Kito., K., Segawa, T., Kuriyama, K., Yamamura, H.I. & Olsen, R.W. pp. 17-30 (New York:Plenum Press).

CORNISH-BOWDEN, A. & KOSHLAND, D.E. (1975). Diagnostic uses of the Hill (Logit and Nernst) plots. *J. Mol. Biol.*, 95, 201-212.

CORR, L. & BURNSTOCK, G. (1991). Vasodilator response of coronary smooth muscle to the sympathetic co-transmitters noradrenaline and

adenosine 5'-triphosphate. *Br. J. Pharmacol.*, **104**, 337-342.

COWAN, W.D. & DANIEL, E.E. (1983). Human female bladder and its noncholinergic contractile function. *Can. J. Physiol. Pharmacol.*, **61**, 1236-1246.

CRAGGS, M.D. & STEPHENSON, J.D. (1982). The effects of parasympathetic blocking agents on bladder electromyograms and function in conscious and anaesthetized cats. *Neuropharmacol.*, **21**, 695-703.

CRAGGS, M.D. & STEPHENSON, J.D. (1986). A non-cholinergic urinary bladder mechanism in New World Primates. *J. Physiol. (London)*, **377**, 78P.

CUATRECASAS, P. & HOLLENBERG, M.D. (1976). Membrane receptor and hormone action. *Adv. Protein Chem.*, 30,251-451.

CUNNANE, T.C. & MANCHANDA, R. (1988). Electrophysiological analysis of the inactivation of sympathetic transmitter in the guinea-pig vas deferens. *J. Physiol. (London)* , **404**, 349-364.

CUSACK, N.J. & HOURANI, S.M.O. (1984). Some pharmacological and biochemical interactions of the enantiomers of adenylyl 5'-(β,γ-methylene)diphosphate with the guinea-pig urinary bladder. *Br. J. Pharmacol.*,, **82**, 155-159.

CUSACK, N.J. & HOURANI, S.M.O. (1990). Subtypes of P_2-

purinoceptors: studies using analogues of ATP. in *Biological Actions of Extracellular ATP, Ann-als of the New York Academy of Sciences*, vol. 603, eds. Dubyak, G.R. and Fedan, J.S. pp. 172-181 (New York:The New York Academy of Sciences).

DANZIGER, R.S., RAFFAELI, S., MORENO-SANCHEZ, R., SAKAI, M., CAPOGROSSI, M.C., SPURGEON, H.A., HANSFORD, R.G. & LAKATTA, E.G. (1988). Extracellular ATP has a potent effect to enhance cytosolic calcium and contractility in single ventricular myocyte. *Cell Calcium*, 9, 193-199.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U, VAN DER MERWE, P.A. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology*, 126, 80-87.

DE MAY, J.G. & VANHOUTTE, P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J. Physiol. (London)* , 316, 247-355.

DE YOUNG, M.B. & SCARPA, A. (1989). ATP receptor -induced Ca^{2+} transients in cardiac myocytes: sources of mobilized Ca^{2+}. *Am. J. Physiol.*, 257, C750-C758.

DEAN, D.M. & DOWNIE, J.W. (1978). Contribution of adrenergic and "purinergic" neurotransmission to contraction in rabbit detrusor. *J. Pharmacol. Exp. Ther.*, 207, 431-445.

DECKERT, J., MORGAN, P.F., BISSERBE, J.-C., JACOBSON, K.A., KIRK, K.L., DALY, J.W. & MARANGOS, P.J. (1988). Autoradiographic localization of mouse brain adenosine receptors with an antagonist ([^{3}H]xanthine amine congener)ligand probe. *Neurosci. Lett.*, **86**, 121-126.

DELAHUNTY, T.M., CRONIN, M.J. & LINDEN, J. (1988). Regulation of GH_3-cell function via adenosine A_1 receptors: Inhibition of prolactin release, cyclic AMP production and inositol phosphate generation. *Biochem. J.*, **255**, 69-77.

DEN HERTOG, A., NELEMANS, A., & VAN DEN AKKER, J. (1989). The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of the guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **166**, 531-534.

DONALD, F.M.S. (1985). A non-adrenergic, non-cholinergic (NANC) excitatory responses in frog atria. *Br. J. Pharmacol.*, **85**, 211P.

DRUNRY, A.N., SZENT-GYÖGRYI, A. (1929). The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. *J. Physiol. (London)*, **68**, 213-237.

DUBYAK, G.R. (1991). Signal transduction by P_2-purinergic receptors for extracellular ATP. *Am. J. Respir. Cell Mol. Biol.* **4**, 295-300.

DUBYAK, G.R. & FEDAN, J.S. (eds) (1990). *Biological Actions of Extracellular ATP. Annals of the New York Academy of Sciences*, vol. 603, (New York:The New York Academy of Sciences).

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, 93, 243-245.

EHLERT, F.J., ROESKE, W.R. & YAMAMURA, H.I. (1984). Muscarinic receptor [^{3}H]ligand binding methods. in *Brain Receptor Methodologies, Part A: General Methods and Concepts. Amines and Acetylcholine*, eds. Marangos, P.J., Campbell, I.C. & Cohen, R.M. pp. 339-355 (Orlando:Academic Press).

ERB, L., HOOVER, R.K. & WEISMAN, G.A. (1992). Identification of cell-surface nucleotide-binding proteins in mouse fibroblasts using BBzATP and BzUTP: correlation with P_2 receptor activities. *Int. J. Purine Pyrimidine Res.*, 3, 74.

EVANS, R.J., DERKACH, V., SURPRENANT, A. (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature*, 357, 503-505.

EVANS, W.H. (1979). Preparation and characterisation of mammalian plasma membranes. in *Laboratory Techniques in Biochemistry and Molecular Biology*, vol. 7, eds. Work, T.W. and Work, E. pp. 1-266 (Amsterdam:North-Holland).

FEDAN, J.S. & LAMPORT, S. J. (1990). P_2-purinoceptor antagonists. in *Biological Actions of Extracellular ATP, The Ann-als of the New York Academy of Sciences. Vol. 603,* eds. Dubyak, G.R. & Fedan, J.S., pp. 182-197 (New York:The New York Academy of Sciences).

FEDAN, J.S., HOGABOON, G.K., WESTFALL, D.P. & O'DONNELL, J.P. (1982). Comparison of contractions of the smooth muscle of the guinea-pig vas deferens induced by ATP and related nucleotides. *Eur. J. Pharmacol.*, **81**, 193-204.

FEDAN, J. S., HOGABOOM, G. K., O'DONNELL, J. P., JENG, S. J., & GUILLORY, R. J. (1985). Interaction of [^{3}H]arylazido aminopropionyl ATP ([^{3}H]ANAPP$_3$) with P_2-purinergic receptors in the smooth muscle of the isolated guinea-pig vas deferens. *Eur. J. Pharmacol.* **108**, 49-61

FEDAN, J. S., HOGABOOM, G. K. & O'DONNELL, J. P. (1986). Further comparison of contractions of the smooth muscle of the guinea-pig isolated vas deferens induced by ATP and related analogs. *Eur. J. Pharmacol.*, **129**, 279-291.

FEDAN, J.S., DAGIRMANJIAN, J.P., ATTFIELD, D. & CHIDECKEL, E.W. (1990). Evidence that the P_{2X} purinoceptor of the smooth muscle of the guinea pig vas deferens is an ATP^{4-} receptor. *J. Pharmacol. Exp. Ther.*, **255**, 46-51.

FLAVAHAN, N.H. & VANHOUTTE, P.M. (1986). Sympathetic purinergic vasoconstriction and thermosensitivity in a canine cutaneous vein.

J. Pharmacol. Exp. Ther., 239, 784-789.

FOORTES, P.A.G., ELLORY, J.C. & LEW, V.L. (1973). Suramin: a potent ATPase inhibitor which acts on the inside surface of the sodium pump. *Biochim. Biophys. Acta*, 318, 262-272.

FORSBERG, E.J., FEUERSTEIN, G., SHOHAMI, E. & POLLARD, H.B. (1987). Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells by means of P_2-purinergic receptors. *Proc. Natl. Acad. Sci. USA.*, 84, 5630-5634.

FRIEL., D.D. (1988). An ATP-sensitive conductance in single smooth muscle cells from the rat vas deferens. *J. Physiol. (London)*, 401,361-380.

FRIEL, D.D. & BEAN, B.P. (1988). Two ATP-activated conductance in bullfrog atrial cells. *J. Gen. Physiol.*, 91, 1-27.

FUJJI, K., (1988). Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig, rabbit and pig urinary bladder. *J. Physiol.*, 404, 39-52.

FYFFE, R.E.W. & FERL, E.R. (1984). Is ATP a central synaptic mediator for certain primary afferent fibres from mammalian skin? *Proc. Natl. Acad. Sci. USA.*, 81, 6890-6893.

GADDUM, J.H. & HOLTZ, P. (1933). The localization of action of drugs on the pulmonary vessels of dogs and cats. *J. Physiol.*, **77**, 139-158.

GAVISH, M., GOODMAN, M.M. & SNYDER, S.H. (1982). Solubilized adenosine receptors in the brain: regulation by guanine nucleotides. *Science*, **215**, 1633-1635.

GILLESPIE, J.H. (1933). The biological significance of the linkages in adenosine triphosphate acid. *J. Physiol. (London)*, **80**, 345-359.

GOODMAN, R.R. & SNYDER, S.H. (1982). Autoradiographic localization of adenosine receptors in rat brain using [^{3}H]cyclohexyladenosine. *J. Neurosci.*, **2**, 1230-1241.

GOODMAN, R.R., COOPER, M.J., GAVISH, M. & SNYDER, S. (1982). Guanine nucleotide and cation regulation of the binding of [^{3}H]cyclohexyladenosine and [^{3}H]diethylphenylxanthine to adenosine A_1 receptors in brain membranes. *Mol. Pharmacol.*, **21**, 329-335.

GOODMAN, R.R., KUHAR, M.J., HESTER, L. & SNYDER, S.H. (1983). Adenosine receptors: autoradiographic evidence for their location on axon terminals of excitatory neurons. *Science*, **230**, 967-969.

GORDON, J.L., (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309-319.

GORDON, J.L. (1990). The effect of ATP on endothelium. in *Biological Actions of Extracellular ATP, Annals of the New York Academy of Sciences, vol. 603*, eds. Dubyak, G.R. and Fedan, J.S. pp. 46-52 (New York:New York Academy of Sciences).

GREEN, H.N. & STONER, H.B. (1950). *Biological Actions of the Adenine Nucleotides*, (London:Lewis).

GREENBERG, D.A., U'PRICHARD, D.C., SHEEHAN, P. & SNYDER, S.H. (1978). Alpha-Noradrenergic receptors in the brain: differential effects of sodium on binding of [^{3}H]agonists and [^{3}H]antagonists. *Brain Res.*, **140**, 378-384.

GYLFE, E. & HELLMAN, B. (1987). External ATP mimics carbachol in initiating calcium mobilization from pancreatic β-cells conditioned by previous exposure to glucose. *Br. J. Pharmacol.*, **92**, 281-289.

HAGA, T., HAGA, K. & HULME, E.C. (1990). Solubilization, purification, and molecular characterization of receptors: principles and strategy. in *Receptor Biochemistry: A Practical Approach*, ed: Hulme, E.C. pp. 1-50 (Oxford:IRL Press).

HÄGGBLAD, J., ERIKSSON, H. & HEILBRONN, E. (1985). ATP-induced cation influx in myotubes in additive to cholinergic agonist action. *Acta Physiol. Scand.*, **125**, 389-393.

HÄGGBLAD, J. & HEILBRONN, E. (1988). P_2-purinoceptor-stimulated phosphoinositide turnover in chick myotubes. *FEBS Lett.*, **235**, 133-136.

HANTRAYE, P., KAIJIMA, M., PRENANT, C., GUIBERT, B., SASTRE, J., COUZEL, M., NAQNET, R., COMAR, D. & MAZIERE, M. (1984). Central type benzodiazepine binding sites: a positron emission tomography study in the baboon's brain. *Neurosci. Lett.*, **48**, 115-120.

HARDEN, T.K., STEPHENS, L., HAWKINS, P.T. & DOWNES, C.P. (1987). Turkey erythrocyte membranes as a model for regulation of phosphalipase C by guanine nucleotides. *J. Biol. Chem.*, **262**, 9057-9061.

HARDEN, T.K., HAWKINS, P., STEPHENS, L., BOYER, L. & DOWNES, C.P. (1988). Phosphoinositide hydrolysis by guanosine 5'-[γ-thio]triphosphate-activated phospholipase C of turkey erythrocyte membranes. *Biochem. J.*, **252**, 583-593.

HARDEN, T.K., BOYER, J.L., BROWN, H.A., COOPER, CC.L., JEFFS, R.A. & MARTIN, M.W. (1990). Biochemical properties of a P_{2Y}-purinergic receptor. in *Biological Actions of Extracellular ATP*, Annals of New York Academy of Sciences, vol., 603, eds. Dubyak, G.R. & FEDAN, J.S. pp. 256-266 (New York: The New York Academy of Sciences).

HARRIS, W.E., SWANSON, P.D. and STAHL, W.L. (1973). Ouabain binding sites and the $(Na^+ + K^+)$-ATPase of brain microsomal

membranes. *Biochim. Biophys. Acta*, 298, 680-689.

HENDERSON, V.E. & ROEPKE, M.E. (1934). The rôle of acetylcholine in bladder contractile mechanisms and in parasympathetic ganglia. *J. Pharmacol. Exp. Ther.*, 51, 97-111.

HERKENHAM, M. (1984). Autoradiographic demonstration of receptor distributions. in *Brain Receptor Methodologies, Part A General Methods and Concepts. Amines and Acetylcholine*, eds. Marangos, P.J., Campbell, I.C. & Cohen, R.M. pp. 127-152 (Orlando:Academic Press).

HERKENHAM, M. (1988). Receptor autoradiography: optimizing anatomical resolution. in *Receptor Localization: Ligand Autoradiography, Receptor Biochemistry and Methodology, vol. 13*, eds. Leslie, F. & Altar, C.A. pp. 37-47 (New York:Alan R. Liss).

HILL, A.V. (1909). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J. Physiol. (London)*, 40, iv-vii.

HILLS, J., MELDRUM, L.A., KLARSKOV, P. & BURNSTOCK, G. (1984). A novel non-adrenergic, non-cholinergic nerve-mediated relaxation of the pig bladder neck: an examination of possible neurotransmitter candidates. *Eur. J. Pharmacol.*, 99, 287-290.

HINDMARSH, J.R., IDOWU, O.A., YEATES, W.K. & ZAR, M.A. (1977).

Pharmacology of electrically evoked contractions of human bladder. *Proc. Br. Pharmacol. Soc.*, 115P.

HOGABOON, G.K., O'DONNELL, J.P. & FEDAN, J.S. (1980). Purinergic receptors: photoaffinity analog of adenosine triphosphate is a specific adenosine triphosphate antagonist. *Science*, **208**, 1273-1276.

HOITING, B., MOLLEMAN, A., DUIN, M., DEN HERTOG, A. & NELEMANS, A. (1990a). P_2 purinoceptor-mediated inositol phosphate formation in relation to cytoplasmic calcium in DDT_1 MF-2 smooth muscle cells. *Eur. J. Pharmacol. (Mol. Pharmacol. Sec.)*, **189**, 31-39.

HOITING, B., MOLLEMAN, A., NELEMANS, A. & DEN HERTOG, A. (1990b). P_2-purinoceptor-activated membrane currents and inositol tetrakisphosphate formation are blocked by suramin. *Eur. J. Pharmacol.*, **181**, 127-131.

HOLTON, P. (1959). The liberation of adenosine triphosphate on antidromic stimulation of the sensory nerves. *J. Physiol. (London)*, **145**, 494-504.

HOLTON, F.A. & HOLTON, P. (1953). The possibility that ATP is a transmitter at sensory nerve endings. *J. Physiol. (London)*, **119**, 50-51P.

HOLTON, F.A. & HOLTON, P. (1954). The capillary dilator substances in dry powders of spinal roots; a possible role of adenosine

triphosphate in chemical transmission from nerve endings. *J. Physiol. (London)*, **126**, 124-140.

HONORÉ, F., FOURNIER, F., COLLIN, T., NARGEOT, J. & GUILBAULT, P. (1991). Functional expression of P_{2Y} purinoceptor in *Xenopus* oocyte injected with brain mRNA. *Pflügers Arch.*, **418**, 447-452.

HOURANI, S.M.O. (1984). Desensitization of the guinea-pig urinary bladder by the enantiomers of adenylyl 5'-(β,γ-methylene) diphosphate and by substance P. *Br. J. Pharmacol.*, **82**, 161-164.

HOURANI, S.M.O., WELFORD, L.A. & CUSACK, N.J. (1985). L-AMP-PCP, an ATP receptor agonist in guinea-pig bladder is inactive on taenia coli. *Eur. J. Pharmacol.*, **108**, 197-200.

HOURANI, S.M.O., LOIZOU, G.D. & CUSACK, N.J. (1986). Pharmacological effects of L-AMP-PCP on ATP receptors in smooth muscle. *Eur. J. Pharmacol.* **131**, 99-103.

HOURANI, S.M.O., WELFORD, L.A., LOIZOU, G.D. & CUSACK, N.J. (1988). Adenosine 5'-(2-fluorodiphosphate) is a selective agonist at P_2-purinoceptors mediating relaxation of smooth muscle. *Eur. J. Pharmacol.*, **147**, 131-136.

HOURANI, S.M.O. & CHOWN, J.A. 1989. The effects of some possible inhibitors of ectonucleotidases on the breakdown and pharmacological effects of ATP in the guinea-pig urinary bladder.

Gen. Pharmacol., 4, 413-416.

HOUSTON, D.A., BURNSTOCK, G. & VANHOUTTE, P.M. (1987). Different P_{2Y}-purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. *J. Pharmacol. Exp. Ther.*, **241**, 501-506.

HOWSON, W., TAYLOR, E.M., PARSONS, M.E., NOVELLI, R., WILCZYNSKA, M.A. & HARRIS, D.T. (1988). Synthesis and biological evaluation of ATP analogues acting at putative purinergic P_{2X}-receptor (on the guinea-pig bladder). *Eur. J. Med. Chem.*, 23, 433-439.

HOYLE, C.H.V. (1992). Transmission: Purine. in *Autonomic Neuroeffector Mechanisms*, eds. Burnstock, G. and Hoyle, C.H.V. pp.367-407 (Chur:Harwood Academic).

HOYLE, C.H.V. & BURNSTOCK, G. (1986). Evidence that ATP is a neurotransmitter in frog heart. *Eur. J. Pharmacol.*, **114**, 239-240.

HOYLE, C.H.V., CHAPPLE, C. & BURNSTOCK, G. (1989). Isolated human bladder: evidence for an adenine dinucleotide acting on P_{2X}-purinoceptors and for purinergic transmission. *Eur. J. Pharmacol.*, **174**, 115-118.

HOYLE., C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617-621.

HOYLE, C.H.V. & Burnstock, G. (1991). ATP receptors and their physiological roles. in *Adenosine in the Nervous System*, ed. Stone, T.W. pp. 43-75 (London:Academic Press).

HUANG, H.-M & SUN, G.Y. (1988). Effects of ATP on phosphatidylinositol-phospholipase C and inositol 1-phosphate accumulation in rat brain synaptosomes. *J. Neurochem.*, 50, 366-374.

HUME, R.I. & HONIG, M.G. (1986). Excitatory action of ATP on embryonic chick muscle. *J. Neurosci.*, 6, 681-690.

HUME, R.I. & THOMAS, S.A. (1988). Multiple actions of adenosine 5'-triphosphate on chick skeletal muscle. *J. Physiol. (London)*, 406, 503-524.

HUSTED, S., SJöGREN, C. & ANDERSSON, K-E. (1983). Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. *J. Urol.*, 130, 392-398.

HÜTTEMANN, E., UKENA, D., LENSCHOW, V. & SCHWABE, U. (1984). R_a adenosine receptors in human platelets: characterization by 5'-N-ethylcarboxamido [^{3}H]adenosine binding in relation to adenylate cyclase activity. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 325, 226-233.

INNIS, R.B. & SNYDER, S.H. (1980). Distinct cholecystokinin receptors in brain and pancreas. *Proc. Nat. Acad. Sci. USA.*, **77**, 6917-6921.

INNIS, R.B., MANNING, D.C., STEWWART, J.M. & SNYDER, S.H. (1981). [^{3}H]Bradykinin receptor binding in mammalian tissue membranes. *Proc. Nat. Acad. Sci. USA.*, **78**, 2630-2634.

INOUE, K., NAKAZAWA, K., FUJIMORI, K., WATANO, T. & TAKANAKA, A. (1992). Extracellular adenosine 5'-triphosphate-evoked glutamate release in cultured hippocampal neurons. *Neurosci. Lett.*, **134**, 215-218.

INOUE, T, & KANNAN, M.S. (1988). Nonadrenergic and noncholinergic excitatory neurotransmission in the rat intrapulmonary artery. *Am. J. Physiol.* **245**, 1142-1148.

INOUE, R. & BRADING, A.F. (1990). The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **100**, 619-625.

INOUE, R. & BRADING, A.F. (1991). Human, pig and guinea-pig bladder smooth muscle cells generate similar inward currents in response to purinoceptor activation. *Br. J. Pharmacol.*, **103**, 1840-1841.

IRVING, H.R. & EXTON, J.H. (1987). Phosphatidylcholine breakdown in rat liver plasma membranes. *J. Biol. Chem.*, **262**, 3440-3343.

JACOBSON, K.A., DALY, J.W. & MANGANIELLO, V. (eds.) (1990). *Purines in Cellular Signalling: Targets for New Drugs*. (New York:Springer-Verlag).

JAHR, C.E. & JESSEL, T.M. (1983). ATP excites a subpopulation of rat dorsal horn neurones. *Nature*, 304, 730-733.

JEFFS, R.A., COOPER, C.L. & HARDEN, T.K. (1991). Solubilization of a guanine nucleotide-sensitive form of the P_{2Y}-purinergic receptor. *Mol. Pharmacol.* 40, 85-92.

JOHNS, A. (1979). The effect of magnesium-free solutions on the responses of the guinea pig urinary bladder to adenosine 5'-triphosphate and nerve stimulation. *Can. J. Pharmacol.* 57, 1320-1323.

JOHNS, A. & PATON, D.M. (1977). The effect of indomethacin on atropine-resistant transmission in rabbit and monkey urinary bladder: evidence for involvement of prostaglandins in transmission. *Prostaglandins*, 13, 245-254.

KASAKOV, L. & BURNSTOCK, G. (1983). The use of slowly degradable analog, α,β-methylene ATP, to produce desensitisation of the P_2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *Eur. J. Pharmacol.*, 86, 291-294.

KENNEDY, C. & BURNSTOCK, G. (1985a). ATP produces vasodilation via P_1-purinoceptors and vasoconstriction via P_2-purinoceptors in the isolated rabbit central ear artery. *Blood Vessels*, **22**, 145-155.

KENNEDY, C. & BURNSTOCK, G. (1985b). Evidence for two types of P_2-purinoceptor in longitudinal muscle of the rabbit portal vein. *Eur. J. Pharmacol.*, **111**, 49-56.

KENNEDY, C., DELBRO, D. & BURNSTOCK, G. (1985). P_2-Purinoceptors mediate both vasodilation (via the endothelium) and vasoconstriction of the isolated rat femoral artery. *Eur. J. Pharmacol.* **107**, 161-168.

KEPPENS, S. & DE WULF, H. (1985). P_2-purinergic control of liver glycogenolysis. *Biochem. J.*, **231**, 797-799.

KEPPENS, S. & DE WULF, H. (1986). Characterization of the liver P_2-purinoceptor involved in the activation of glycogen phosphorylase. *Biochem. J.*, **240**, 367-371.

KEPPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1989). Characterization of purinoceptors present on human liver plasma membranes. *FEBS Lett.*, **248**, 137-140.

KERMODE, J.C. (1989). The curvilinear Scatchard plot: experimental artifact or receptor heterogeneity? *Biochem. Pharmacol.*, **38**, 2053-2060.

KLARSKOV, P. (1987). Non-cholinergic, non-adrenergic inhibitory nerve-responses of bladder outlet smooth muscle in vitro. *Br. J. Urol.*, **60**, 337-342.

KLARSKOV, P., GERSTENBERG, T.C., RAMIREZ, D. & HALD, T. (1983). Non-cholinergic, non-adrenergic nerve mediated relaxation of trigone, bladder neck and urethral smooth muscle in vitro. *J. Urol.*, **129**, 848-850.

KLOTZ, I.M. (1982). Numbers of receptor sites from Scatchard graph: fact or fantasies. *Science*, **217**, 1247-1249.

KLOTZ, K.N., LOHSE, M.J. & SCHWABE, U. (1986). Characterization of the solubilized A_1 adenosine receptor from rat brain membranes. *J. Biochem.*, **46**, 1258-1534.

KNIGHT, G.E., HOYLE, C.H.V. & BURNSTOCK, G. (1990). Glibenclamide antagonises the responses to ATP, but not adenosine or adrenaline, in the gastric ligament of the starfish *Asterias Rubens*. *Comp. Biochem. Physiol.*, **97C**, 363-367.

KNIGHT, G.E., HOYLE, C.H.V. & BURNSTOCK, G. (1992). Effects of adenine nucleosides and nucleotides on the isolated heart of the snail *Helix Aspersa* and the slug *Arion Ater*. *Comp. Biochem. Physiol.*, **101C**, 175-181.

KOLB, H.-A. & WAKELAM, M.J.O. (1983). Transmitter-like action of ATP on patched membranes of cultured myoblasts and myotubes. *Nature*, 303, 621-623.

KREUTZBERG, G.W., REDDINGTON, M. & ZIMMERMANN, H. (eds.) (1986). *Cellular Biology of Ectoenzymes*, pp. 305-307 (Berlin Heidelberg: Springer-Verlag).

KRISHTAL, O.A. & MARCHENKO, S.M. (1984). ATP-activated ionic permeability in the somata membrane of mammalian sensory neurons. *Neirofiziologiya*, 16, 327-336.

KRISHTAL, O.A., MARCHENKO, S.M. & PIDOPLICHKO, V.I. (1983). Receptor for ATP in the membrane of mammalian sensory neurones. *Neurosci. Lett.*, 35, 41-45.

KRISHTAL, O.A., MARCHENKO, S.M., OBUKHOV, A.G. & VOLKOVA, T.M. (1988a). Receptors for ATP in rat sensory neurons: the structure-function relationship for ligands. *Br. J. Pharmacol.*, 95, 1057-1062.

KRISHTAL, O.A., MARCHENKO, S.M. & OBUKHOV, A.G. (1988b). Cation channels activated by extracellular ATP in rat sensory neurons. *Neurosci.*, 27, 995-1000.

KU, H-H., CLEVELAND, W.L. & ERLANGER, B.F. (1987). Monoclonal antibodies to adenosine receptor by an auto-anti-idiotypic approach. *J. Immun.* ,139, 2376-2384.

KUHAR, M.J. (1985). Receptor localization with the microscope. in *Neurotransmitter Receptor Binding*, 2nd ed., eds, Yamamura, H.I., Enna, S.J. & Kuhar, M.J. pp. 153-176 (New York:Raven Press).

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, 227, 680-685.

LAMBRECHT, G., FRIEBE, T.P., WINDSCHEIF, U., HILDEBRANDT, C., SPATZ-KÜMBEL, G., BÄUMERT, H.G. & MUTSCHLER, E. (1992a). PPADS selectively blockes P_2-purinoceptor-mediated responses in rabbit vas deferens. *Int. J. Purine Pyrimidine Res.*, 3, 85.

LAMBRECHT, G., FRIEBE, T., GRIMM, U., WINDSCHEIF, U., BUNGARDT, E., HILDEBRANT, C., BÄUMERT, H.G., SPATZ-KÜMBEL, G. & MUTSCHLER, E. (1992b). PPADS, a novel functionally selective antagonist of P_2 purinoceptor-mediated responses. *Eur. J. Pharmacol.*, 217, 217-219.

LANGLEY, J.N. (1878). On the physiology of the salivary secretion. *J. Physiol. (London)*, 1, 339-369.

LANGLEY, J.N. (1905). On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari. *J. Physiol. (London)*, 33, 374-413.

LANGLEY, J.N. & ANDERSON, H.K. (1895). The innervation of the

pelvic and adjoining viscera. Part II. The bladder. *J. Physiol. (London)*, **19**, 71-84.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly-equilibrating but competitive antagonist at P_{2X}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645-649.

LEFKOWITZ, R.J., ROTH, J., PRICER, W. & PASTAN, I. (1970). ACTH receptors in the adrenal: specific binding of ACTH-^{125}I and its relation to adenyl cyclase. *Proc. Nat. Acad. Sci. U.S.A.*, **65**, 745-752.

LEGSSYER, A., POGGIOLI, J., RENARD, D. & VASSORT, G. (1988). ATP and other adenine compounds increase mechanical activity and inositol triphosphate production in rat heart. *J. Physiol. (London)*, **401**, 185-199.

LEVIN, R.M., SHOFER, F.S. & WEIN, A.J. (1980). Cholinergic, adrenergic and purinergic response of sequential strips of rabbit urinary bladder. *J. Pharmacol. Exp. Ther.*, **212**, 536-540.

LEVIN, R. M., JACOBY, R. & WEIN, A.J. (1981). Effect of adenosine triphosphate on contractility and adenosine triphosphatase activity of the rabbit urinary bladder. *Mol. Pharmacol.*, **19**, 525-528.

LEVIN, R. M., JACOBY, R. & WEIN, A.J. (1983). High-affinity, divalent ion-specific binding of ^{3}H-ATP to homogenate derived from

rabbit urinary bladder, *Mol. Pharmacol.*, 23, 1-7.

LEVIN, R.M., LONGHURST, P.A., KATO, K., McGUIRE, E.J., ELBADAWI, A. & WEIN, A.J., (1990), Comparative physiology and pharmacology of the cat and rabbit urinary bladder. *J. Urol.*, 143, 848-852.

LEWIS, M.E., PATEL, J., MOON EDLEY, S. & MARANGOS, P.J. (1981). Autoradiographic visualization of rat brain adenosine receptors using N^6-cyclohexyl[^{3}H]adenosine. *Eur. J. Pharmacol.*, 73, 109-110.

LIBERT, F., PARMENTIER, M., LEFORT, A., DINSART, C., VAN SANDE, J., MAENHAUT, C., SIMONS, M-J., DUMONT, J.E. & VASSART, G. (1989). Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science*, 224, 569-572.

LIBERT, F., SCHIFFMANN, S.N., LEFORT, A., PARMENTIER, M., GÉRARD, C., DUMONT, J-E., VANDERHAEGHEN, J-J. & VASSART, G. (1991). The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO. J.* 10, 1677-1682.

LINDEN, J., TUCKER, L. & LYNCH, K.R. (1991). Molecular cloning of adenosine A_1 and A_2 receptors. *Trends Pharmacol. Sci.*, 12, 326-328.

LIU, S.F., McCORMACK, D.G., EVANS, T.W., & BARNES, P.J. (1989a). Characterization and distribution of P_2-purinoceptor subtypes in rat pulmonary vessels. *J. Pharmacol. Exp. Ther.*, 251, 1204-1210.

LIU, S.F., McCORMACK, D.G., EVANS, T.W., & BARNES, P.J. (1989b). Evidence for two P_2-purinoceptor subtypes in human small pulmonary arteries. *Br. J. Pharmacol.*, 98, 1014-1020.

LOTAN, I., DASCAL, N., COHEN, S. & LASS Y. (1986). ATP-evoked membrane responses in *Xenopus* oocytes. *Pflügers Arch.*, 406, 158-162.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275.

LUKACSKO, P. & KRELL, R.D. (1982). Response of the guinea-pig urinary bladder to purine and pyrimidine nucleotides. *Eur. J. Pharmacol.*, 80, 401-406.

MAAYANI, S. & WEINSTEIN, H. (1980). "Specific binding" of ^{3}H-phencyclidine: artifacts of the rapid filtration method. *Life Sci.* 26, 2011-2022.

MacKENZIE, I. & BURNSTOCK, G. (1984). Neuropeptide action on the guinea-pig bladder, a comparison with the effects of field stimulation and ATP. *Eur. J. Pharmacol.* 105, 85-94.

MacKENZIE, I., MANZINI, S. & BURNSTOCK, G. (1988). Regulation of voltage-dependent excitatory responses to α,β-methylene ATP, ATP and non-adrenergic nerve stimulation by dihydropyridines in the

guinea-pig vas deferens. *Neurosci.*, 27, 317-332.

MAENHAUT, C., VAN SANDE, J., LIBERT, F., ABRAMOWITZ, M., PARMENTIER, M., VANDERHAEGEN, J-J., DUMONT, J.E., VASSART, G. & SCHIFFMANN, S. (1990), RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* 173, 1169-1178.

MAGGI, C.A., SANTICIOLI, P. & MELI, A. (1984). Postal development of myogenic contractile activity and excitatory innervation of rat urinary bladder. *Am. J. Physiol.*, 247, R972-R978.

MAHAN, L.C., McVITTIE, L.D., SMYK-RANDALL, E.M., NAKATA, H., MONSMA, F.J. Jr., GERFEN, C.R. & SIBLEY, D.R. (1991). Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.*, 40, 1-7.

MAMALAKI, C., BARNARD, E.A. & STEPHENSON, F.A. (1989). Molecular size of the γ-aminobutyric acid $_A$ receptor purified from mammalian cerebral cortex. *J. Neurochem.*, 52, 124-134.

MARCHENKO, S.M., VOLKOVA, T.M. & FEDOROV, O.I. (1987). ATP-activated ionic permeability in smooth muscle cells isolated from the guinea-pig urinary bladder. *Neirofiziologiya*, 19, 95-100.

MARIEN, M.R. & ALTAR, C.A. (1988). Quantitative receptor autoradiography: from localization to function. in *Receptor*

Localization: Ligand Autoradiography, Receptor Biochemistry and Methodology, vol. 13, eds. Leslie, F.M. & Altar, C.A. pp. 67-94 (New York:Alan R. Liss, Inc).

MARTIN, T.W. & MICHAELIS, K. (1989). P_2-Purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. *J. Biol. Chem.*, **264**, 8847-8856.

MATSUURA, H. & EHARA, T. (1992). Two ionic conductances activated by extracellularly applied ATP in guinea-pig heart cells. *J. Physiol. (London), Proc. Physiol. Soc.*, **446**, 154P.

MATLIB, M.A., CRANKSHAW, J., GARFIELD, R.E., CRANKSHAW, D.J., KWAN, C-Y., BRANDA, L.A. and DANIEL, E.E. (1979). Characterization of membrane fractions and isolation of purified plasma membranes from rat myometrium. *J. Biol. Chem.*, **254**, 1834-1840.

MATTIASSON, A., ANDERSSON, K-E. & SJÖGREN, C. (1985). Adrenergic and non-adrenergic contraction of isolated urethral muscle from rabbit and man. *J. Urol.*, **133**, 298-303.

MAZIERE, M., COMAR, D., GODOT, J.M., COLLARD, P., CCEPEDA, C. & NAQUET, R. (1981)., in vivo characterization of myocardium muscarinic receptors by positron emission tomography. *Life Sci.*, **29**, 2391-2397.

McGONIGLE, P. & MOLINOFF, P.B. (1989). Quantitative aspects of drug-receptor interactions. in *Basic Neurochemistry: Molecular,*

Cellular and Medical Aspects, 4th Ed., eds. Siegel, G.J. et al. pp. 183-200 (New York:Revan Press).

McMILLIAN, M.K., SOLTOFF, S.P., CANTLEY, L.C., TALAMO, B.R. (1987). Extracellular ATP elevates intracellular free calcium in rat parotid acinar cells. *Biochem. Biophysiol. Res. Commun.*, **149**, 523-530.

McPHERSON, G.T., MOLENAAR, P., MALTA, E. and RAPER, C. (1985). Influence of assay buffer on dissociation constants of drugs at β-adrenoceptor subtypes. *Eur. J. Pharmacol.*, **119**, 93-100.

McPHERSON, G.T. (1983). A practical computer-based approach to the analysis of radioligand binding experiments. *Comp. Prog. Biomed.*, **17**, 107-114.

MELDRUM, L.A. & BURNSTOCK, G. (1983). Evidence that ATP acts as a co-transmitter with noradrenaline in sympathetic nerves supplying the guinea-pig vas deferens. *Eur. J. Pharmacol.*, **92**, 161-163.

MILLER, J.A., CURELLA, P. & ZAHNISER, N.R. (1988). A new densitometric procedure to measure protein levels in tissue slices used in quatitative autoradiography. *Brain Res.*, **447**, 60-66.

MIYAHARA, H., & SUZUKI, H. (1987). Pre-and post-junctional effects of adenosine triphosphate on noradrenergic transmission in the rabbit ear artery. *J. Physiol. (London)*, **389**, 423-440.

MODAK, M.J. & GILLERMAN-COX, E. (1982). Biochemistry of terminal deoxynucleotidyl transferase. *J. Biol. Chem.*, **257**, 15105-15109.

MORISHITA, H. & FURUKAWA, T. (1989). Low calcium and calcium antagonists potentiate the contraction of guinea-pig vas deferens induced by ATP: a permissive role for P_2-purinoceptors. *Eur. J. Pharmacol.*, **164**, 507-513.

MORRIS, A.J., WALDO, G.L., DOWNES, C.P. & HARDEN, T.K. (1990). A receptor and G-protein-regulated polyphosphoinositide-specific phospholipase C from turkey erythrocytes. *J. Biol. Chem.*, **265**, 13508-13514.

MOSS, H.E. and BURNSTOCK, G. (1985). A comparative study of electrical field stimulation of the guinea-pig, ferret and marmoset urinary bladder. *Eur. J. Pharmacol.*, **114**, 311-316.

MUNSON, P.J. (1984). Heterogeneous receptors and binding curve analysis in neurobiology. in *Brain Receptor Methodologies, Part A: General Methods, Concepts. Amines and Acetylcholine*, eds. Marangos, P.J., Campbell, I.C. & Cohen, R.M. pp. 33-48 (Orlando:Academic Press).

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerised approach for characterization of ligand-binding system. *Anal. Biochem.*, **107**, 220-239.

MUNSON, P.J. & RODBARD, D. (1983). Number of receptor sites from Scatcard and Klotz graphs: a constructive critique. *Science*, 220, 979-981.

NAKATA, H. (1989). Purification of A_1 adenosine receptor from rat brain membranes. *J. Biol. Chem.*, 264, 16545-16551.

NATAKA, H. (1990). A_1 adenosine receptor of rat testis membranes: purification and partial characterization. *J. Biol. Chem.*, 256, 671-677.

NATAKA, H. & FUJISAWA, H. (1983). Solubilization and partial characterization of adenosine binding sites from rat brainstem. *FEBS. Lett.*, 158, 93-97.

NAKAZAWA, K. & MATSUKI, N. (1987). Adenosine triphosphate-activated inward current in isolated smooth muscle cells from rat vas deferens. *Pflügers Arch.*, 409, 644-646.

NALLY, J.E. & MUIR, T.C. (1990). The selectivity of α,β-methylene ATP (α,β-MeATP) and suramin as purinergic antagonists in the rabbit saphenous artery. In *Purines in Cellular Singaling: Targets for New Drugs*. Eds. Jacobson, K.A., Daly, J.W. & Manganiello, V., pp. 416- (New York:Springer-Verlag).

NANOFF, C., FRIESSMUTH, M., TUISL, E. & SCHÜTZ, W. (1990). P_2, but not P_1-purinoceptors mediate formation of 1,4,5-inositol

triphosphate and its metabolites via a pertusis toxin-insensitive pathway in the rat renal cortex. *Br. J. Pharmacol.*, **100**, 63-68.

NEUHAUS, R., REBER, B.F.X. & REUTER, H. (1991). Regulation of bradykinin- and ATP-activated Ca^{2+}-permeable channels in rat pheochromocytoma (PC12) cells. *J. Neurosci.*, **11**, 3984-3990.

NONOTTE, I., MATHIEU, M-N. & CHEVILLARD, C. (1989). P2-purinergic-induced ionised calcium elevation in the human promyelocytic cell line HL60. *Br. J. Pharmacol.*, **97**, 238P (Proc. Suppl.).

NORBY, J.G., OTTOLENGHI, P. & JENSEN, J. (1980). Scatchard plot: common misinterpretation of binding experiments. *Anal. Biochem.*, **102**, 318-320.

OKAJAMA, F., TOKUMITSU, Y., KONDO, Y., & UI, M. (1987). P_2-purinergic receptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of inositol triphosphate in rat hepatocytes. *J. Biol. Chem.*, **262**, 13483-13490.

OLAH, M.E., JACOBSON, K.A. & STILES, G.L. (1990). Purification and characterization of bovine cerebral cortex A_1 adenosine receptor. *Arch. Biochem. Biophys.*, **283**, 440-446.

OLAH, M.E., REN, H., OSTROWSKI, J., JACOBSON, K.A. & STILES, G.L. (1992). Cloning, expression, and characterization of the unique bovine A_1 adenosine receptor. *J. Biol. Chem.*, **267**, 10764-10770.

PALACIOS, J.M., NIEHOFF, D.L. & KUNAR, M.J. (1981). Receptor autoradiography with tritium-sensitive film: potential for computerized densitometry. *Neurosci. Lett.*, 24, 111-116.

PALEA, S., CORSI, M., PIETRA, C., GAVIRAGHI, G., ARTIBANI, W. & TRIST, D.G. (1992). Pharmacological characterization of purinoceptors in isolated human urinary bladder. *Int. J. Purine Pyrimidine Res.*, 3, 51.

PASTERNAK, G.W., SNOWMAN, A.M. & SNYDER, S.H. (1975). Selective enhancement of [^{3}H]opiate agonist binding by divalent cations. *Mol. Pharmacol.*, **11**, 735-744.

PASTERNAK, G.W. & SNYDER, S.H. (1975). in *Neurosciences Research Program Bulletin, vol. 13: Opiate Receptor Mechanisms*, eds. Snyder, S.H. and Matthysse, S. p.27 (Cambridge:MIT Press).

PAULMICHL, M. & LANG, F., (1988). Enhancement of intracellular calcium concentration by extracellular ATP and UTP in madin Darby canine kidney cells. *Biochem. Biophys. Res. Commun.*, **156**, 1139-1143.

PEARCE, B., MURPHY, S., JEREMY, J., MORROW, C. & DANDONA, P. (1989). ATP-evoked Ca^{2+} mobilisation and prostanoid release from astrocytes: P_2-purinergic receptors linked to phosphoinositide hydrolysis. *J. Neurochem.*, 52, 971-977.

PERRY, B.D. & U'PPRICHARD, D.C. (1984). α-Adrenergic receptors in neural tissues: methods and applications of radioligand binding assays. in *Brain Receptor Methodologies, Part A General Methods and Concepts. Amines and Acetylcholine*, eds. Marangos, P.J., Campbell, I.C. & Cohen, R.M. pp. 255-283 (Orlando:Academic Press).

PERT, C.B. & SNYDER, S.H. (1973). Properties of opiate-receptor binding in rat brain. *Proc. Nat. Acad. Sci. USA.*, **70**, 2243-2247.

PERT, C.B., PASTERNAK, G. & SNYDER, S.H. (1973). Opiate agonists and antagonists discriminated by receptor binding in brain. *Science*, **182**, 1359-1361.

PERT, C.B. & SNYDER, S.H. (1974). Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol. Pharmacol.*, **10**, 868-879.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.*, **83**, 346-356.

PETERSON, G.L. (1979). Review of the Folin protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.*, **100**, 201-220.

PHANEUF, S., BERTA, P., CASANOVA, J. & CAVADORE, J.-C. (1987). ATP stimulates inositol phosphates accumulation and calcium

mobilization in a primary culture of rat aortic myocytes. *Biochem. Biophys. Res. Commun.*, **143**, 454-460.

PIANET, I., MERLE, M. & LABOUESSE, J. (1989). ADP and indirectly, ATP are potent inhibitors of cAMP production in intact isoproterenol-stimulated C6 glioma cells. *Biochem. Biophys. Res. Commun.*, **163**, 1150-1157.

PIROTTON, S. & RASPE, E., DEMOLLE, D., ERNEUX, C. & BOEYNAEMS, J.-M. (1987). Involvement of inositol 1,4,5-triphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. *J. Biol. Chem.*, **262**, 17461-17466.

POLLACK, A.E., PETERFREUND, R., RIVKEES, S., WEAVER, D., REPPERT, S.M. & FINK, J.S. (1992). The rat A_2 adenosine receptor: molecular cloning, exclusive coexpression with striatal D_2 receptors and mechanisms of transcriptional regulation. *Int. J. Purine Pyrimidine Res.*, 3, 14.

POTTER, P. & WHITE, T.D. (1980). Release of adenosine 5'-triphosphate from synaptosomes from different regions of rat brain. *Neurosci.*, 5, 1351-1356.

PUCÉAT, M., CLÉMENT, O., SCAMPS, F. & VASSORT, G. (1991). Extracellular ATP-induced acidification leads to cytosolic calcium transient rise in single rat cardiac myocytes. *Biochem. J.*, **274**, 55-62.

QUIRION, R., HAMMER, R.P.,Jr., HERKENHAM, M. & PERT, C.B. (1981). Phencyclidine (angel dust)/σ "opiate" receptor: visualization by trisium-sensitive film. *Proc. Nat. Acad. Aci. USA.*, 78, 5881-5885.

RAINBOW, T.C., BLEISCH, W.V., BIGEON, A. & McEWEN, B.S. (1982). Quantitative densitometry of neurotransmitter receptors. *J. Neurosci. Methods*, 5, 127-138.

RALEVIC, V., & BURNSTOCK, G. (1988). Actions mediated by P_2-purinoceptor subtypes in the isolated perfused mesenteric bed of the rat. *Br. J. Pharmacol.*, 95, 637-645.

RALEVIC, V., BURNSTOCK, G. (1991). Roles of P_2-purinoceptors in the cardiovascular system. *Circulation*, 84, 1-14.

RAPAPORT, E. (1990). Mechanisms of anticancer activities of adenine nucleotides in tumor-bearing hosts. in *Biological Actions of Extracellular ATP, Annuals of the New York Academy of Sciences, vol. 603*, eds. Dubyak, G.R. and Fedan, J.S. pp. 142-150 (New York:the New York Academy of Sciences).

REILLY, W.M., BURNSTOCK, G. (1987). The effect of ATP analogues on the spontaneous electrical and mechanical activity of rat portal vein longitudinal muscle. *Eur. J. Pharmacol.*, 138, 319-325.

REPPERT, S.M., WEAVER, D.R., STEHLE, J.H. & KIVKEES, S.A. (1991). Molecular cloning and characterization of a rat A_1-adenosine

receptor that is widely expressed in brain and spinal cord. *Mol. Endocrinol.*, **5**, 1037-1048.

RIBEIRO, J.A. & SEBASTIAO, A.M. (1986). Adenosine receptors and calcium: basis for proposing a third (A_3) adenosine receptor. *Prog. Neurobiol.*, **26**, 179-209.

RICE, W.R. & SINGLETON, F.M. (1987). P_{2Y}-purinoceptor regulation of surfactant secretion from rat isolated alveolar type II cells is associated with mobilization of intracellular calcium. *Br. J. Pharmacol.*, **91**, 833-838.

RICE, W.R. & SINGLETON, F.M. (1989). Reactive blue 2 selectively inhibits P_{2Y}-purinoceptor-stimulated surfactant phospholipid secretion from rat isolated alveolar type II cells. *Br. J. Pharmacol.*, **97**, 158-162.

RONCA-TESTONI, S. GALBANI, P. & GAMBACCIANI, M. (1984). Some properties of a purinergic receptor solubilized from human uterus membranes. *FEBS Lett.*, **172**, 335-338.

RUGGIERI, M.R., WHITMORE, K.E. & LEVIN, R.M. (1990). Bladder purinergic receptors. *J. Urol.*, **144**, 176-181.

SACHS, G., JACKSON, R.J. and RABON, E.C. (1980). Use of plasma membrane vesicles. *Am. J. Physiol.*, **238**, G151-G164.

SALT, T.E. & HILL, R.G. (1983). Excitation of single sensory neurons in the rat caudal trigeminal nucleus by iontophoretically applied adenosine 5'-triphosphate. *Neurosci. Lett.*, 35, 53-57.

SALTER, M.W. & Henry, J.L. (1985). Effects of adenosine 5'-monophosphate and adenosine 5'-triphosphate on functionally identified units in the cat spinal dorsal horn, evidence for a differential effect of adenosine 5'-triphosphate on nociceptive vs non-nociceptive units. *Neurosci.*, 15, 815-825.

SALVATORE, C.A., LUNEAU, C.J., JOHNSON, R.G. & JACOBSON, M.A. (1992). Molecular cloning and characterization of human A_1 and A_2 adenosine receptors. *Int. J. Purine Pyrimidine Res.*, 3, 82.

SAVILLE, V.L. & BURNSTOCK, G. (1988). Use of reserpine and 6-hydroxydopamine supports evidence for purinergic cotransmission in the rabbit ear artery. *Eur. J. Pharmacol.*, 155, 271-277.

SCAMPS, F. & VASSORT, G. (1990). Mechanism of extracellular ATP-induced depolarization in rat isolated ventricular cardiomyocytes. *Pflügers Arch.*, 417, 309-316.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, 51, 660-672.

SCHUBERT, P. & KREUTZBERG, G.W. (1987). Pre-and post-synaptic effects of adenosine on neuronal calcium fluxes. in *Topics and Perspectives in Adenosine Research*, eds. Gerlach, E. and Becker,

B.F. pp. 521-532 (Berlin:Springer-Verlag)

SCHWABE, U. (1985). Use of radioligands in the identification, classification, and study of adenosine receptors. In *Methods Used in Adenosine Research*, ed. Paton, D.M. pp. 239-278 (New York:Plenum Press).

SEBASTIAO, A.M. & RIBIERO, J.A. (1988). On the adenosine receptor and adenosine inactivation at the rat diaphragm neuromuscular junction. *Br. J. Pharmacol.*, 94, 109-120.

SEBASTIAO, A.M. & RIBIERO, J.A. (1989). 1,3,8- and 1,3,7-Substituted xanthines: relative potency as adenosine receptor antagonists at the frog neuromuscular junctions. *Br. J. Pharmacol.*, 96, 211-219.

SHINOZUKA, K., BJUR, R. & WESTFALL, D.P. (1988). Characterization of prejunctional purinoceptors on adrenergic nerves of the rat caudal artery. *Naunyn Schmiedeberg's Arch. Pharmacol.*, 338, 221-227.

SIBLEY, G.N.A., (1984), A comparison of spontaneous nerve-mediated activity in bladder muscle from man, pig and rabbit. *J. Physiol.*, 354, 431-443.

SIMON, E.J., HILLER, J.M. & EDELMAN, I. (1973). Sterospecific binding of the potent narcotic analgesic [^{3}H]etorphine to rat-

brain homogenate. *Proc. Nat. Acad. Sci. USA.*, **70**, 1947-1949.

SIMON, J., SZÜCS, M., BENYHE, S., BORSODI, A., ZEMAN, P., & WOLLEMANN, M., (1984). Solubilization and characterization of opioid binding sites from frog (*Rana esculenta*) brain. *J. Neurochem.*, **43**, 957-963.

SJÖGREN, C., ANDERSSON, K-E., HUSTED, S., MATTIASSON, A. & MOLLER-MADSEN, B. (1982). Atropine resistance of transmurally stimulated isolated human bladder muscle. *J. Urol.*, **128**, 1368-1371.

SNEDDON, P, & BURNSTOCK, G. (1984). Inhibition of excitatory junction potentials in guinea-pig vas deferens by α,β-methylene ATP: further evidence for ATP and noradrenaline as cotransmitters. *Eur. J. Pharmacol.*, **100**, 85-90.

SNEDDON, P, & BURNSTOCK, G. (1985). ATP as a co-transmitter in the rat tail artery. *Eur. J. Pharmacol.*, **106**, 149-152.

SNEDDON, P. & WESTFALL, D.P. (1984). Pharmacological evidence that adenosine triphosphate and noradrenaline are cotransmitters in the guinea-pig vas deferens. *J. Physiol. (London)*, **347**, 561-580.

SNEDDON, P., WESTFALL, D.P. & FEDAN, J.S. (1982). Cotransmitters in the motor nerves of the guinea-pig vas deferens: Electrophysiological evidence. *Science*, **218**, 693-695.

SPEAKMAN, M.J., WALMSLEY, D. & BRADING, A.F. (1988). An in vitro

pharmacological study of the human trigone--a site of non-adrenergic, non-cholinergic neurotransmission. *Br. J. Urol.*, **61**, 304-309.

STEHLE, J.H., RIVKEES, S.A., LEE, J.J., WEAVER, D.R., DEEDS, J.D. & REPPERT, S.M. (1992). Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. *Mol. Ednocrinol.*, **6**, 384-393.

STEINBERG, T.H., BUISMAN, H.P., GREENBERG, S., DI VIRGILIO, F. and SILVERSTEIN, S.C. (1990). Effects of extracellular ATP on mononuclear phagocytes. in *Biological Actions of Extracellular ATP, Annuals of the New York Academy of Sciences, vol. 603*, eds. Dubyak, G.R. and Fedan, J.S. pp. 120-129 (New York:New York Academy of Sciences).

STILE, G.L. (1985). The A_1 adenosine receptor: solubilization and characterization of a guanine nucleotide-sensitive form of the receptor. *J. Biol. Chem.*, **260**, 6728-6732.

STONE, T.W., (1981). Physiological roles for adenosine and ATP in the nervous system. *Neuroscience*, **6**, 523-535.

STONE, T.W. (1991). (ed.) *Adenosine in the Nervous System*, (London:Academic Press).

SU, C.. (1975). Neurogenic release of purine compounds in blood

vessels. *J. Pharmacol. Exp. Ther.*, **195**, 159-166.

SU, C., (1978). Purinergic inhibition of adrenergic transmission in rabbit blood vessels. *J. Pharmacol. Exp. Ther.* **204**, 352-361.

TATHAM, P.E.R., CUSACK, N.J. & GOMPERTS, B.D. (1988). Characterization of the ATP^{4-}-receptor that mediates permeabilization of rat mast cells. *Eur. J. Pharmacol.*, **147**, 13-21.

TAYLOR, D.A., WEISE, S., FAISON, E.P. & YARBROUGH, G.G., (1983). Pharmacological characterization of purinergic receptors in the rat vas deferens. *J. Pharmacol. Exp. Ther.* **224**, 40-45.

THEOBALD, R.T. Jr. (1983). The effect of arylazido aminopropionyl ATP on atropine resistant contractions of the cat urinary bladder. *Life Sci.*, **32**, 2479-2484.

THOMAS, S.A. & HUME, R.I. (1990). Permeation of both cations and anions through a single class of ATP-activated ion channels in developing chick skeletal muscle. *J. Gen. Physiol.*, **95**, 569-589.

THOMAS, S.A., ZAWISA, M.J., LIN, X. & HUME, R.I. (1991). A receptor that is highly specific for extracellular ATP in developing chick skeletal muscle *in vitro*. *Br. J. Pharmacol.*, **103**, 1963-1969.

TRUSSELL, L.O. & JACKSON, M.B. (1987). Dependence of an adenosine-

activated potassium current on a GTP-binding protein in mammalian central neurons. *J. Neurosci.*, 7, 3306-3316.

TUCKER, A.L., LINDEN, J., ROBEVA, A.S., D'ANGELO, D.D. & LYNCH, K.R. (1992). Cloning and expression of a bovine adenosine A_1 receptor cDNA. *FEBS Lett.*, 297, 107-111.

UNNERSTALL, J.R., NIEHOFF, D.L., KUNAR, M.J. & PALACIOS, J.M. (1982). Quantitative receptor autoradiography using ^{3}H-Ultrofilm application to multiple bensodiazepine receptors. *J. Neurosci. Methods*, 6, 59-73.

U'PRICHARD, D.C. & SNYDER, S.H. (1980). Interactions of divalent cations and guanine nucleotides at α-nonadrenergic receptors binding sites in bovine brain. *J. Neurochem.*, 34, 385-394.

USDIN, T.B., CREESE, I. & SNYDER, S.H. (1980). Regulation of cations of [^{3}H]spiroperidol binding associated with dopamine receptors of rat brain. *J. Neurochem.*, 34, 916-922.

VAN CALKER, D., MÜLLER, M. & HHAMPRECHT, B., (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurosci.*, 33, 999-1005.

WAGNER, H.N., BURNS, H.D., DANNALS, R.F., WONG, D.F., LANGSTROM, B., DUELFER, T., FROST, J.J., RAVERT, H.T., LINKS, J.M., ROASENBLUM, S.B., LUKAS, S.E., KRAMER, A.V. & KUHAR, M.J. (1983).

Imaging dopamine receptors in the human brain by positron emission tomography. *Science*, 221, 1264-1266.

WAKUI, M. & INOMATA, H. (1985). Evidence for an increase in membrane conductance during adenosine triphosphate-induced depolarization in the guinea-pig vas deferens. *Pflügers Arch.* 430, 112-114.

WARD, F.A., WALKER, B.A.M. & HAGENLOCKER, B.E. (1990). Functional consequences of interactions between human neutrophils and ATP, ATPγS, and adenosine. in *Biological Actions of Extracellular ATP, Annuals of the New York Academy of Sciences, vol. 603*, eds. Dubyak, G.R. and Fedan, J.S. pp. 108-119 (New York:New York Academy of Sciences).

WARLAND, J.J.I., & BURNSTOCK, G. (1987). Effects of reserpine and 6-hydroxydopamine on the adrenergic and purinergic components of sympathetic nerve responses of the rabbit saphenous artery. *Br. J. Pharmacol.*, 92, 871-880.

WEDD, A.M. (1931). The action of adenosine and certain related compounds on the coronary flow of the perfused heart of the rabbit. *J. Pharmacol. Exp. Ther.*, 41, 355-366.

WEILAND, G.A. & MOLINOFF, P.B. (1981). Quantitative analysis of drug-receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.*, 29, 313-330.

WEISMAN, G.A., ERB, L. & HOOVER, R.K. (1992). Are P_{2Z} receptors coupled to calcium channels in mammalian cells? *Int. J. Purine Pyrimidine Res.*, 3, 18.

WELFORD, L.A., CUSACK, N.J. & HOURANI, S.M.O. (1987). The structure-activity relationships of ectonucleotidases and of excitatory P_2-purinoceptors: evidence that dephosphorylation of ATP analogues reduces pharmacological potency. *Eur. J. Pharmacol.*, 141, 123-130.

WELFORD, L.A. & ANDERSON, W.H. (1988). Purine receptors and guinea-pig trachea: evidence for a direct action of ATP. *Br. J. Pharmacol.*, 95, 689-694.

WESSEL, D. & FLÜGGE, U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.*, 138, 141-143.

WESTFALL, D.P., HOGABOON, G.K., COLBY, J., O'DONNELL, J.P. & FEDAN, J.S. (1982). Direct evidence against a role of ATP as the nonadrenergic noncholinergic inhibitory neurotransmitter in guinea-pig taenia coli. *Proc. Natl. Acad. Sci. USA.*, 79, 7041-7045.

WHITE, T.D. (1988). Role of adenine compounds in autonomic neurotransmission. *Pharmacol. Ther.*, 38, 129-168.

WHITE, T.D., CHAUDHRY, A., VOHRA, M.M., WEBB, D., & LESLIE, R.A. (1985). Characteristics of P_2 (nucleotide) receptors mediating contraction and relaxation of rat aortic strips: possible physiological relevance. *Eur. J. Pharmacol.*, **118**, 37-44.

WIERASZKO, A. & SEYFRIED, T.N. (1989). ATP-induced synaptic potentiation in hippocampal slices. *Brain Res.*, **491**, 356-359.

WIERASZKO, A., GOLDSMITH, G. & SEYFRIED, T.N. (1989). Stimulation-dependent release of adenosine triphosphate from hippocampal slices. *Brain Res.*, **485**, 244-250.

WIKLUND, N.P. & GUSTAFSSON, L.E. (1988). Indications for P_2-purinoceptor subtypes in guinea-pig smooth muscle. *Eur. J. Pharmacol.*, **148**, 361-370.

WILLIAMS, M. (1990). Purine nucleosides and nucleotides as central nervous system modulators. in *Biological Actions of Extracellular ATP, Annuals of the New York Academy of Sciences, vol. 603*, eds. Dubyak, G.R. and Fedan, J.S. pp. 93-107 (New York:New York Academy of Sciences).

WOOD, B.E., SQUIRE, A., O'CONNOR, S.E. & LEFF, P. (1989). ADP-β-F is not a selective P_{2Y}-receptor agonist in rabbit jugular vein. *Br. J. Pharmacol.*, **98**, supplement, 749P.

YAMAMOTO, R., CLINE, W.H. Jr., TAKASAKI, K. (1988). Reassessment of the blocking activity of prazosin at low and high

concentrations on sympathetic neurotransmission in the isolated rat mesenteric vasculature of rats. *J. Auton. Nerv. Syst.*, **8**, 303-309.

YATANI, A. TSUDA, Y., AKAIKE, N. & BROWN, A.M. (1982). Nanomolar concentrations of extracellular ATP activate membrane Ca channels in snail neurones. *nature*, **296**, 169-171.

YOUNG, W.S.III & KUHAR, M.J. (1979). A new method for receptor autoradiography: [^{3}H]opioid receptors in rat brain. *Brain Res.*, **179**, 255-270.

ZHAO, D. & DHALLA, N.S. (1990). [^{35}S]ATPγS binding sites in the purified heart sarcolemma membrane. *Am. J. Physiol.*, **258**, C185-C188.

ZHENG, J-S., CHRISTIE, A., LEVY, M.N. & SCARPA, A. (1992). Extracellular ATP elicits I_{ATP} and increase I_{Ca} through different mechanisms in rat ventricular myocytes. *FASEB. J.*, **6**, A978.