EFFECTS OF SOME AGENTS ON RECEPTOR- AND CELL VOLUME-CONTROLLED CHANGES IN THE IONIC PERMEABILITY OF ISOLATED GUINEA-PIG HEPATOCYTES

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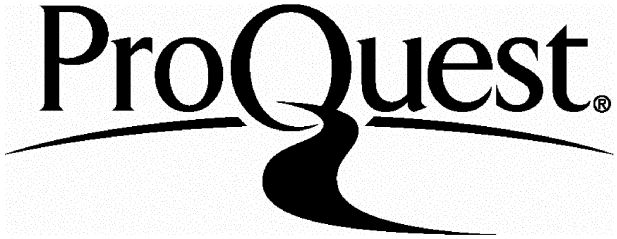

ABSTRACT

Sodium-linked amino acid transport causes an increase in the membrane permeability of liver cells to potassium. This effect on permeability is generally attributed to the concomitant rise in cell volume produced by the inward movement of solute. It is thought to form the basis of the subsequent regulatory volume decrease. The first part of this study examined the pharmacology and electrical characteristics of cell volume regulation in isolated guinea-pig hepatocytes using intracellular recording techniques and the whole cell variant of the patch clamp technique. In patch clamp studies, cell swelling was induced by the application of hydrostatic pressure to the shank of the patch pipette. This produced a large outward K^+ current in voltage clamped cells, apparently without a rise in cytosolic free Ca^{2+} concentration. Spectral analysis of the current noise during this response suggested that the K^+ channel involved has a unitary conductance of 7pS.

In microelectrode recordings, the volume activated potassium conductance (G_V) is sometimes triggered as a consequence of electrolyte leakage from the electrode and the influx of osmotically obliged water. This experimental artefact was turned to advantage in the current study to test the ability of a variety of agents to inhibit G_V. G_V demonstrated a similar sensitivity to cetiedil to the potassium conductances that subserve the Gardos effect in red blood cells and the volume regulatory decrease described in lymphocytes subjected to hypotonic stress. Its insensitivity to oligomycin A and oxpentifylline, however, sets G_V apart from these cetiedil sensitive conductances.

The second part of this study looks at various aspects of the hormonal regulation of liver cell membrane permeability. The changes in membrane conductance evoked by P_2-purinoceptor activation were

investigated first. Previous workers have demonstrated that Ca^{2+} mobilizing agonists trigger Ca^{2+}-activated K^+ and Cl^- permeabilities ($P_{K(Ca)}$ and $P_{Cl(Ca)}$) in the liver. In the present study, it was shown that whereas low concentrations of ATP exclusively activate the Ca^{2+}-gated K and Cl channels, larger concentrations also induce a transient depolarizing current which flows through a separate conductive pathway that does not depend on intracellular Ca^{2+}.

The mechanisms coupling hormonal receptor stimulation to the activation of $P_{K(Ca)}$ and $P_{Cl(Ca)}$ were addressed next. Fast oscillations in membrane conductance were observed when the hepatocytes were stimulated by submaximal concentrations of either the Ca^{2+} mobilizing agonist ATP or the cAMP-dependent agonist salbutamol. The cellular basis for this oscillatory activity was explored by employing the whole cell patch technique to introduce the putative second messengers cAMP and inositol 1,4,5 trisphosphate (IP_3) into the cells. The results obtained suggest the release of intracellular Ca^{2+} by both cAMP and IP_3 can be pulsatile.

Finally, the potentiating effect of salbutamol on the membrane response to ATP, described previously in liver slices and cell suspensions, was re-examined at the single cell level. The dose-response relationship of single cells to ATP was found to shift to the left in the presence of salbutamol, so that near threshold concentrations of this agonist became sufficient in some cells to set in motion the series of cyclical fluctuations in cytosolic calcium, normally associated with more intense stimulation. These results are discussed in the context of Berridge's two pool model of intracellular calcium homeostasis.

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1.1. INTRODUCTION

Both procaryotic and eucaryotic cells contain aqueous channels that when open, allow ions to passively diffuse across the membrane at a rate as much as a factor of ten less than that of diffusion through pure water. Frequently channels discriminate between ion species, allowing certain ions to pass readily, while retarding or rejecting others. This selectivity is achieved partly through steric constraints imposed by the channel's internal dimensions and the size and hydration of the permeant ion, and partly by the charge distribution within the channel.

The channel pore may be opened or closed by a gating mechanism working under the instructions of a sensor which signals changes in, for instance, membrane potential or tension or the levels of a given ligand. The sensor may coexist in the same macromolecule as the port and the gate. The most obvious examples are the voltage-activated Na^+, K^+ and Ca^{2+} channels found in excitable tissue and the receptors at the fast chemical synapses that bind acetylcholine, glutamate, glycine or γ-aminobutyric acid, but the recently identified stretch-activated Ca^{2+} channels in the choroid plexus (Christensen, 1987) and the stretch-activated K^+ channels in renal epithelium (Sackin, 1989) may also be gated by intrinsic sensors.

In addition, channel activity can be governed by remote sensors, usually via intracellular, second messenger molecules. The sensor protein may control a Ca^{2+} flux or the synthesis of cAMP, cGMP or a polyinositol phosphate. This second messenger then diffuses through the cytoplasm and, sometimes with the assistance of macromolecules, can instruct channel gates to open or close. The channel can therefore be in a different part of the cell than the sensor. Such a remote sensor mechanism is clearly present in retinal rods, where light strikes

rhodopsin activating transducin and thence a phosphodiesterase, leading to the breakdown of cGMP and consequently to the closure of cation permeable channels in the surface membranes of the outer segment (see Hille, 1991 for reference). In atrial myocytes, the muscarinic receptor also recruits a G protein, Gi, to gate an inwardly rectifying K^+ channel but through a membrane delimited pathway. Here the activated G protein is believed to interact directly with nearby channels turning on the K^+ current without the involvement of a cytoplasmic second messenger (Soejima & Noma, 1984; Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985; Breitwieser & Szabo, 1985).

The available evidence suggests that the gating mechanisms that control the ionic permeability of the liver make use of both intrinsic and remote sensors. The voltage-activated K^+ channels identified recently in avian hepatocytes (Marchetti, Premant & Brown, 1988) can be assumed to have their own intrinsic sensors on the basis of their ability to gate normally in excised patches in the absence of any second messengers, while a whole host of receptor species coupled to phospholipase C are recognised to serve as remote sensors for Ca^{2+}-activated K^+ and Cl^- conductances in all the animals studied so far (but for the rat). The present study focuses on two in particular of these permeability mechanisms in guinea-pig hepatocytes. One is coupled to purinoceptor activation and the other to cell swelling.

1.2 THE LIVER

1.2.1 The physiological functions of the liver

The liver is the largest organ in the body. The remarkable range of biochemical functions it performs in intermediary metabolism and its strategic location, interposed as it is between the gastrointestinal tract and the systemic circulation, enables it to play a vital role in processing

nutrients absorbed from the gut and transforming them into materials needed by the other specialized tissues of the body.

One of its most important functions is the maintenance of the normal blood glucose concentration. Liver cells take up glucose from the blood and by means of a series of enzymatic reactions polymerise it to give glycogen, the storage form of carbohydrates. Simpler compounds such as lactic acid, glycerol and pyruvic acid, can also be converted into glucose and thence to glycogen. As the need arises, glycogen is broken down to glucose again by the enzyme phosphorylase which is activated via a kinase by the hormones adrenaline and glucagon. In addition the liver plays a key role in the metabolism and transport of lipids and in the maintenance of lipid levels in the circulating blood. The lipids in the blood plasma come from ingested food, from mobilization of fat depots in adipose tissue or from synthesis from carbohydate or protein in the liver. The main vehicle for the transport of lipids from whatever source is lipoprotein and it is in the liver that the transformation of lipids into lipoproteins takes place. The liver is also the site of synthesis of plasma proteins, of amino acid deamination and of the interconversion of amino acids.

An important excretory function of the liver is the elimination of a variety of drug molecules, the steroid hormones and bilirubin. These substances are subjected to oxidation, hydroxylation and/or conjugation with glucoronate in the liver by the cytochrome P450 system located in the endoplasmic reticulum and by gluconyltransferases. The reaction products are then excreted in the bile secreted by the parenchymal cells. As well as acting as a vehicle for excretion, bile and in particular the bile salts, play an important part in digestion, emulsifying ingested fat.

1.2.2 The histological organisation of the liver: the lobule

The smallest functional unit of the liver is the lobule (see Fig 1.1). Within each lobule, hepatocytes are arranged in sheets or plates normally one cell thick. The plates are radially disposed with respect to a central vein, a branch of the hepatic vein. Blood is carried from branches of the hepatic artery and the portal vein running in the portal triad, across the plates to the central vein in a series of interconnecting sinusoids. The sinusoidal walls consist predominantly of endothelial cells together with a smaller number of fixed macrophages, termed Kupffer cells.

The endothelial cells have typical overlapping junctions in some places, but in others the attenuated margins of neighbouring cells may be separated by intercellular openings of 0.1-0.5μm. In electron micrographs it can be seen that the thin peripheral portions of the endothelial cells are fenestrated. Without a basal lamina, this discontinuous endothelium allows macromolecules of up to 0.5μm in diameter to pass freely into the perisinusoidal space of Disse lying between the sinusoids and liver cells. The exchange of substances between the parenchymal cells and the blood of the portal vein is further facilitated by the presence of microvilli where the parenchymal cells abut the space of Disse.

Within the plates, adjacent hepatocytes are separated by a narrow intercellular cleft which widens at the site of the bile canaliculus to form a canalicular space, 0.5-1.0μm in diameter. This empties into a thin walled ductule which runs over the surface of the plate to the bile ducts of the portal triad. On either side of the canaliculus the membranes of the opposing cells come into close contact and form a "tight" junction comparable with zonula occludens of other epithelia. This seals the commisures of the canaliculus and prevents its contents

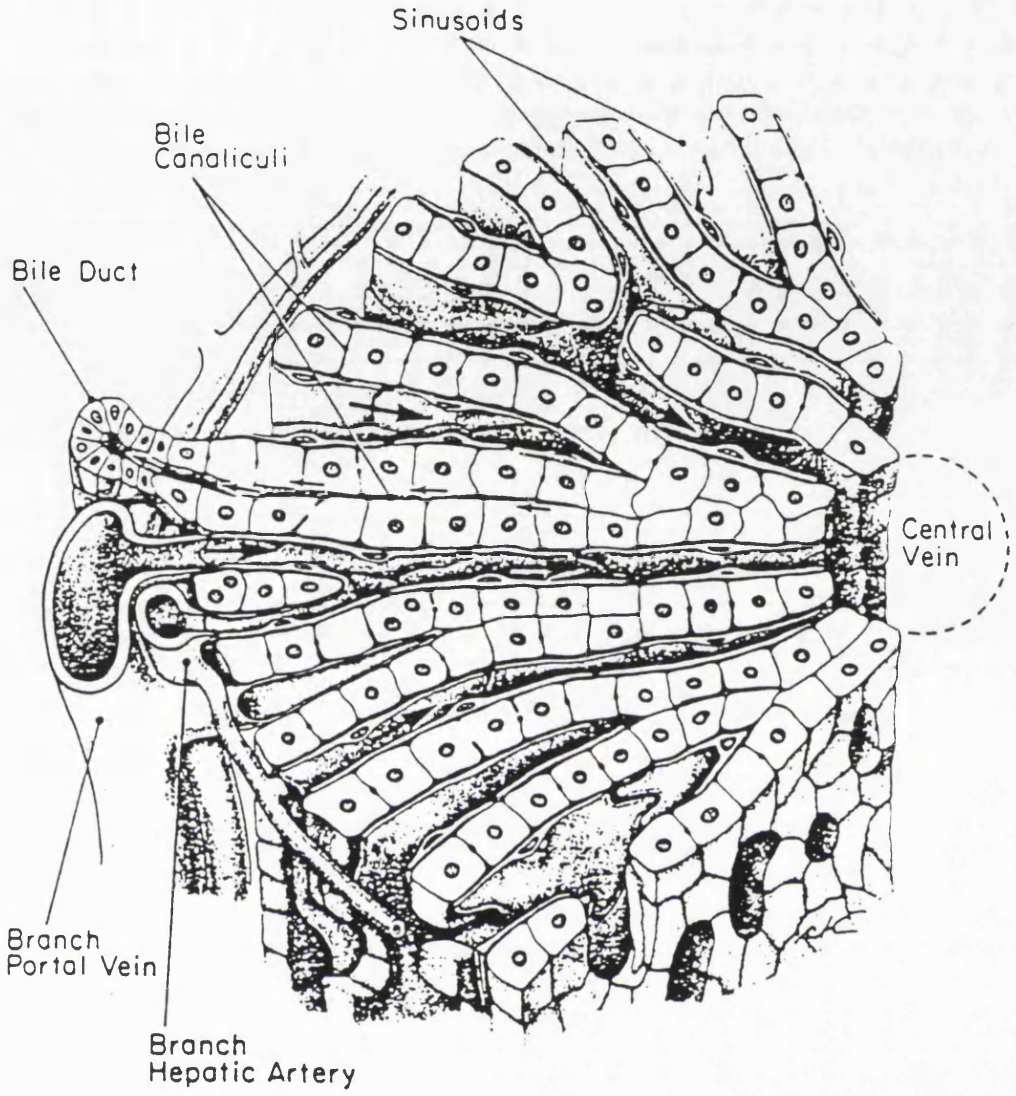


Fig. 1.1 Schematic drawing illustrating the histological organisation of a liver lobule. The plates of hepatocytes are radially disposed with respect to a central vein. Blood is carried from branches of the hepatic artery and portal vein across the plates to the central vein in sinusoids. Bile is secreted by the liver cells into the canaliculi which drain into the bile duct. Reproduced from Bloom, W. & Fawcett, D.W. (1975) "A textbook of histology". 10th Edition, Pub. W.B. Saunders.

escaping in bulk into the extracellular space. Nevertheless this tight junction may constitute a primary route of paracelluiar ion and water transfer (Boyer, 1980).

In addition to the zonulae occludens adjacent to the canaliculus, a number of gap junctions are found on the boundaries between adjoining parenchymal cells. These sites of low electrical resistance permit not only small ions, but also putative second messengers such as inositol 1,4,5 trisphosphate and cAMP to pass between cells (Lawrence *et al.*, 1978; Saez *et al.*, 1989) and thus provide for coordination of the cells' physiological activities. Interestingly, the number of gap junctions which exist between cells in a given area shows an inverse relationship to the density of its nervous innervation (for references see Lautt, 1983).

1.2.3 The innervation of the liver

A direct innervation of the liver parenchyma was first postulated by Pfluger as long ago as 1869, but its existence was not demonstrated unequivocally until the application of fluorescence and electron microscopy. In the guinea-pig liver, fluorescent staining for catecholamines reveals an extensive network of catecholaminergic nerves spreading from the perivascular plexus through the whole lobule, with almost every hepatocyte making contact with a fluorescent varicosity or nerve ending (Metz & Forssmann, 1980). These results are confirmed in electronmicrographs. Contact between hepatocytes and adrenergic nerves has also been noted throughout the lobule of the livers of rabbits, cats, dogs and humans (Forssmann and Ito, 1977). By contrast, the adrenergic innervation of mouse and rat liver is largely restricted to the triads and probably the smooth muscle of the portal vein and hepatic artery branches, with few nerve fibres even penetrating the

parenchyma.

In the rat, cholinergic nerves form a plexus, separate and distinct from the adrenergic nerves, which courses adjacent to the vasculature in the portal areas. While Sutherland (1964) and Skaaring and Bierring (1976) suggest that the nerves also penetrate the lobule to innervate liver cells, no evidence of this was found by Satler *et al.* (1974) or by Reilly *et al.* (1978) using procedures which were more carefully controlled. The latter investigators, however, did demonstrate that some of these fibres were not contiguous with the vasculature and passed adjacent to the hepatic cells that abutted the portal area. Histochemically, some of these fibres appeared to terminate on these hepatic cells as end bulbs of Hed. Virtually nothing has been reported on distribution of cholinergic nerves in livers of other species. But more pertinent to the present study, the possibility that either the liver receives a purinergic supply or that ATP acts as a cotransmitter to either acetylcholine or noradrenaline in this tissue has not been addressed to date.

1.2.3.1 The function of hepatic nerves

The physiological significance of the autonomic innervation of the liver parenchyma has been discussed in depth in a review by Lautt (1983). The relative importance of the reflex stimulation of sympathetic nerves supplying the liver and the circulating hormones glucagon and adrenaline in the control of blood glucose is a subject of hot debate. While Seydoux *et al.* (1979) suggested that the carbohydrate metabolism of the liver is controlled by its own nerve supply rather than by circulating catecholamines, the contrary view has since been expressed by Jarhult *et al.* (1980) who concluded that the hepatic nerves make only a minor contribution to the development of

hyperglycemia. However, the potentiating effect of circulating glucagon, and probably adrenaline, on the glycogenolytic response to perivascular nerve stimulation described by Beckh, Hartmann and Jungermann (1982) argues for a more significant role for the intrahepatic sympathetic nerves in the control of plasma concentration of glucose.

The physiological function of the parasympathetic nerves is similarly open to speculation. One idea put forward by Lautt (1980) is that they serve to reflexly prepare the liver for the influx of glucose that follows an oral glucose load, through the activation of glycogen synthetase.

1.3 THE SUITABILITY OF THE ISOLATED LIVER CELLS AS AN EXPERIMENTAL MATERIAL

In the present study intracellular recordings were made from single guinea-pig hepatocytes in short term culture. The use of isolated cells allows the increases in membrane conductance caused by agonists and by changes in cell volume to be quantified - information that is not readily obtainable in liver slices and strips, because of the syncytial nature of the tissue. A further advantage of this approach is that the cells are freely accessible to applied drugs and thus the diffusion delays that are inevitable when drug effects are studied by means of intracellular recording from cells within liver slices are largely avoided. What should not be overlooked though are the changes that may occur in the liver cell when it is isolated from its normal environment.

1.3.1 The polarity of the isolated cell

Cell isolation inevitably entails loss of the morphological and functional polarity of the cell, and this loss is followed by structural changes in

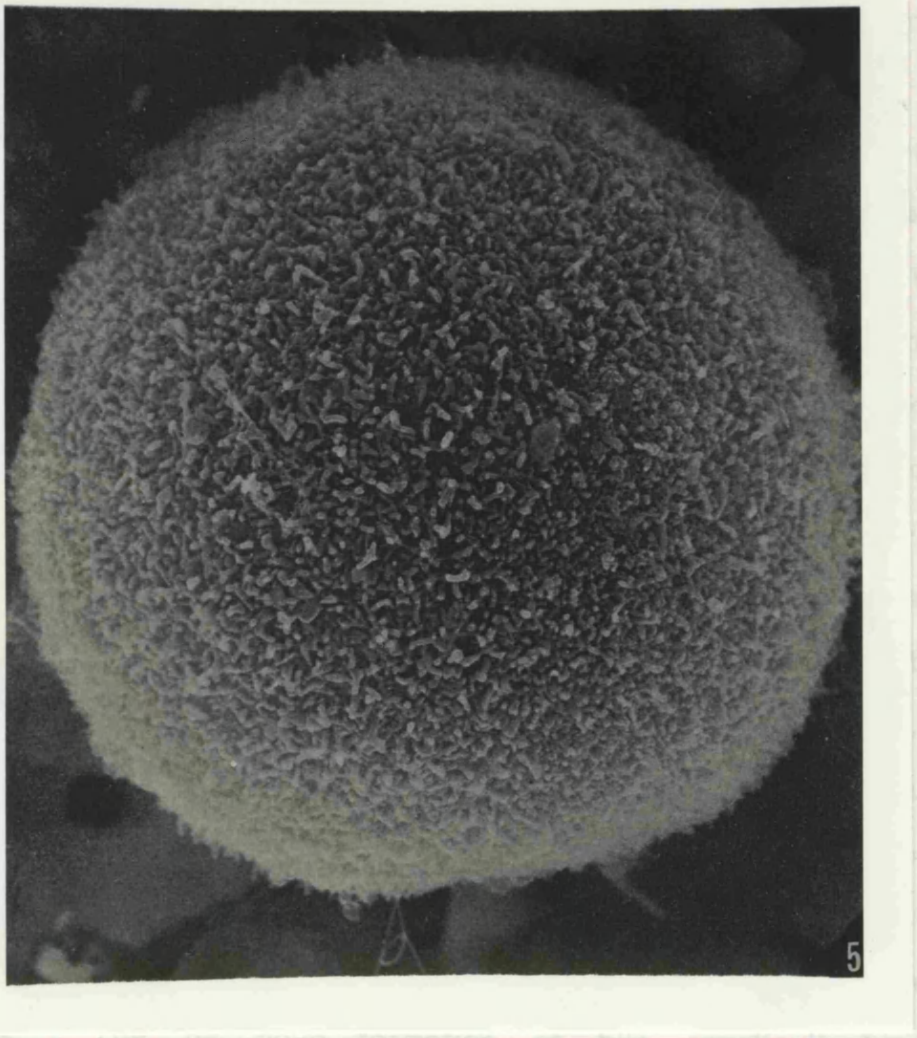


Fig. 1.2 Electron micrograph of an isolated hepatocyte. Cell is covered with microvilli. There is no evidence of any surface differentiation within field of view. Magnified × 6600. Reproduced from Drochmans, Wanson, May & Bernaert (1978).

the cell itself. Shortly after starting the isolation procedure the microvilli, which are confined to the canalicular and the sinusoidal membrane surface of parenchymal cells *in situ*, begin to spread progressively over the cell; the cell loses its polyhedral shape and takes on a spherical appearance (see Fig. 1.2). Despite this membrane randomization, the Mg^{2+}-ATPase activity still circles each freshly isolated cell in a belt-like arrangement reminiscent of the location of the bile canniculi on the surface of the liver cell in the intact tissue. However, within four hours biliary polarity also disappears. The Golgi complex and lysosomes are dispersed and the only evidence of Mg^{2+}-ATPase activity is in cell-surface membrane between undissociated couplets and groups of cells where it coincides with what appears to be the functional equivalent of the bile caniculus (Graf, Gautam & Boyer, 1984). But hepatocytes maintained in primary culture gradually put out processes and at points of contact bile canicull may form between adjacent cells, sealed off by tight junctions. This is accompanied by the reorganization of cytoplasmic vesicles in each cell to give Golgi complexes. The *de novo* formation of bile canaliculi has been investigated by Gebhardt (1986).

1.3.2 Ion distribution across the cell membrane measured *in vivo* and *in vitro*

Table 1.1 compares values of the intracellular concentrations of the major inorganic ions obtained in a variety of liver preparations. The most noticeable change *in vitro* is a marked increase in intracellular chloride and sodium in the isolated perfused liver and liver slices. These changes in transmembrane ionic distribution are maintained under *in vitro* conditions for hours which led to the suggestion by Haylett and Jenkinson (1972*a*) that in slices a new steady state may be achieved

Table 1.1 Comparison of intracellular concentrations of inorganic ions (expressed in mmol l^{-1} cell water) in the intact liver, liver slice and dispersed cells.

	$[Na^+]$	$[K^+]$	$[Cl^-]$	Reference
rat liver *in situ*	22	165	22	Williams & Woodbury (1971)
dog liver *in situ*	35	174	23	Lambotte (1977)
isolated perfused rat liver	16.4	113	36.4	Claret & Mazet (1972)
guinea-pig liver slice	73	144	66	Haylett & Jenkinson (1972*a*)
isolated rat hepatocytes	25	139	42	Reynes & Benos (1983)
isolated guinea-pig hepatocyes	30	123	—	De Witt & Putney (1984)
isolated rat hepatocye couplets	16.3	117	23.6	Graf, Henderson, Krumpholz & Boyer (1987)

through an alteration in membrane permeability.

In contrast, the intracellular levels of both ions and metabolites fall in isolated cells immediately following dispersion, but rapidly approach their *in vivo* values during incubation in an oxygenated medium. The ability of dispersed hepatocytes to hold the concentrations of intracellular ions at levels closer to those *in vivo* has been taken to reflect the metabolic superiority of this preparation to the liver slice. In keeping with this notion the rates of oxygen consumption, gluconeogenesis, urea and ketone production and the adenosine nucleotide levels in isolated hepatocytes approximate those in intact liver.

1.3.3 The membrane potential of liver cells measured *in vivo* and *in vitro*

Membrane potentials recorded intracellularly from single hepatocytes and couplets isolated from rat and guinea pig liver by collagenase digestion range typically from -25 to -40mV (Green, Dale & Haylett, 1972; Graf, Gautam & Boyer, 1984; Graf, Henderson, Krumpholz & Boyer, 1987; Field & Jenkinson, 1987). This compares with *in vivo* values of between -31 to -60mV reported in the rat and dog (Schanne & Coraboeuf, 1966; Williams, Withrow & Woodbury, 1971; Lambotte, 1977; Kernan & MacDermot, 1980; Meyer, Yancey & Revel, 1981; Fitz & Scharschmidt, 1985).

The tendency for the recorded membrane potentials to be lower in isolated cells than in the intact organ has been attributed in part to the membrane leaks introduced by micropuncture which shunts the membrane potential more effectively the higher the total cell membrane resistance. Compared to the isolated cell, the total input resistance in intact liver is very low due to extensive current flow into neighbouring

cells through low resistance intracellular communications. Hence, the cell membrane potential of the impaled cell in the intact organ may be held to values close to normal by its intact neighbours. This in itself cannot be the complete story because even when the leaks are negligible, as is the case in whole cell current clamped guinea-pig hepatocytes, the average membrane potential recorded is only -25mV (Capiod & Ogden, 1989*a*).

Another possible explanation for the disparity between *in vivo* and *in vitro* values of membrane potential is suggested by early work on the perfused liver. A number of groups (Claret and Mazet, 1972; Folke, 1972; Dambach & Friedmann, 1974) have noted that when rat liver is perfused with an artificial physiological medium the membrane potential drops to between -26 and -46mV. This effect may be reversed indefinitely by supplying the liver with blood from a living donor rat (Folke, 1972). This has given rise to the idea that some blood bourne factor is responsible for maintaining the membrane potential at its *in vivo* level. Of the likely candidates noradrenaline, adrenaline and glucagon can perhaps be excluded on the grounds that, at least in the unstressed animal, they are not present in the blood plasma in sufficient quantities to evoke a Ca^{2+}-activated increase in P_K in the liver cell membrane (Cocks, Jenkinson & Koller, 1984) and by inference, to influence the resting membrane potential. The answer may instead lie, at least partly, in the amino acids and sugars which can reach millimolar concentrations in the portal vein and thus trigger the volume activated P_K described by Bakker-Grunwald (1983) and Kristensen & Folke (1984) (see also section 1.5). In keeping with this notion, very negative potentials (-70 to -80mV) have been recorded from single hepatocytes when using microelectrodes filled with 4M potassium acetate (Petzinger & Bigalke, 1986; Kawanishi, Blank, Harootunian, Smith & Tsien, 1989), a

filling solution which would be expected to cause cell swelling. However, this is not the only interpretation which can be placed on these results, since the same filling solution could in theory also produce some intracellular alkalinization and activate the pH-sensitive K^+ conductance recently identified in rat hepatocytes (Fitz, Trouillot & Scharschmidt, 1989).

1.4 CAN THE GOLDMAN CONSTANT FIELD EQUATION ADEQUATELY DESCRIBE THE RESTING MEMBRANE POTENTIAL OF THE LIVER CELLS?

The Goldman constant field equation (Goldman, 1943) is based on the most widely accepted model for ion permeation through biological membranes. It is usually expressed as

$$E_r = \frac{RT}{F} \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o} \qquad (1.1)$$

where E_r = the resting membrane potential

R = the universal gas constant

T = the absolute temperature

F = Faraday's constant

P = the permeability constant for the ionic species denoted by the subscript

[X] = the concentration of each ionic species, with subscripts i and o denoting the intracellular and extracellular compartments respectively.

This equation can be simplified considerably if it can be assumed that Cl^- ions are passively distributed across the cell membrane. A passive distribution of Cl^- has in fact been demonstrated in perfused dog (Lambotte, 1977) and rat liver (Claret & Mazet, 1972; Fitz &

Scharschmidt, 1987) and in isolated rat hepatocytes (Bear, Petrunka & Strasberg, 1985). By contrast, in the rat liver *in vivo*, Williams, Withrow & Woodbury (1971) observed an intracellular chloride concentration lower than what would have been predicted by passive distribution, but the difference was so small that it could easily be put down to an error incurred in estimating the extracellular space.

It seems reasonable then to assume that the membrane potential of the liver cell is adequately described by the abbreviated form of the Goldman equation (Hodgkin, 1958)

$$E_r = \frac{RT}{F} \ln \frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[K]_i} \qquad (1.2)$$

where α is the ratio P_{Na}/P_K.

By substituting the values of E_r and the intracellular and extracellular ionic concentrations obtained experimentally into equation 1.2, α can be estimated to be between 0.1 and 0.2 in rat liver *in vivo* (Williams *et al.*, 1971) 0.17 in dog liver *in vivo* (Lambotte, 1977) and 0.24 in guinea-pig liver slices (Haylett & Jenkinson 1972*a*). It is preferable, however, to examine the effects of changing extracellular ion concentrations on membrane potential and then to compare the results with the behaviour predicted by the Goldman constant field equation. Haylett & Jenkinson (1972*a*) adopted this approach with guinea-pig liver slices. The data they obtained was best fitted with α constrained to 0.23, but a marked deviation of the observed values from the theoretical curve was seen at the higher concentration of external K^+. This discrepancy may be accounted for by a dependence of P_K on $[K^+]$ (Claret & Mazet, 1972; Graf & Petersen, 1974, 1978; Graf, Henderson, Krumpholz & Boyer, 1987).

At variance with the work of Haylett and Jenkinson (1972*a*) and

Graf and Petersen (1974), the transmembrane flux studies of Claret and Mazet (1972) yielded a value of 0.53 for α. However, when this value was substituted into the abbreviated form of the Goldman equation (eqn. 1.2), the theoretical value of E_r obtained exceeded the measured membrane potential by 10 mV. This difference may perhaps be attributed to the activity of the Na pump.

As in many tissues the unequal distribution of Na^+ and K^+ across the plasma membrane is maintained in the liver primarily by an ouabain-sensitive Na^+-K^+-ATPase pump located at the basolateral membrane (Blitzer & Boyer, 1978) extruding Na^+ in exchange for K^+. By contrast K^+ efflux and Na^+ influx are predominantly passive except for a small contribution to transmembrane Na flux made by an amiloride sensitive Na^+/H^+ exchange (Arias & Forgac, 1984); a Na^+/HCO_3^- co-transport (Renner, Lake, Bruce, Scharschmidt, Zimmerli & Meier, 1989) and a glycoside insensitive Na^+/Na^+ exchange mechanism (Claret, 1978).

If it can be assumed to a first approximation that just the Na^+-Ka^+ ATPase pump operates electrogenically in the liver than the modified form of the Goldman equation suggested by Mullins and Noda (1963)

$$E_r = \frac{RT}{F} \log \frac{r\,[K^+]_i + \alpha[Na^+]_i}{r\,[K^+]_o + \alpha[Na^+]_o} \qquad (1.3)$$

where r is the coupling ratio or the number of Na^+ ions carried out per K^+ ion carried in, can be invoked to account for hepatic membrane potential. Fitting their data with eqn. 1.3, Claret and Mazet found that it was necessary to assume that 3 Na^+ ions must be extruded and 2 K^+ ions must be taken up per cycle of the pump, to explain the disparity between the measured and theoretical values of E_r. The Na^+ pump was also considered by Haylett & Jenkinson (1972a) and Graf and Petersen

(1974) to be electrogenic in character. The latter study attempted to quantify the direct contribution of the Na^+ pump to the resting potential of mouse liver segments. The authors concluded from their experiments that under conditions of extreme Na^+ loading and maximal stimulation with extracellular K^+, the electrogenic component of membrane potential may be more than 10mV, but normally the pump contributes at most 2mV to the resting membrane potential (see also Graf *et al.*, 1987). This implies that the results of Claret & Mazet (1972) were obtained under conditions in which the Na^+ pump was maximally stimulated.

It should be added that Heller & Van der Kloot (1974) have argued that the liver cell membrane potential cannot be adequately described by even the modified form of the Goldman equation given by Mullins & Noda (1963) on the grounds that it cannot explain the effects of changing $[Na]_o$ on V_r. However, they did not take into consideration the possibility that P_K may change with $[Na^+]_o$, as has since been shown (Graf & Petersen, 1978).

1.5 THE RELATIONSHIP BETWEEN MEMBRANE PERMEABILITY AND CELL VOLUME

Bakker-Grunwald (1983) and Kristensen and Folke (1984) have shown that Na^+-coupled entry of alanine is accompanied by a quinine-sensitive increase in the K^+ permeability of rat liver cells. Current evidence suggests that it is the rise in cell volume caused by the intracellular accumulation of alanine that serves as the trigger for this change in membrane permeability. Thus the membrane response to alanine can be mimicked by modest extracellular hypotonicity and inhibited, at least partially, by the addition of poorly permeant solutes to the external media in sufficient quantities to prevent cell swelling (Bakker-Grunwald, 1983; Kristensen & Folke, 1984).

From the viewpoint of homeostatic regulation, this so-called "volume-activated" K^+ permeability may answer several purposes during alanine uptake:

(1) It prevents cellular K^+ accumulation during the enhanced Na^+-K^+ pumping which follows the influx of Na^+ with alanine.

(2) It makes a decisive contribution to cell volume regulation, subserving the regulatory volume decrease (RVD) which returns the swollen cells towards its original size.

(3) The accompanying tendency toward hyperpolarization increases the driving force for the Na^+ coupled influx of alanine and the efflux of Cl^- and other anions, necessary to maintain electroneutrality.

Whether a change in anion permeability also contributes to liver cell regulation is not yet clear. The persistence of RVD and the accompanying K^+ efflux in the nominal absence of external HCO_3^- obviously argues against an active involvement of this ion in RVD. Corsanti, Gleeson & Boyer (1990) have recently demonstrated that RVD was inhibited by 40% in Cl^- depleted hepatocytes and by almost 90% after DIDS treatment. These results suggest Cl^- is involved in RVD, but do not shed light on the mechanism by which the Cl^- efflux takes place. A role for Cl^- in liver cell volume regulation has recently been confirmed (Haddad, Beck, Boyer & Graf, 1991).

1.5.1 A role for Ca^{2+} in the volume-activated increase in potassium permeability?

The link between cell swelling and the increase in K^+ permeability is poorly understood. In cell attached recordings from rat liver cells. Ca^{2+}-activated K^+ channels and stretch activated non-selective cation ($NSC^+(SA)$) channels have been identified by Bear & Petersen (Bear & Petersen, 1987; Bear, 1990). Both are stimulated by L-alanine.

Moreover, examination of the time courses for current activation reveals that NSC^+(SA) channels are activated approximately 30s before the K^+ channel, corresponding to a transient elevation in cytosolic Ca^{2+} (Bear, 1990). Such results could be taken to suggest that Ca^{2+} influx through the NSC^+(SA) channel is required for the activation of the K^+ channels effective in cell volume regulation (see e.g. Christensen, 1987). However, several workers have failed to demonstrate a role for Ca^{2+} in K^+ channel activation in rat hepatocytes (Burgess *et al.*, 1981; Sawanobori, Takanashi, Hiraoka, Iida, Kamisawa & Maezawa, 1989) and Bear (1990) in fact has herself found that in the absence of external Ca^{2+} hepatoma cells are still capable of maintaining their volume in the face of hypotonic stress. This issue thus remains unresolved.

1.6 THE INFLUENCE OF HORMONES ON ION MOVEMENTS

Early evidence for a hormonal action on K^+ movements came from the work of D'Silva (1936) who showed the liver to be the primary source of extra plasma K^+ in adrenaline induced hyperkalemia. The net loss of K^+ from the liver is now recognized to reflect an increase in the K^+ permeability of the hepatocyte membrane in response to α adrenoceptor receptor stimulation. This was first suggested by Haylett and Jenkinson (1972*a*,*b*) on the basis of intracellular recordings and measurements of ^{42}K flux from guinea pig liver slices. The effect has since been amply confirmed by electrophysiological studies on mouse and guinea-pig liver segments (Graf & Petersen, 1978; Egashira, 1980*a*; Karashima, 1981) and also by measurements of K^+ and Rb^+ fluxes in suspensions of dispersed hepatocytes (Burgess, Claret & Jenkinson, 1981; DeWitt & Putney, 1984).

The permeability change evoked by α receptor stimulation is not restricted to K^+. Thus, particularly in cells with a high resting

membrane potential in liver slices, noradrenaline can produce a biphasic response, with a small depolarization preceding the dominant hyperpolarization mediated by K^+ ions (Egashira, 1980*a*; Karashima, 1981). Ionic substitution experiments on liver segments have suggested that the initial depolarizing component of the response is in fact due to an increase in membrane permeability to Na^+ and Cl^-. However, more recent studies using intracellular and whole cell patch clamp techniques to record from single liver cells in primary culture (Field & Jenkinson, 1987; Caplod & Ogden, 1989*a*) have concluded that an increase in P_{Cl} and P_K alone could adequately account for the main features of the observed rise in membrane conductance induced by noradrenaline.

1.6.1 The involvement of Ca^{2+} in hormone mediated changes in liver membrane permeability

The demonstration that Ca^{2+} could regulate the activity of phosphorylase b kinase, a key enzyme in the glycogenolytic cascade, in skeletal muscle (Heilmeyer, Meyer, Haschke & Fischer, 1970) prompted Haylett in 1976 to investigate the relationship between $^{45}Ca^{2+}$ and $^{42}K^+$ movements and glucose release in guinea-pig and rabbit slices stimulated with either the α-agonist, amidephrine or the β adrenoceptor agonist, isoprenaline. The amidephrine-induced efflux of $^{42}K^+$ and glucose was paralleled by an equally rapid efflux of $^{45}Ca^{2+}$. By comparison isoprenaline had little, if any, effect on either the $^{42}K^+$ or $^{45}Ca^{2+}$ efflux. Together these results were taken to suggest that the α receptor mediated changes in P_K and glycogenolysis were both consequences of a rise in cytosolic Ca^{2+}.

Direct evidence for this elevation in $[Ca^{2+}]_i$ has since been obtained using the Ca^{2+}-sensitive dyes Quin2 (Charest, Blackmore, Berthon & Exton, 1983; Berthon, Binet, Mauger & Claret, 1984) and

aequorin (Woods, Cuthbertson & Cobbold, 1986; 1987). The rise in cytosolic Ca^{2+} in aequorin loaded rat hepatocytes (Charest, Prpic, Exton & Blackmore, 1985) and the increase in P_K (as assessed either by changes in membrane potential or by net movements Rb^+; DeWitt & Putney, 1984; Egashira, 1980*b*; Karashima, 1981) become transient in preparations bathed in a low Ca^{2+} medium but are restored on the addition of Ca^{2+}. This implies that the K^+ release response occurs in two phases; an initial phase due to the Ca^{2+} mobilization from internal stores, whilst a more sustained K^+ loss draws upon extracellular Ca^{2+}.

Further support for the role of Ca^{2+} is found in two studies by Burgess, Claret & Jenkinson (1979,1981). The Ca^{2+} ionophore A23187 was initially shown by these investigators to cause a rapid loss of K^+ from dispersed hepatocytes. In their later experiments it was demonstrated that this increase in P_K could be blocked by apamin, a neurotoxin which has since been well characterised as a selective inhibitor of Ca^{2+}-activated P_K mechanisms in certain excitable tissues, with little effect on other types of K^+ channel (Hughes, Romey, Duval, Vincent & Lazdunski, 1982; Hughes, Schmidt, Romey, Duval, Frelin & Lazdunski, 1982; Romey & Lazdunski, 1984; Seagar, Granier & Couraud, 1984; Brown & Sepulveda, 1985; Kawai & Watanabe, 1986).

Field and Jenkinson (1987) have subsequently reported that the action of noradrenaline on P_{Cl} can also be mimicked by A23187. More direct evidence for the activation of both the K^+ and Cl^- conductances by intracellular Ca^{2+} comes from experiments conducted in the same laboratory in which voltage clamped liver cells were internally perfused with buffered Ca^{2+} solutions (Capiod, Field, Ogden & Sandford, 1987). Very recently a small conductance K^+ channel has been identified in patches excised from guinea-pig hepatocytes. This channel is activated by raising the Ca^{2+} concentration at the internal membrane face over

the physiological range and blocked by apamin applied externally (Capiod & Ogden, 1989*b*).

In keeping with the notion that Ca^{2+} is a key intermediary, the effect on P_K is just as readily seen with other Ca^{2+} mobilizing agonists such as ATP (Jenkinson & Koller, 1977; Burgess *et al.*, 1981) and angiotensin II (Weiss & Putney, 1978; Cocks, Jenkinson & Koller, 1984) as with noradrenaline. What is perhaps more surprising is that the increase in P_K is also seen in a proportion of guinea-pig cell and slice preparations on the addition of low concentrations of the cAMP dependent hormones, isoprenaline and salbutamol (Haylett & Jenkinson, 1972*a*; Cocks *et al.*, 1984). Recent Quin 2 studies have shown that isoprenaline and cAMP analogues can elevate $[Ca^{2+}]_i$ in rat liver cells (Combettes, Berthon, Binet & Claret, 1986). It seems reasonable then to suppose that any effect on P_K mediated by cAMP is also due to an increase in cytosolic Ca^{2+} activating $P_{K(Ca)}$. In keeping with this, it is blocked by apamin.

Interestingly with combined administration of low doses of cAMP dependent and Ca^{2+} dependent agonists a marked potentiation is observed whether the response is measured in terms of changes in membrane potential in liver slices (Jenkinson & Koller, 1977) or changes in K^+ or $^{86}Rb^+$ movements in cells suspensions (Cocks *et al.*, 1984; Burgess, Dooley, McKinney, Nanberg & Putney, 1986). This interaction does not involve increases in the formation of either cAMP (Cocks *et al.*, 1984) or inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$ see below) (Poggioli, Mauger & Claret, 1986; Burgess *et al.*, 1986) but is thought to be due to a greater increase in cytosolic $[Ca^{2+}]$ (Morgan, Charest, Blackmore & Exton, 1984; Poggioli *et al.*, 1986).

1.6.2 The association between receptor occupation and PI turnover

It is now well recognised that a rise in cytosolic calcium is not the primary response to the activation of calcium mobilizing receptors. The first indication that the breakdown of membrane phopspholipids might be involved in signal transduction came from the work of Hokin and Hokin (1954). These investigators reported that the secretory response of the exocrine pancreas to muscarinic stimuli was associated with an increase in phosphatidyl inositol (PI) metabolism. In his 1975 review, Michell noted the association of enhanced PI turnover with Ca^{2+}-mobilizing receptors and presented arguments that this biochemical reaction might somehow serve to couple receptor activation to the release of Ca^{2+} from intracellular pools.

The details of the biochemical pathway linking receptor occupation to an increase in PI breakdown have since been defined in a number of tissues including the liver. It is generally accepted that the stimulation of a cell surface receptor initiates the hydrolysis of a relatively minor membrane phospholipid, phosphatidyl inositol 1,4,5-bisphosphate (Ptd Ins P_2) by a phospholipase C (PLC) to give two second messengers 1,2 diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins $(1,4,5)P_3$).

There is a substantial body of evidence, reviewed e.g. by Cockcroft (1987), that the receptor controls PLC activity through a GTP binding protein(s), a situation somewhat analogous to the receptor activation of adenylate cyclase. The first direct demonstration that Ca^{2+} mobilizing receptors couple to PLC through a G protein in the liver came from Exton's laboratory (Uhing *et al.,* 1986). They found that a crude liver plasma membrane preparation produced Ins $(1,4,5)P_3$ in reponse to vasopressin, but only in the presence of GTP (and Mg^{2+}). The stable analogues of GTP, GTPγS and GppNHp each stimulated Ins $(1,4,5)P_3$ formation and most importantly potentiated the effects of vasopressin.

Moreover GDP-γ-S completely abolished stimulation of PLC by both guanine nucleotides and the hormone.

1.6.3 IP_3 - a second messenger for Ca mobilization

The first clear indication that IP_3 might be responsible for the mobilization of intracellular Ca^{2+} came from the observation that IP_3 stimulated the release of Ca^{2+} from permeabilized pancreatic acinar cells (Streb *et al.*, 1983). In the following year Burgess, Godfrey, McKinney, Berridge, Irvine & Putney (1984*a*) demonstrated the ability of IP_3 to release Ca^{2+} from saponin treated hepatocytes. In the same study these workers described a rapid breakdown of Ptd Ins P_2 in hepatocytes, but with a latency which seemed rather too great to account for the mobilization of intracellular Ca^{2+} stores. However, subsequent reports by Irvine, Letcher, Lander and Downes (1984) that a significant proportion of the IP_3 formed by parotid glands in response to carbachol in fact represented an inactive isomer Ins(1,3,4) P_3, prompted Burgess, McKinney, Irvine & Putney (1985) to re-examine the kinetics of IP_3 production. The second series of experiments revealed that Ins(1,4,5,)P_3 formation is stimulated by angiotensin with no apparent lag in contrast to the Ca^{2+} response and the formation of Ins(1,3,4)P_3 which are both delayed for at least a second.

In keeping with the notion that Ins (1,4,5)P_3 functions as the second messenger in the release of Ca^{2+} from the intracellular stores in the liver, a close correlation has been shown to exist between the initial rates of increase of cytosolic free Ca^{2+} concentration induced by the action of vasopressin in hepatocytes and that of Ptd Ins P_2 breakdown and the production of IP_3 (Thomas, Alexander & Williamson, 1984). Other supporting evidence that Ins 1,4,5 P_3 is the relevant second messenger includes:

(1) Ptd Ins P_2 breakdown does not require cytosolic [Ca^{2+}] to be in excess of resting levels (Charest *et al.,* 1985), although elevated [Ca^{2+}] may favour the reaction (Melin, Sundler & Jergil, 1986) and

(2) Ins(1,4,5)P_3 is rapidly metabolized to products which do not release Ca^{2+} from the IP_3-sensitive Ca^{2+} pool, though the metabolite, Ins(1,3,4,5)P_4 may provide Ins(1,4,5)P_3 with access to other IP_3-insensitive Ca^{2+} pools (Changya, Gallacher, Irvine, Potter & Petersen, 1989*a*) and assist it in the mobilization of extracellular Ca^{2+} (Morris, Gallacher, Irvine & Petersen, 1987).

1.6.4. The hormone sensitive Ca^{2+} pool

The notion that both the endoplasmic reticulum and the mitochondria contributed to the Ca^{2+} signal stemmed from early studies of the distribution of cell Ca^{2+} which indicated that these organelles contain the bulk of the sequestered Ca^{2+} and the knowledge that these organelles possess well developed Ca^{2+} transport systems (Bygrave, 1978*a,b*; Becker, Fiskum & Lehninger, 1980). In addition fractionation studies using $^{45}Ca^{2+}$ prelabelled cells (Kimura *et al.,* 1982) and rapid fractionation of the liver utilizing a Percoll density gradient (Reinhart, Taylor & Bygrave, 1982*a,b*) suggested that the Ca^{2+} content of both mitochondria and endoplasmic reticulum enriched fractions decreased following the administration of hormones to isolated cells or perfused liver. Other workers though have questioned the likelihood of mitochondrial involvement, on the basis of data suggesting that normal, healthy cells do not generally contain appreciable quantities of Ca^{2+} in their mitochondria (Burgess, McKinney, Fabiato, Leslie & Putney, 1983; Somlyo, Bond & Somlyo 1985; Bond, Vadasz, Somlyo & Somlyo, 1987).

The view that the endoplasmic reticulum coincides with the hormone sensitive Ca^{2+} pool gained popularity with reports from a

number of laboratories that inhibition of mitochondrial function did not effect the ability of Ins(1,4,5)P_3 to mobilise Ca^{2+} from a variety of permeabilised cells including hepatocytes (Burgess, Godfrey, McKinney, Berridge, Irvine & Putney, 1984*a*; Burgess *et al.*, 1983; Joseph, Thomas, Williams, Irvine & Williamson, 1984) neutrophils (Prentki, Wollheim & Lew, 1984) and pancreatic acinar cells (Streb, Irvine, Berridge & Schulz, 1983). Also Streb and co-workers (1984) carried out a detailed analysis of Ins (1,4,5)P_3-induced Ca^{2+} release in subcellular fractions of exocrine pancreas, and compared this activity with specific enzymatic markers for the plasma membrane, mitochondria and endoplasmic reticulum. A strong positive correlation was obtained between Ins(1,4,5)P_3 induced Ca^{2+} release and the endoplasmic reticulum marker enzyme cytochrome C NADPH reductase, whereas for both the plasma membrane and mitochondrial markers, the correlation was negative. Spat and co-workers (1986) subsequently identified a high affinity binding site for IP_3 in rat liver microsomal fractions.

However, it should be mentioned that recent results obtained in rat hepatocytes have challenged the idea that the endoplasmic reticulum is the target pool for IP_3. Firstly, Guillemette, Balla, Baukal and Catt (1988) have reported IP_3 receptor numbers are greater, and the level of IP_3 induced Ca^{2+} release higher, in plasma membrane fractions than in microsomes. Secondly an immunocytochemical approach using antibodies raised against calsequestrin and Ca^{2+}-Mg^{2+}-ATPase has shown that Ca^{2+} can be accumulated by an Ins(1,4,5)P_3 sensitive vesicular system (the "calcisome") distinct from the endoplasmic reticulum (Volpe, Krause, Hashimoto, Zorzato, Pozzan, Meldolesi & Lew, 1988).

Whatever its exact location, only part of the intracellular Ca^{2+} pool appears to be regulated by Ins(1,4,5)P_3. The addition of IP_3 to permeabilised hepatocytes releases on average just 30% of the

sequestered $.Ca^{2+}$; the remainder is released by ionophores (Burgess *et al.*, 1984). This implies that there is an $Ins(1,4,5)P_3$ insensitive Ca^{2+} pool which is a separate membrane compartment, probably with distinct Ca^{2+}-pumping characteristics (Thévenod, Dehlinger-Kemmer, Kemmer, Christian, Potter and Schultz, 1989).

1.6.4.1 Does the IP_3 insensitive pool contribute to Ca^{2+} signal initiated by $Ins(1,4,5)P_3$?

Studies on permeabilized cells and cell fractions have shown that GTP can release Ca^{2+} from both pools independently of $Ins(1,4,5)P_3$ (Chueh & Gill, 1986) and that this release can be blocked by treatments that have no effect on the $Ins(1,4,5)P_3$-sensitive system. These properties closely resemble the GTP dependent control of vesicular protein transport through the Golgi complex (Bourne, 1988) and with the relatively constant levels of GTP in cells, the physiological significance of GTP-induced calcium release in intact cells is doubtful. It has been suggested, however, that GTP may somehow enlarge the $Ins(1,4,5)P_3$-sensitive pool at the expense of the $Ins(1,4,5)P_3$-insensitive pool (Ghosh, Mullaney, Tarazi & Gill, 1989) and this process may be controlled by $Ins(1,3,4,5)P_4$ (Irvine, Moor, Pollock, Smith & Wregget, 1988). The $Ins(1,4,5)P_3$-insensitive pool may also help to amplify and propagate the calcium signal derived from the $Ins(1,4,5)P_3$-sensitive store by a mechanism of calcium-induced calcium release (Bussa, Ferguson, Joseph, Williamson & Nuccitelli, 1985).

1.6.5 The mobilization of intracellular Ca^{2+} by $Ins(1,4,5)P_3$

The permeabilization of the internal Ca^{2+} stores by $Ins(1,4,5)P_3$ is an extremely rapid process. Laser photolysis of inactive caged $Ins(1,4,5)P_3$ opens Ca^{2+} dependent K^+ channels in the plasma membrane

of patch clamped liver cells after a delay of just 200ms (Ogden, Capiod, Trentham & Walker, 1990) and when Ca^{2+} release from permeabilised hepatocytes is measured directly with quin2, a Ca^{2+} response may be detected as early as 20ms after the addition of $Ins(1,4,5)P_3$ (Champeil, Combettes, Berthon, Doucet, Orlowski & Claret, 1989).

The kinetics and the temperature insensitivity (Joseph & Williamson, 1986) of the Ca^{2+} release induced by $Ins(1,4,5)P_3$ favour a ligand-gated Ca^{2+} channel over a cation exchange mechanism. However, the presence of IP_3 sensitive Ca^{2+} channels has so far only been demonstrated directly in excitable tissues (Ehrlich & Watras, 1988; Maeda, Kawasaki, Nakade, Yokota, Taguchi, Kasai, Mikoshiba, 1991; Mayrleitner, Chadwick, Timeran, Fleischer & Schindler, 1991).

In a recent study of the fast kinetics of the mobilization of internal Ca^{2+} stores in permeabilized basophilic leukemia cells, Meyer, Holowka and Stryer (1988) concluded that at least three molecules of $Ins(1,4,5)P_3$ must be bound to open a Ca^{2+} channel. A somewhat lower cooperativity, however, has since been reported in broadly similar experiments conducted on rat hepatocytes by Champeil *et al.*, (1989).

A characteristic feature of the $Ins(1,4,5)P_3$ receptor is that it does not desensitize. This is perhaps most clearly illustrated by the inability of cells to resequester Ca^{2+} mobilized by inositol (1,4,5) trisphosphorothioate (Taylor, Berridge, Cook and Potter, 1989), an analogue which is resistant to metabolism by either the $Ins(1,4,5)P_3$-5-phosphatase (Willcocks, Potter, Cooke & Nahorski, 1988) or the $Ins(1,4,5)P_3$-3-kinase (Taylor *et al.*, 1989). Any decline in the release of Ca^{2+} can usually be attributed instead either to the rapid metabolism of $Ins(1,4,5)P_3$ (Stauderman, Harris & Lovenberg, 1988) or to the transfer of the mobilized Ca^{2+} to an $Ins(1,4,5)P_3$ insensitive pool.

The $Ins(1,4,5)P_3$ induced release of Ca^{2+} can be modulated by a

variety of physiological and pharmacological agents. Classical Ca^{2+} channel blockers have no effect (Joseph & Williamson, 1986) but release is inhibited by cinnarizine and flunarizine (Seiler, Arnold & Stanton, 1987) and by the K^+ channel blocker tetraethylammonium (TEA) (Shah & Pant, 1988). In the absence of K^+, Ins(1,4,5)P_3 is incapable of mobilizing intracellular Ca^{2+} though other monovalent cations (Na^+»$Tris^+$>Li^+) can substitute for K^+, which appears to function as a counterion to neutralise the charge developed by the efflux of Ca^{2+} (Joseph & Williamson, 1986).

1.6.5.1 The mobilization of intracellular Ca^{2+} by cAMP

Very recently cAMP has been reported to modify the binding properties of the Ins(1,4,5)P_3 receptor in rat liver, but this was not related by Mauger, Claret, Pietri and Hilly (1989) to any biological effect. cAMP can in fact mobilize intracellular calcium on its own accord from the Ins(1,4,5)P_3 sensitive pool in the liver cells (Mauger & Claret, 1986). The mechanism by which cAMP controls Ca^{2+} movements in this compartment remains to be elucidated but it is already obvious that the action of cAMP is not mediated by PI breakdown, since it does not alter the basal IP_3 fraction of the liver cell (Pittner & Fain, 1989) and it is not effected by heparin (Capiod, Sandford, Combettes, Noel, Jenkinson & Claret, 1990) which inhibits the binding of Ins(1,4,5)P_3 to its purified receptor (Supattapone *et al.,* 1988) and the mobilization of Ca^{2+} by Ins(1,4,5)P_3 from permeabilized cells (Hill, Berggren & Boynton 1987).

1.6.6. The mobilization of extracellular Ca^{2+}

In addition to mobilizing intracellular Ca^{2+}, many agonists can provide an influx of external Ca^{2+} to support a maintained response. Both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 have been implicated in controlling

the influx of Ca^{2+} into the cell (see e.g. Kuno, & Gardner, 1987; Morris, Gallacher, Irvine & Petersen, 1987; Penner, Matthews & Neher, 1988; Changya, Gallacher, Irvine, Potter & Petersen, 1989*b*) but just how these inositol phosphates may function is uncertain because the route of Ca^{2+} entry into nonexcitable cells is unknown. In Putney's capacitance model (Putney, 1986; 1990), IP_3 is postulated to trigger an increase in P_{Ca} indirectly by depleting the intracellular Ca^{2+} pools. Much of the original evidence for this model infact came from studies comparing the kinetics of the Ca^{2+} entry triggered by lowering $[Ca^{2+}]_i$ and that by agonists in rat liver cells (Poggioli, Mauger, Guesdon & Claret, 1984), but the relative importance of this mechanism in the hepatocyte has recently been thrown into question by reports that Ca^{2+} influx can be dissociated from Ca^{2+} release with the ER Ca^{2+} pump inhibitor tBUBHQ (Kass, Llopis, Chow, Duddy & Orrenius, 1990).

An alternative hypothesis is that Ca^{2+} flows into the cytosol through Ca^{2+} channels in the plasma membrane gated by either one of the polyinositol phosphates or Ca^{2+} itself. Indeed $Ins(1,4,5)P_3$ has been found to release Ca^{2+} from plasma membrane vesicles from liver (Guillemette, Balla, Baukal & Gatt, 1988). Results subsequently obtained with $Ins(1,4,5)PS_3$ by Hansen, Siemens and Williamson (1990) indicate that the action of $Ins(1,4,5)P_3$ on P_{Ca} does not require its metabolism and are consistent with an $Ins(1,4,5)P_3$ operated Ca^{2+}-channel. However, this data could just as readily be explained in terms of the Ca^{2+} permeable channel recently described in endothelial cells, which can be opened by either Ca^{2+} or $Ins(1,3,4,5)P_4$ (Luckhoff & Clapham, 1992). Clearly further investigations will be required before the hormone stimulated influx of Ca^{2+} in the liver can be assigned unequivocally to the activity of any one second messenger operated channel.

1.7 THE AIMS OF THE PRESENT STUDY

The primary objective of this study was to obtain more information about the ion permeabilities of both resting and activated liver cells. The aim of the first series of experiments was to characterise further the volume-dependent K^+ permeability first described by Bakker-Grunwald (1983) and Kristensen & Folke (1984). Cell swelling as a result of electrolyte leakage from the electrode into the cytoplasm is a well recognised problem when recording intracellularly from small cells. In hepatocytes, its occurrence can be recognised by a hyperpolarization (as P_K rises) associated with a fall in membrane conductance. These effects have been used here to advantage to identify pharmacological agents that inhibit the volume-activated K^+ permeability present in the liver cell. In addition the whole cell patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) has been employed to record changes in membrane conductance as cell volume was deliberately increased by the application of gentle hydrostatic pressure to the shank of a patch pipette. A large outward current was observed under these conditions, and the spontaneous fluctuations in this current ("current noise") were analysed to estimate the properties of the underlying unitary events.

In the second main series of experiments the hormonal control of intracellular calcium in hepatocytes was investigated by monitoring the Ca^{2+} dependent conductances, $P_{K(Ca)}$ and $P_{Cl(Ca)}$ (Capiod, Field, Ogden & Sandford, 1987; Field & Jenkinson, 1987). The validity of this approach has been confirmed in cultured epithelial cells (Yada, Oiki, Ueda & Okada, 1986), L cells (Ueda, Oiki & Okada, 1986), hamster eggs (Igusa & Miyazaki, 1986) and more recently guinea-pig hepatocytes (Ogden, Capiod, Walker & Trentham, 1990) where changes in membrane current or potential (due to the opening of K^+ channels) and

intracellular Ca^{2+} have been shown to occur in phase with each other. It may in fact be argued that such an indirect technique which makes use of an endogenous calcium reporter is preferable to a method that employs an artificial indicator which may buffer intracellular Ca^{2+} and distort the delicate mechanisms responsible for regulatory intracellular Ca^{2+} (see e.g. Monk, Reynolds, Thomas and Williamson, 1988).

The present work re-examines at a single cell level the nature of the Ca^{2+} response to ATP and salbutamol applied individually and the interactions which have been reported to occur between these agonists in liver slices (Jenkinson & Koller, 1977) and suspensions of isolated liver cells (Cocks, Jenkinson & Koller, 1984). In particular, the cellular basis of the pattern of the Ca^{2+} response to receptor stimulation has been addressed in whole cell experiments where the rapid exchange of cell and electrode contents, which occurs shortly after the establishment of the whole cell configuration (Marty & Neher, 1983; Lamb, Matthews & Torre, 1986), has been exploited to introduce the putative second messengers cAMP and IP_3 directly into the liver cell.

Finally, some experiments on the nature of the receptors involved in the Ca^{2+}-mobilizing action of ATP are described, together with a preliminary study of a fast inward current which was observed in response to relatively large concentrations of this agonist.

CHAPTER 2

MATERIALS AND METHODS

2.1 PREPARATION OF ISOLATED HEPATOCYTES

2.1.1 Hepatocyte isolation

Hepatocytes were prepared from male Hartley guinea-pigs (200-450g) by collagenase digestion, adopting the method developed by Howard and Pesch (1968) and Berry and Friend (1969), and subsequently modified by Seglen (1973). The guinea-pigs were anaesthetised with pentobarbitone (Sagatal, 30mg/kg), fentanyl (Sublimaze, 0.1mg/kg) and droperidol (Droleptan, 10mg/kg) given as successive intraperitoneal injections.

The abdomen was opened and the intestines displaced to one side to reveal the portal vein. Side branches entering the vein were tied off. Loose ties were placed around the upper and lower ends of the vein. The vein was then cannulated, and the liver was gently flushed with 15-30ml Ca^{2+}-free Eagle's Minimum Essential Medium (MEM). The omission of Ca^{2+} during this and the following stage is thought to facilitate cell dissociation by promoting the separation of desmosomes between adjacent cells (Drochmans, Wanson, May & Bernaert *et al.*, 1978). The liver was rapidly dissected from the animal and transferred to a water jacketed perfusion chamber. The cannula was cut about 2cm from the point of insertion into the portal vein and connected to the perfusion apparatus, with the weight of the liver supported by a perforated perspex platform which allowed the perfusate to drain away from the bottom of the chamber to waste (Fig. 2.1). The liver was first perfused with Ca^{2+}-free solution under gravity at a rate of approximately 40ml/min^{-1}. After 10 minutes perfusion with the Ca^{2+}-free MEM, this was replaced by a high Ca^{2+} (5.1 mM) MEM solution containing collagenase. Due to the expense of the enzyme this solution was recirculated until the liver became soft (4-7 minutes). The liver was

then disconnected from the perfusion system and transferred to a Petri dish, where the gall bladder, blood vessels and superficial connective tissue were dissected away. The liver was minced with a razor blade, then transferred to a 250ml conical flask with the remaining collagenase solution and incubated in a shaking water bath (ca. 60 strokes/min) at 37°C for approximately 4 minutes. The suspension was gassed continuously during this period and all subsequent incubations by passing a constant stream of 95% O_2/5% CO_2 over the surface of the medium. At the end of the incubation 10ml of new born calf serum was added to reduce further digestion by the collagenase and the cell suspension was filtered through 50μm nylon mesh to remove undissociated tissue. The cells were washed twice in normal MEM by gentle centrifugation at 50×g (550rpm, MSE bench centrifuge) for 2 minutes and then resuspended in supplemented Eagle's MEM solution (see below). After a further 30 minutes incubation in a shaking bath maintained at 37°C, the cells were resuspended in supplemented Eagle's medium.

2.1.2 Solutions

The basic "minimum essential medium" (MEM) used for hepatocyte isolation had the following composition (mM): NaCl 116; KCl 5.4; $MgSO_4$ 0.81; NaH_2PO_4 0.96; $NaHCO_3$ 25; $CaCl_2$ 1.8 and glucose 0.56. A Ca^{2+} free variant was prepared by omitting the $CaCl_2$, and including 0.52mM EGTA.

For cell dissociation, collagenase (from *Clostridium histolyticum*) was dissolved at a concentration of 0.33mg/ml in normal MEM containing an additional 3.3mM $CaCl_2$, ie, a total of 5.1mM.

Once isolated, the cells were suspended in Eagle's MEM prepared from a powder obtained initially from Wellcome Reagents and

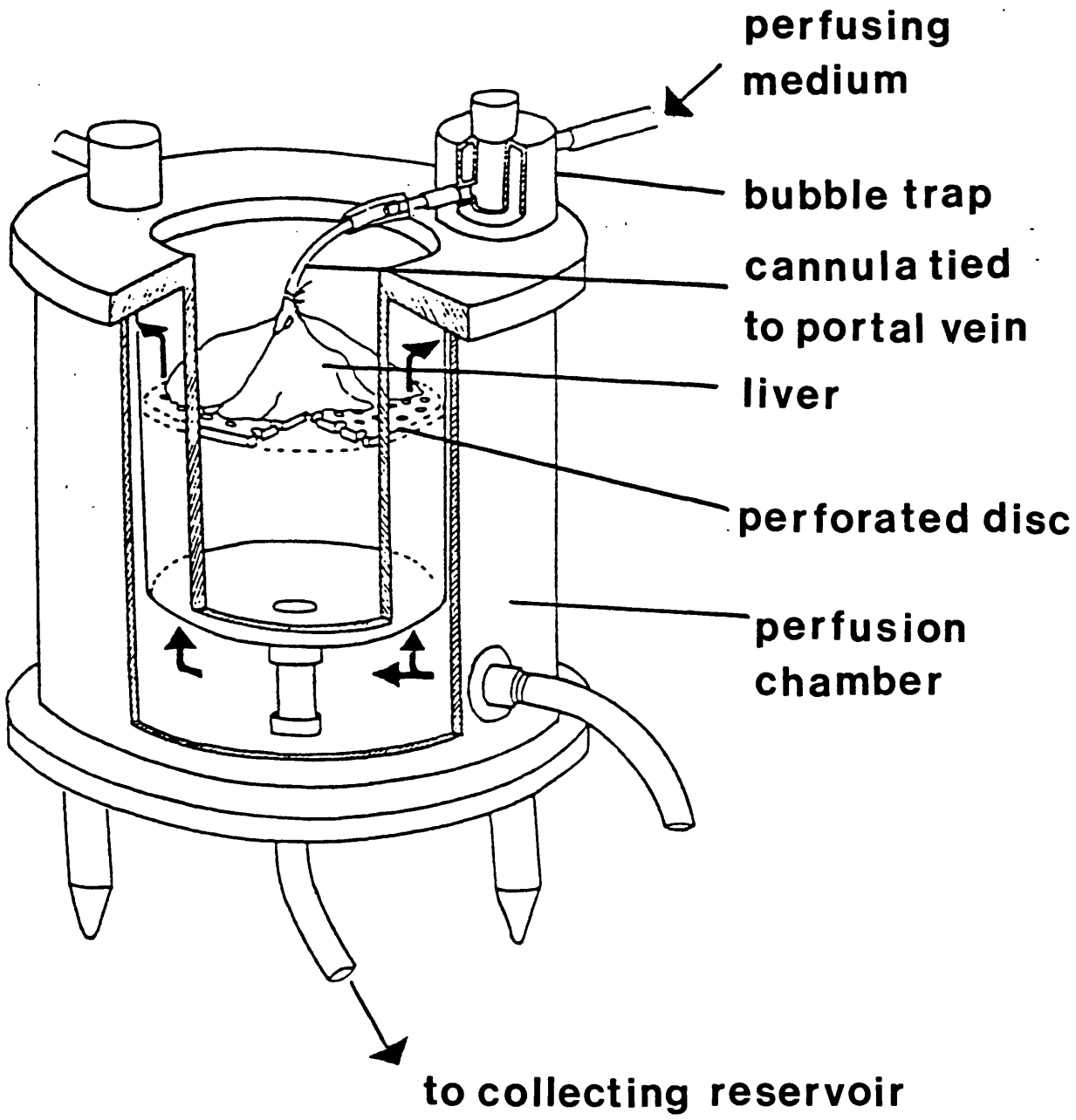


Fig. 2.1 Water jacketed chamber used in the isolation of single liver cells. The liver rests on a perforated perspex disc, while it is perfused via the portal vein, under gravity, with Ca^{2+}-free MEM or a collagenase solution from reservoirs, held 0.75 metres above the perfusion chamber.

→ indicates the direction with which the liver is perfused.

➡ indicates the direction that water pre-warmed to 37°C circulates through the water jacket.

Diagram reproduced with kind permission of Neil Castle.

subsequently from Gibco. This medium (see Table 2.1 for composition) was supplemented with 2% bovine serum albumin (BSA fraction V), 10% new born calf serum and penicillin and streptomycin (200,000 units/litre).

Throughout the isolation procedure all the above solutions were warmed to 37°C and gassed with 95% O_2/5% CO_2, to maintain a pH of 7.4.

2.1.3 Viability of cells

The viability of the isolated hepatocyes was assessed by their ability to exclude Trypan blue. 20μl of cell suspension was mixed with an equal volume of Trypan blue (0.4%, Gibco) and examined under bright field illumination to determine the proportion of cells in which the cytoplasm was stained by the dye. Cell death/damage renders the plasma membrane leaky allowing the dye to enter the cells and stain intracellular organelles. Typically 70-90% of guinea pig hepatocytes excluded the dye.

2.2 SHORT TERM CULTURE OF HEPATOCYTES

Ten minutes after the final suspension, cells were plated out in 35mm Falcon Petri dishes, at a density of approximately 7×10^5 cells in 2ml of supplemented Eagle's medium, and incubated at 37°C in an atmosphere of 5% CO_2/95% O_2. Frequently groups of undissociated cells could be identified in the cell suspension. As the cells progressively flattened out in primary culture neighbouring cells sometimes appeared to become coupled and the cell margins became increasingly difficult to resolve. Recently Graf *et al.* (1987) have demonstrated that rat hepatocytes isolated as couplets retain the electrical coupling known to exist in the intact liver (Penn, 1966; Haylett & Jenkinson, 1972*a*). For

the purpose of this study it was important that most of the cells were unassociated, so care was taken to ensure the cell density was low.

Usually after one hour the cells had become attached to the Petri dishes. With intracellular and whole cell techniques, satisfactory recordings were obtained most readily after 2-6 hours incubation, before the cells became excessively flat. All the results presented in this thesis were obtained from cells incubated for 2-10 hours at 37°C.

2.3 INTRACELLULAR RECORDING

2.3.1 Recording medium

Before beginning recording, the supplemented Eagle's medium was replaced by a solution containing (mM): NaCl 137; KCl 4.4; $CaCl_2$ 2.5; $MgSO_4$ 0.81; N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 8; glucose 0.2%; BSA 1%; adjusted to pH 7.4 with NaOH and filtered through a 0.22μm Millipore filter.

For "chloride free" experiments chloride was replaced on an equimolar basis with the larger organic anions methylsulphate and isethionate which are thought to be less membrane permeant (e.g. Field and Jenkinson, 1987). The composition of the chloride free solution (solution B) is given in Table 2.2.

2.3.2 Recording conditions

The tissue culture dish was inserted into a machined recess in an aluminium block. The block was mounted on the stage of a Zeiss Larborlux II microscope, fitted with a long working distance objective. Holes drilled through the base of the recess allowed the cells to be viewed under bright field conditions at 340x magnification. The block was heated using a d.c. power supply which had been modified so that the temperature recorded by a thermistor probe inserted into the block

could be maintained thermostatically. The block was kept at 38-40°C which corresponded to a bath temperature of 35-37°C.

To limit evaporation a thin film of liquid paraffin (Spectrosol, BDH Chemicals) was floated on top of the recording medium. Long, fine tipped microelectrodes, such as those employed in this study, are particularly susceptible to mechanical interference and when the layer of paraffin was in place, microelectrode movement could sometimes be detected even in the absence of any obvious external disturbance. This vibration of the microelectrode tip may have resulted from a resonance which was related in some way to the presence of the paraffin-water interface. In keeping with this, the degree of resonance depended critically on the relative proportions of recording medium and liquid paraffin used. Microelectrode movement was found to be minimal with a combination of 6ml of recording medium and 1ml of liquid paraffin, and this was used routinely.

The recording apparatus was housed in a Faraday cage on a vibration isolation table (MICRO-g Technical Manufacturing Corporation). To increase the mechanical stability of the recording assembly a large paving stone was used as a table top on which was placed a 1" thick steel plate bearing the microscope.

2.3.3. Microelectrodes

Microelectrodes were fabricated from 1.0mm O.D. borosilicate glass capillaries containing a filament, (Clark Electromedical GC-100F-15), using a two stage horizontal puller (Narashige PN-3). Microelectrodes were back-filled just before use with a solution of 1M K citrate and 10mM KCl, titrated with citric acid to pH6 and filtered through a 0.22μm Millipore filter. Usable microelectrodes filled with this solution had a resistance of 120-150MΩ. Less stable intracellular recordings were obtained with

identical microelecrodes filled with either 1M K methylsulphate (pH6) or 4M K acetate (pH6). Electrical contact with the microelectrode filling solution was made with a silver/silver chloride pellet (Clark Electromedical EH-3MS). 10mM KCl was therefore included in the filling solution to ensure electrochemical reversibility and stability of the half-cell potential.

The microelectrode holder was mounted on a hydraulic microdrive (Narashige MO-103) which provided remote fine control of the microlectrode's movements in three dimensions, including along the axis of the microelectrode. The driven unit of the micromanipulator was in turn mounted on a Zeiss sliding manipulator which afforded coarse control of the microelectrode's movement, again in three dimensions.

2.3.4 The measurement of membrane potential and input resistance using a single microelectrode

The single-microelectrode 'bridge' method (see Purves 1981) was employed. Changes in membrane potential and conductance were followed with a Neurolog 102G DC amplifier. The signal from the Neurolog was recorded on magnetic tape (Racal store 4DS) and displayed on a digital oscilloscope (Gould OS 4020) and on a pen recorder (Ormed MX 216 or Rikadenki RW-11T). A standard voltage source was inserted between the bath electrode and earth to allow the voltage traces to be calibrated.

2.4 WHOLE CELL PATCH CLAMP RECORDING

2.4.1 Extracellular medium

The chloride-containing recording medium (solution C, Table 2.2) used was identical in composition to that for intracellular recording, except that BSA was omitted, since it hinders the formation of high

resistance seals between cell membrane and pipette.

2.4.2 Recording conditions

Patch clamp recordings were made at 35-37°C. The Falcon dish was housed in a heated aluminium block, which was mounted on the stage of an inverted microscope (Nikon, Diaphot), fitted with a long working distance objective. The hepatocytes were perfused continuously under gravity at approximately 4 ml/min with HEPES buffered saline, preheated to 40°C in a water jacketed reservoir. Fluid was drained from the bath with the aid of a peristaltic pump. Recording conditions were otherwise the same as for intracellular work.

2.4.3. Patch pipettes

Patch pipettes were fabricated from thin walled borosilicate capillary tubing (Clark Electromedical GC 150 TF), using a two stage vertical puller (List, LM 3PA). Pipettes were coated to within 50μm of the tip with Sylgard (Dow Corning) which was then heat-cured. The pipettes were fire-polished and back filled immediately prior to use. The standard intracellular solution contained: 145mM KCl, 8mM HEPES and 50μM EGTA, titrated to pH7.1 with KOH and filtered through a 0.22μm Millipore filter. Pipettes filled with this solution had resistances of 2-8MΩ.

A filling solution with a higher Ca^{2+} buffering capacity might have been preferable in some respects to that used in the current study. Marty and Neher (1983) report that reducing the concentration of EGTA below 0.1mM can lead to difficulties in establishing the whole cell configuration and to instability of the access resistance. However, in preliminary experiments it was found that the inclusion of 1.1mM EGTA and 0.1mM $CaCl_2$ inhibited the whole cell response to either 10μM ATP or

intracellular dialysis with cAMP in salbutamol-sensitive preparations (see later). A further modification to the filling solution which might be desirable would be to reduce the Cl^- concentration to a more physiological level. High intracellular Cl^- concentrations lead to a rapid deterioration of perfused squid axons and inhibit Ca^{2+} dependent exocytosis from "leaky" bovine adrenal medullary cells (Baker, 1984), though Marty and Neher (1983) report that there is no noticeable difference in whole cell recordings obtained from chromaffin cells using pipettes containing isotonic chloride, sulphate, glutamate or aspartate.

2.4.4 Whole cell recording from hepatocytes

Tight-seal whole cell recording techniques were used (Hamill *et al.*, 1981; Marty and Neher, 1983). Whole cell currents and potentials were measured with a LM-EPC-7 patch clamp amplifier (List Electronics). The amplifier probe was insulated from ground and mounted on the micromanipulator. A PTFE adaptor was used to make electrical contact between the silver-silver chloride pellet in the suction pipette holder (Clark Electromedical, EH-2MS) and the probe. During the final stages of my work, when the equipment became available, a patch clamp software system (pClamp 5.0) was sometimes used in conjunction with a Labmaster data acquisition board (AD and DA conversion) and a microcomputer (Dell 200) to generate step changes in holding potential. Currents were low pass filtered at 10kHz at the output of the patch clamp amplifier and stored on a FM tape recorder (Racal Store 4DS).

Problems may be encountered when voltage clamping hepatocytes with patch pipettes because of the relatively large size of the cells. The consequent large cell capacitance and whole cell currents can severely limit the speed and accuracy with which the membrane potential can be clamped. In principle, the error between the clamp potential and

the membrane potential resulting from the pipette current flowing through the series resistance of the pipette-cell junction (R_S), can be automatically compensated for by feeding back a fraction of the current signal to V_{ref}. However, series resistance compensation of this kind can result in instability and in practice the values of R_S often increased during recording, so making compensation imprecise. Corrections for the errors due to the series resistance were therefore made instead using values of R_S determined from the "G series" potentiometer of the patch clamp amplifier, subsequent to capacitance transient compensation. To minimise the voltage errors arising from the series resistance, low resistance pipettes were used and small cells were selected. Taking such precautions also limited the attenuation of the response of the voltage-clamped cell to applied voltage steps (and to currents originating in the cell membrane) by the low pass filter formed by the series resistance and cell capacitance ($f_{CO} = 1/2\pi R_S C$, where C is the cell capacitance).

2.4.5 Intracellular dialysis

The patch pipette and cell constituents exchange rapidly following the establishment of the whole cell recording configuration (Marty and Neher, 1983). The effects of putative second messengers on membrane properties can thus be examined by their inclusion in the pipette filling solution. Inositol 1,4,5-trisphosphate (IP_3), and cyclic adenosine monophosphate (cAMP) were dissolved in the filling solution and stored at -18°C until required. Ca^{2+}-buffers were used to change and stabilise internal Ca^{2+}. BAPTA was used in preference to EGTA because its affinity for Ca^{2+} is independent of pH and it binds and releases Ca^{2+} 2-3 orders of magnitude faster than EGTA (Tsien, 1980). To determine the importance or otherwise of Ca^{2+} in the changes in membrane

permeability that were to be studied, pipettes were sometimes filled with a solution containing 130mM KCl, 10mM K_4 BAPTA and 8mM HEPES. The BAPTA solution was titrated with KOH to pH7.1 and passed through a 0.22μm Millipore filter before use.

The total ionic content of all the intracellular mediums used were adjusted so that they were isotonic with the extracellular medium. Osmolarity was determined by freezing point depression, using an osmometer.

2.4.6 Changes in cell volume

During whole cell recordings cell volume can be increased by the application of gentle positive pressure to the interior of the pipette. This was done by raising the air pressure in a one litre glass jar connected by tight fitting polythene tubing to the "suction" port of the pipette holder via a solenoid-valve (Lee, LFAA 120118HA), activated by a Grass stimulator. A water manometer was placed in parallel.

2.4.7 Noise analysis of volume activated K^+ currents

The activation of ion conductances is often associated with an increase in membrane current or voltage noise due to the random fluctuations in the number of channels open at a particular time (Katz & Miledi, 1972; Anderson and Stevens, 1973). Spectral analysis of the noise variance can provide information on the elementary conductance and gating of the ion channels. For analysis of the noise variance whole cell currents were band pass filtered between 0.5Hz and 1kHz (-3dB, Barr and Stroud EF3 filter) and recorded at a high gain. An unfiltered, low gain current trace was made on a separate channel of the tape to monitor the mean current amplitude. The noise analysis programme used was written by Professor D. Colquhoun and

subsequently modified by Dr. P.T.A. Gray to run on a PDP-11/73 computer; graphics were written by Dr. A.B. Cachelin. The current noise variance trace was amplified and then, to avoid aliasing errors, filtered again at 500Hz (-3dB) so that the final bandwidth was 0.5-500Hz. The unfiltered mean current and the bandpass filtered noise traces were digitized at a rate of 1024Hz. Records usually 30-60s in length were split into 1s blocks for sampling, providing a resolution of 1Hz. Recording artefacts were edited out and mean current amplitudes, mean variance and spectral densities were then calculated for each 1s sample. Net mean current, net variance and spectral density curves were calculated by subtracting the control values obtained in the presence of cetiedil at 100μM (see later).

Net spectral density curves were fitted by a least squares routine with the sum of 2 Lorentzian functions described by the equation:

$$S(f) = \frac{S(0)_1}{1+(f/f_{c_1})^2} + \frac{S(0)_2}{1+(f/f_{c_2})^2} \qquad (2.1)$$

where S(f) is the spectral density at frequency f, $S(0)_1$ is the low frequency cut off, f_c is the 'corner frequency' where the amplitude falls to $^1/_2$ and the subscripts 1 and 2 refer to each of the 2 Lorentzian components.

The noise variance can be determined directly from the digitized data or as the sum of the integrals of each spectral component describing the spectral density curve. In this study the latter was employed. This method yields a slightly higher value since the Lorentzian curves are extrapolated to frequencies outside the bandwidth of the recordings.

For a spectrum described by the sum of 2 Lorentzian functions:

$$VAR_{net(1+2)} = \frac{S(0)_1}{4\tau_1} + \frac{S(0)_2}{4\tau_2} \qquad (2.2)$$

where τ is the time constant related to the characteristic cut off frequency of the Lorentzian curve (f_c) by the expression:

$$\tau = \frac{1}{2\pi f_c} \qquad (2.3)$$

The corrected noise variance determined in this way was used to estimate the single channel current according to the relationship

$$\frac{VAR_{net}}{\bar{I}} = i(1-p) \qquad (2.4)$$

where $\bar{I}$ is the mean whole-cell current, i is the single channel current and p is the open probability of the channels

If $p << 1$

$$i = \frac{VAR_{net}}{\bar{I}} \qquad (2.5)$$

If the single channels behave ohmically, the single channel conductance, γ, can be calculated from:

$$i = \gamma (V - V_{eq}) \qquad (2.6)$$

where V is the clamp potential and V_{eq} is the equilibrium potential for the ion in question.

2.5 DRUG APPLICATION BY PRESSURE EJECTION

During both intracellular and patch recording drugs were applied to individual cells by pressure ejection from small bore pipettes. Pipettes were fabricated from thin walled borosilicate glass (Clark Electromedical, GC150TF-15). The tips of the pipettes were broken down

to a diameter of 2-4μm under microscopic control. The shanks were back filled with drug solution with a fine cannula. The tips were filled by applying pressure to the back of the electrode with a hand held syringe. If solution could not be expressed from the tip, the electrode was assumed to be blocked, and was discarded. The filled electrodes were mounted on a Zeiss sliding plate micromanipulator and connected by tight-fitting polythene tubing to a pressure regulated gas source via a solenoid valve (Lee, LFAA 1200118HA), activated by a Grass stimulator. The pressure pulse was monitored by a pressure transducer (Radiospares 303-337). The output of the pressure transducer was relayed to the tape recorder and displayed on the second channel of the Ormed pen recorder.

With the pipette in place its ability to release solution in response to a pressure pulse was tested again by checking that ejection caused movement of a suitable piece of debris loosely attached to the bottom of the culture dish. To avoid problems of drug leakage from the tip, pipettes were held away from the cells and then brought within 5-10μm from the cells immediately before application, and withdrawn immediately afterwards.

The ejection of fluid from the pipette can be visualised under dark field illumination using fluorescein. Immediately following the onset of the pressure pulse a cloud of dye emanating from the tip of the pipette is seen to engulf the cell, even when the cells are under continuous perfusion. Little dilution of the puffer contents occurs during delivery and to a close approximation the concentration of drug immediately around the cell during the pressure pulse can be assumed to be equal to that in the pipette itself (Choi & Fischbach, 1981). The latency of the pressure ejection system, determined by measuring the lag in the onset of the effects of K^+ rich solutions on hyperpolarized

hepatocytes (Field, 1986) was not more than 200msec when the puffer was placed within 5-10μm of the cell. This is an order of magnitude lower than the latency of the agonist evoked Ca^{2+} response (Berthon, Binet, Mauger & Claret, 1984; Field & Jenkinson, 1987).

To avoid vehicle artefacts drugs were made up in a carrier solution that was identical or as similar as possible to the recording medium. Pressure ejection of carrier solutions had no noticeable effect on the electrical properties of the liver cell, unless the pipette was placed very close to the cell or the ejection pressure exceeded 2pSi. Under these circumstances a slight hyperpolarization accompanied by a small increase in membrane resistance, coincident with the pressure pulse, was frequently observed.

All the results presented in this thesis are expressed as mean $\pm$ standard error (s.e.m.) of the number of observations in parentheses, as a measure of the variability of the measurements.

2.6 SOURCES OF MATERIALS

Amersham International plc: D-myo-inositol 1,4,5-trisphosphate, potassium salt.

BDH Chemicals Ltd. Dorset: Inorganic salts, glucose, sucrose (all of "AnalaR" grade), NaOH and KOH (AVS, carbonate free), liquid paraffin ("Spectrosol"), 1,2-bis(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA).

Bristol-Myers Company, Indiana, USA: Amidephrine mesylate.

Boehringer Corporation (London) Ltd: Collagenase (from *Clostridium histolyticum*).

Eastman-Kodak Company, New York, USA: Sodium isethionate.

GIBCO Reagents Ltd., Paisley: Newborn calf serum (NCS), Tryptan blue stain, penicillin/streptomycin, Eagle's minimum essential medium (MEM).

Hoechst Pharmaceuticals: Pentoxifylline.

Janssen Pharmaceutical Ltd., Oxford: Fentanyl (Sublimaze), droperidol (Droleptan).

Koch-Light, Bucks: Noradrenaline bitarate.

McNeil Pharmaceutical, Pennsylvania, USA Cetiedil citrate and bepridil hydrochloride.

May & Baker Ltd., Essex: Sodium pentobarbitone (Sagatal).

Miles Laboratories Ltd. : Bovine serum albumin fraction V (BSA).

Pfaltz & Bauer, Waterbury, USA: Potassium methylsulphate.

Research Biochemicals, Natick, USA: 2-methylthioadenosine triphosphate, sodium salt.

Sigma Chemical Company, St. Louis, USA: N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid (HEPES), ethylene glycol bis- (β-aminoethyl ether) N,N,N$'$,N$'$-tetraacetic acid (EGTA), adenosine 5$'$-triphosphate, disodium salt (ATP), adenosine 5$'$-diphosphate, sodium salt (ADP), α,β-methylene adenosine 5$'$-triphosphate, lithium salt, adenosine 3$'$,5$'$-cyclic monophosphate, sodium salt (cAMP), salbutamol hemisulphate, adiphenine hydrochloride, oligomycin A.

Wellcome Reagents Ltd.: Eagle's minimum essential medium (MEM).

Apamin was kindly provided by Dr. P.N. Strong.

Table 2.1 Composition of Eagle's MEM

a) Purchased from Wellcome Reagents Ltd.

	mg l^{-1}	mmol l^{-1}
NaCl	-	116
KCl	-	5.4
$CaCl_2$	-	1.8
$MgSO_4$ (anhydrous)	-	-
$MgSO_4.7H_2O$	-	0.81
$NaH_2PO_4.2H_2O$	-	0.96
$NaHCO_3$	-	13
glucose	-	5.6
l-glutamine	-	2.0
l-arginine hydrochloride	-	0.6
l-cystine	-	0.2
l-cystine hydrochloride	-	-
l-histidine hydrochloride (anhydrous)		0.2
l-histidine hydrochloride. H_2O	-	-
dl-isoleucine	-	0.8
dl-leucine	-	0.8
dl-lysine hydrochloride	-	0.4
dl-methionine	-	0.2
dl phenylalanine	-	0.4
dl-threonine	-	0.8
dl-trytophan	-	0.1
l-tyrosine	-	0.2
dl-valine	-	0.8
aneurine hydrochloride	1.0	-
biotin	-	-
choline bitartrate	-	-
choline chloride	1.0	-
folic acid	1.0	-
inositol	2.0	-
nicotinamide	1.0	-
Ca pantothenate	1.0	-
pyridoxal hydrochloride	1.0	-
riboflavin	0.1	-
thiamine hydrochloride	-	-
phenol red	10	-

b) Purchased from GIBCO Reagents Ltd.

	mg l^{-1}	mmol l^{-1}
NaCl	-	116
KCl	-	5.4
$CaCl_2$	-	1.8
$MgSO_4$ (anhydrous)	-	0.81
$MgSO_4.7H_2O$	-	-
$NaH_2PO_4.H_2O$	-	1.0
$NaHCO_3$	-	13
glucose	-	5.6
l-glutamine	-	-
l-arginine hydrochloride	-	0.1
l cystine	-	-
l-cystine hydrochloride	-	0.05
l-histidine hydrochloride (anhydrous)		-
l-histidine hydrochloride H_2O	-	0.1
l-isoleucine	-	0.2
l-leucine	-	0.2
l-lycine hydrochloride	-	0.2
l-methionine	-	0.05
l phenylalanine	-	0.1
l-threonine	-	0.2
l-trytophan	-	0.02
l-tyrosine	-	0.1
l-valine	-	0.2
aneurine hydrochloride	-	
biotin	1.0	-
choline bitartrate	1.8	-
choline chloride	-	-
folic acid	1.0	-
inositol	2.0	-
nicotinamide	1.0	-
d-Ca pantothenate	1.0	-
pyridoxal hydrochloride	1.0	-
riboflavin	0.1	-
thiamine hydrochloride	1.0	-
phenol red	10	-

Table 2.2 Composition of extracellular solutions used in microelectrode and patch clamp experiments. Concentrations are given in mmol l^{-1} unless otherwise stated. When necessary sodium and potassium salts were prepared by titration of the free acid with NaOH or KOH. Solutions were adjusted to pH7.4 with approximately 4mM NaOH and filtered through 0.22μm Millipore filters before use.

	A	B	C
sodium chloride	137	–	137
sodium isethionate	–	137	–
potassium chloride	4.4	–	4.4
potassium methylsulphate	–	4.4	–
calcium chloride	2.5	–	2.5
calcium sulphate	–	2.5	–
magnesium sulphate	0.81	0.81	0.81
HEPES	8	8	8
glucose	11	11	11
albumin (g/100ml)	1	1	–

CHAPTER 3

THE RESTING PROPERTIES OF GUINEA-PIG HEPATOCYTES AND THE CHANGES INDUCED BY CELL SWELLING

3.1 INTRACELLULAR RECORDINGS FROM ISOLATED HEPATOCYTES AT 37°C

The application of intracellular recording techniques to study isolated hepatocytes have been described in detail by Field and Jenkinson (1987). Briefly, in the present study intracellular recordings were made from single, mono- or binucleate guinea-pig hepatocytes, which could be confidently identified as parenchymal cells on the basis of size and morphology. Large liver cells were generally selected for intracellular recording since they proved easier to impale and were less subject to perturbations associated with microelectrode damage and leakage (see later).

Figure 3.1 illustrates the successful impalement of two guinea-pig hepatocytes. Typically the insertion of the microelectrode gave rise to a transient hyperpolarization with the activation of Ca^{2+}-gated K^+ and Cl^- conductances. The membrane potential then declined and the input resistance increased as the membrane sealed around the tip of the microelectrode (see Fig. 3.1A). When the damage caused by impalement was less marked, the initial activation did not occur and electrotonic potentials were observed immediately (see Fig. 3.1B).

3.1.1 Passive electrical properties of guinea-pig hepatocytes impaled with microelectrodes

In the current series of experiments recordings were accepted if the initial penetration-induced hyperpolarization lasted only a few seconds and was followed by "sealing in" to give an input resistance of at least 130MΩ. Membrane potential and resistance were measured so long as they remained stable. Recording was normally abandoned when cells showed a slow hyperpolarization (see later) or the microelectrodes became blocked.

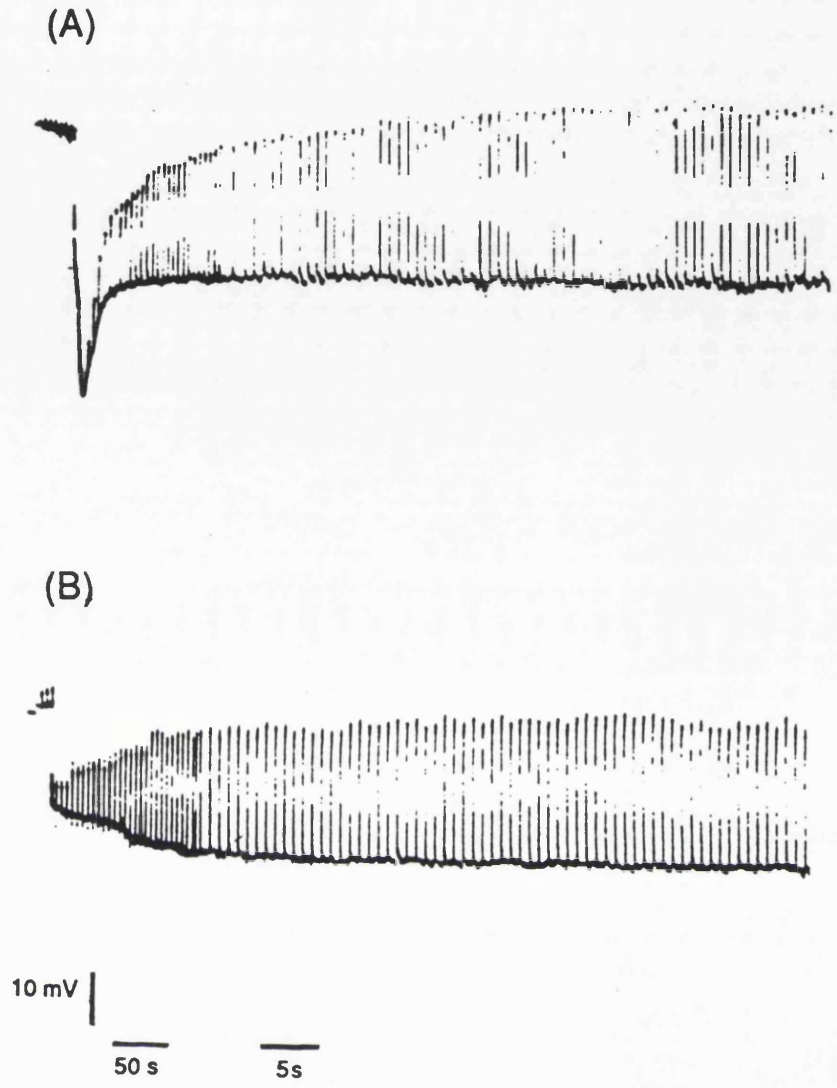


Fig. 3.1 Records illustrating satisfactory impalements of two separate guinea-pig hepatocytes. The vertical deflections reflect electrotonic potentials set up by 100pA current pulses applied through the microelectrode at a rate initially of ≃0.4Hz and subsequently of ≃1.0Hz at which time the paper speed was increased by a factor of 4. The short-lived increase in membrane potential and conductance evident in A, but not in B, is probably attributable to the transient activation of K^+ and Cl^- permeabilities.

Adopting these criteria, the resting membrane potentials and input resistances of guinea-pig hepatocytes measured using intracellular recording techniques were -22.1±1.3mV and 245±32MΩ respectively (mean ± s.e.m.; n=25), before corrections for the shunt introduced by the microelectrode. These values are somewhat higher than those recorded earlier by Field and Jenkinson (1987) under identical conditions. Taking the leak conductance to be 1nS in magnitude (see Marty & Neher, 1983 and Attwell, Werblin & Wilson, 1982) and to have a reversal potential of -5mV (Ince *et al.*, 1986), Ohm's law suggests that the membrane potential of the intact guinea-pig hepatocytes used in the current experiments would in fact have been 27.6mV and the input resistance 323MΩ.

Membrane time constants (τ) were determined in a sample of hepatocytes by fitting the electrotonic potentials with single exponentials. The mean value of τ was 13.5±1.0ms (n=12). Dividing the value of τ for each cell by the corresponding input resistance gave a cell capacitance of 58.0±3.3pF. If it is assumed that the specific membrane capacitance for hepatocytes is 1μF cm^{-2}, then the membrane surface area of a guinea-pig hepatocyte is 5,800μm^2 and the specific membrane resistance is 18.7KΩcm^2, a value close to those obtained using whole cell patch clamp technique in the present study (18.9KΩcm^2) and previously by Capiod and Ogden (1989*a*) (17KΩcm^2).

3.1.2 Slow hyperpolarization of hepatocytes impaled with microelectrodes

In the earlier intracellular experiments of Field and Jenkinson (1987) it was noted that sometimes following sealing in of the microelectrode the resting conductance of the hepatocyte gradually rose over a period of 10-15 minutes, while the membrane potential became increasingly more negative finally reaching a value of between -60 and

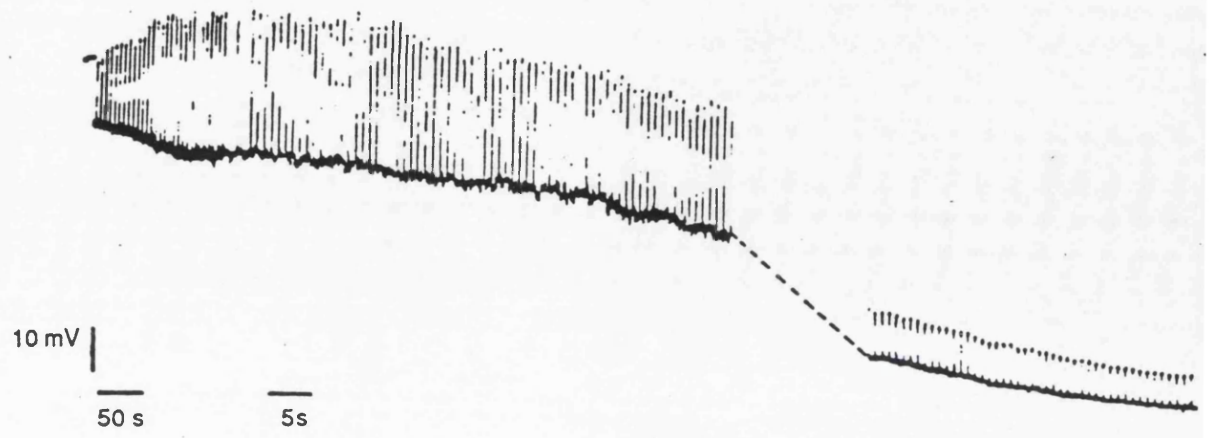


Fig 3.2 Shows the deterioration of an intracellular recording. Recording conditions as in Fig. 3.1; 100pA current pulses. Electrotonic potentials were seen immediately following the insertion of the microelectrode. Their amplitude increased for the first ≈40 sec. With increasing time the membrane potential began to rise and the resistance to fall. At the end of the record the membrane potential had risen to ≈-75mV.

-80mV. The application of quinine (200μM) at this point abolished the increase in conductance and depolarized the cell membrane. This led Field and Jenkinson (1987) to postulate that cell swelling caused by electrolyte leakage had triggered the activation of the quinine-sensitive, volume-activated K^+ conductance described by Bakker-Grunwald (1983) and Kristensen and Folke (1984) in rat hepatocytes which were either actively taking up alanine or subjected to hypo-osmotic stress.

In the present study a similar sequence of events to that described by Field and Jenkinson (1987) was observed in cells impaled with microelectrodes filled with 1M K citrate (see Fig. 3.2), 1M K methylsulphate and 4M K acetate. In a series of recordings made with microelectrodes filled with 1M K citrate, the membrane potential had increased by 48.6$\pm$1.3mV (mean $\pm$ s.e.m., n=10) at the height of the slow hyperpolarization and the conductance by a factor of 2.8$\pm$0.2, corresponding to a rise of 9.7$\pm$0.7nS.

If these changes are indeed linked to alterations in cell volume, it should, in theory, be possible to reverse them by shrinking the cell through exposure to a hypertonic solution. Increasing the osmolarity of the bathing medium with 100mM sucrose though either proved ineffective against the slow hyperpolarization or it caused a small depolarization without a measurable change in input resistance. However, in subsequent experiments conducted in this laboratory (J.H. Sweiry and D.H. Jenkinson, unpublished results; see also Sandford *et al.*, 1992) it has proved possible to reverse the increase in conductance seen in rat liver cells impaled with microelectrodes filled with 750mM K citrate by applying a hypertonic recording solution that contained 80mM mannitol in addition to its normal constituents. Similar results have been obtained by Graf *et al.*, (1988).

A brief attempt was also made in the present study to see whether the channel responsible for the slow hyperpolarization could be activated during Na^+-alanine cotransport. But no satisfactory answer was obtained from these experiments since it was not possible to dissociate the effects, if any, of alanine accumulation on membrane permeability from those of electrolyte leakage.

3.1.3 The pharmacology of the slow hyperpolarization

Although quinine inhibits the volume-activated increase in K^+ conductance in guinea-pig and rat hepatocytes (Field & Jenkinson, 1987; Howard & Wondergem, 1987; Corasanti, Gleeson & Boyer, 1990), it lacks both potency and selectivity. A more satisfactory probe is the antisickling agent cetiedil, which has been demonstrated to block the K^+ permeability changes that underlie volume regulation in lymphocytes (Sarkadi, Mack & Rothestein, 1984; Decoursey, Chaudry, Gupta & Cahalan, 1987). Fig. 3.3A illustrates the effect of cetiedil (10μM) on a liver cell showing marked volume activation, as assessed by a high resting conductance and a strongly negative membrane potential. The addition of cetiedil caused a rapid, reversible fall in conductance with a substantial depolarization, changes clearly consistent with a reduction in K^+ permeability.

The dose-inhibition relationship of the blocking action of cetiedil was established using recordings in which the initial impalement satisfied the criteria laid down in section 3.1.2, and in which the membrane potential had risen to a stable value of greater than -60mV, before the addition of cetiedil. The results are shown in Fig. 3.3B. For each point, the conductance increase in the presence of cetiedil has been expressed as a percentage of the increase in the resting input conductance associated with the slow hyperpolarization previously

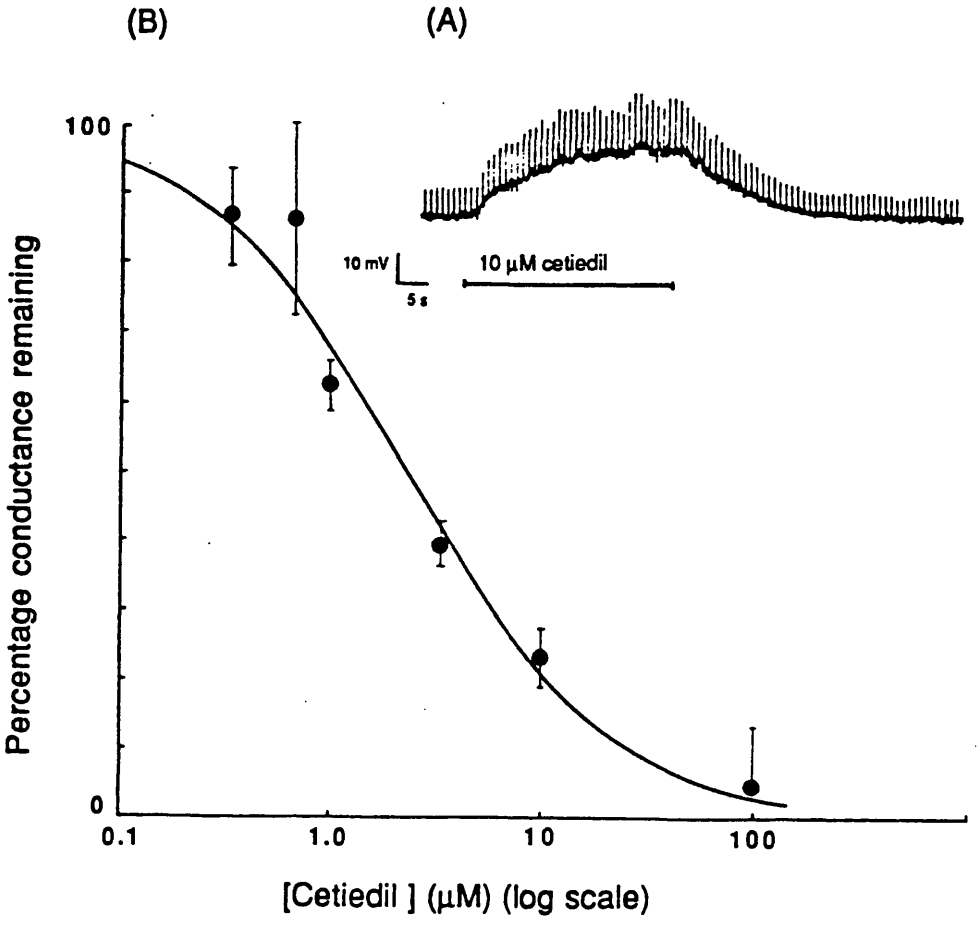


Fig. 3.3(A) shows the effect of cetiedil (10μM, applied during horizontal bar) on the membrane potential and resistance of a single guinea-pig hepatocyte which had hyperpolarized to approximately -70mV. The vertical deflections show the electrotonic potentials set up by the passage of current pulses (100pA) through the recording electrode.

(B) Concentration-response relation for this action. For each point the conductance increase in the presence of cetiedil has been expressed as a percentage of the increase in the resting input conductance associated with the slow hyperpolarization. These percentages were averaged between experiments and the s.e. of the means are indicated by bars. The line has been drawn using the equation 3.1 (see Text).

recorded in the same cell. The data has been fitted with the equation:

$$Y = 100 \times \left(1 - \frac{[L]^{n_H}}{[L]^{n_H} + IC_{50}{}^{n_H}}\right) \qquad \text{eq. 3.1}$$

where Y is the percentage conductance increase observed when the inhibitor is present at a concentration [L]; n_H is the Hill coefficient and IC_{50} the ligand concentration required to produce half maximal inhibition. A least squares logistic fit gives an estimate of 2.4±0.4 (s.d.) μM and 0.9±0.1 (s.d.) for the IC_{50} and Hill coefficient respectively. By contrast both the resting K^+ conductance and the increase in Ca^{2+}-activated K^+ conductance in the hepatocyte cell membrane produced in response to extracellular ATP (10μM) proved insensitive to cetiedil (100μM) (Fig. 3.4).

In addition to cetiedil, oligomycin A has been found to markedly inhibit the regulatory decrease in cell volume which follows the application of hypotonic solutions to lymphocytes and which is at least partly attributable to the opening of a K^+ channel (Sarkadi *et al.*, 1985). Oligomycin A (2-15μM), however, proved ineffective against the slow hyperpolarization of the liver cell and by inference, the volume-activated K^+ conductance.

Cetiedil has also been shown in erythrocytes to suppress the increase in K^+ permeability and the resulting net loss of K^+ that follow a rise in cytosolic Ca^{2+} (the Gardos effect) (see e.g. Christopherson & Vestergaard-Bogind, 1985). Oxypentifylline is also active in this regard (Bilto, Player, West, Ellory & Stuart, 1987). However, when oxypentifylline was tested against the volume-activated conductance in the hepatocyte no effect was seen even at concentrations (100μM) that would have maximally inhibited the Gardos effect in red blood cells.

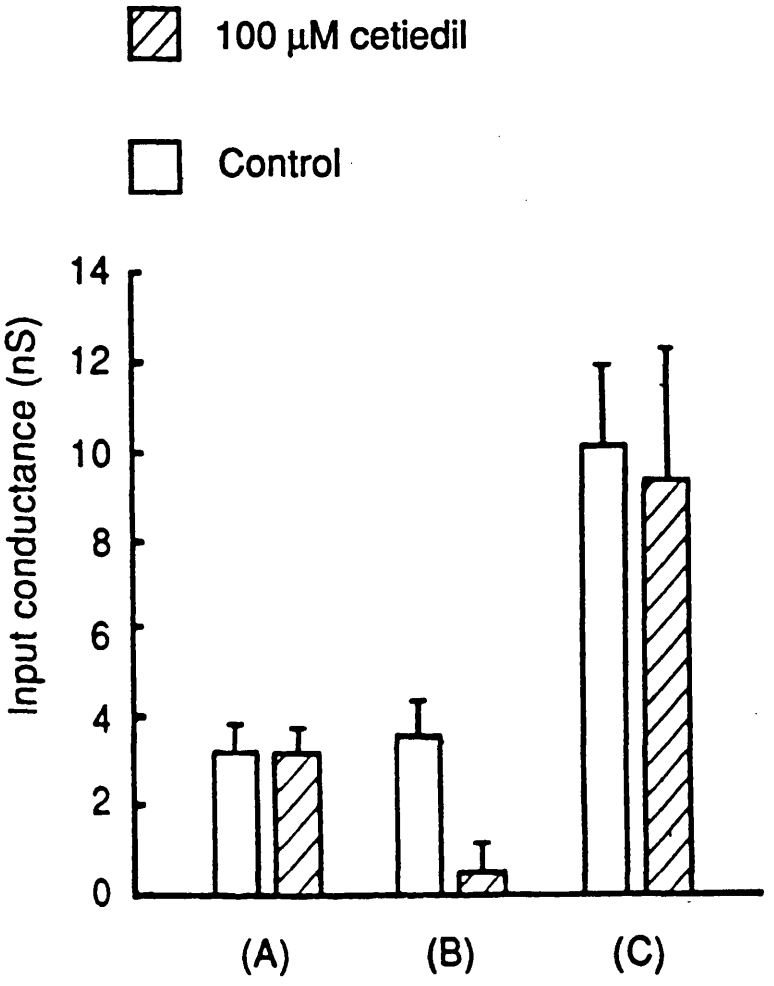
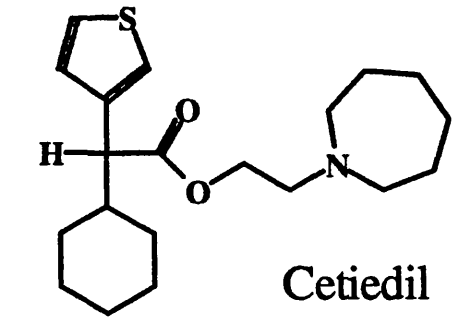
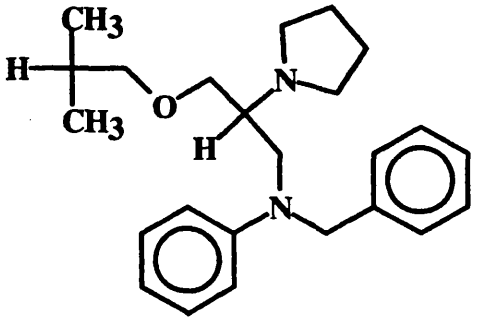


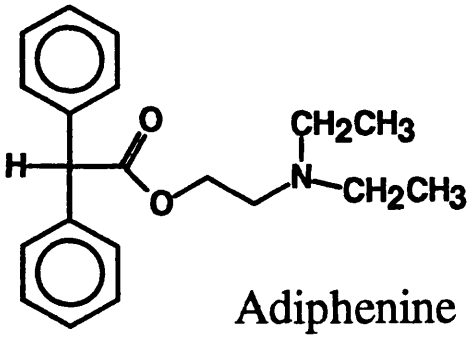
Fig. 3.4 Comparison of the effects of cetiedil (100μM) on the resting conductance (A), the volume-activated K^+ conductance (B) and Ca^{2+}-activated K^+ conductance evoked by ATP (C). In A and B, the cells were bathed in normal bathing fluid and in C, chloride-free. Results are means ± s.e.m. of 3-5 experiments.



Cetiedil



Bepridil



Adiphenine

Fig. 3.5 The structures of the agents that proved effective against the slow hyperpolarization.

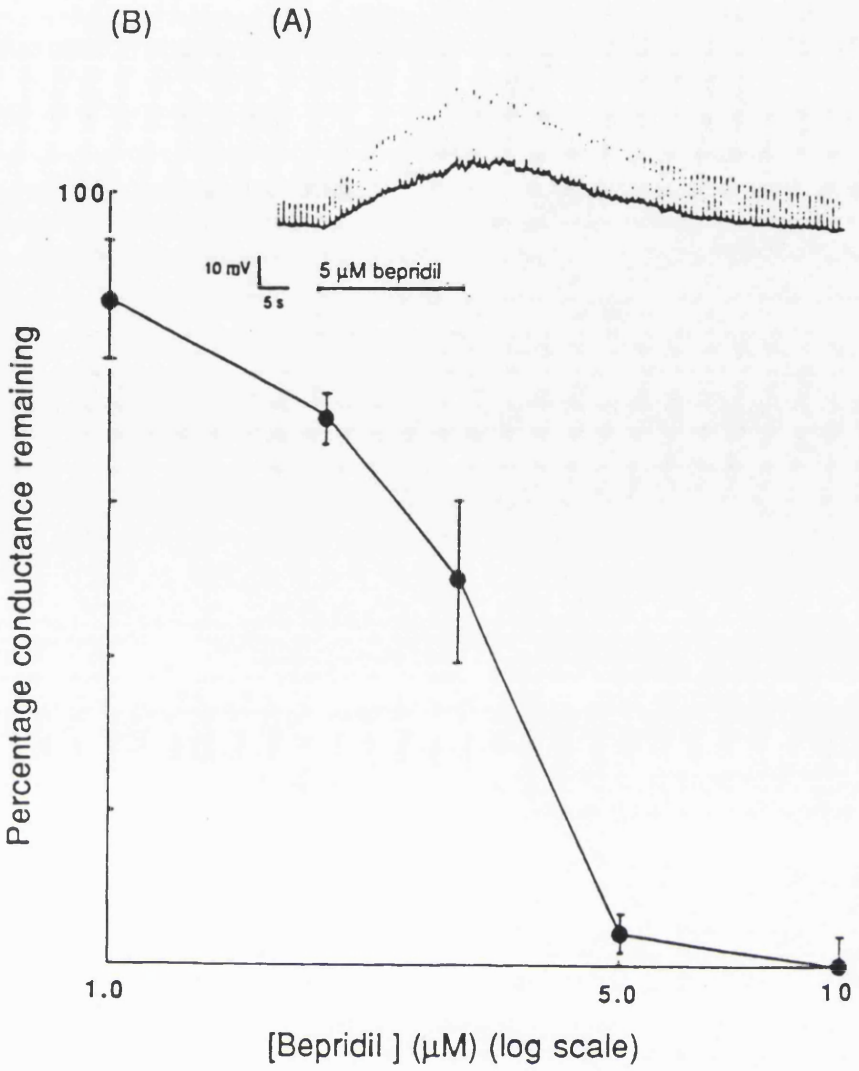


Fig. 3.6(A). The effect of bepridil applied at 5µM on the membrane potential and resistance of a single guinea-pig hepatocyte which had hyperpolarized to ≃ -70mV. The vertical deflections show the electrotonic potentials set up by the passage of current pulses (100pA) through the recording electrode.

(B) Concentration-response relation for this action. For each point the conductance increase in the presence of bepridil has been expressed as a percentage of the increase in the resting input conductance associated with the slow hyperpolarization. These percentages were averaged between experiments and the s.e.m. of the means are indicated by bars.

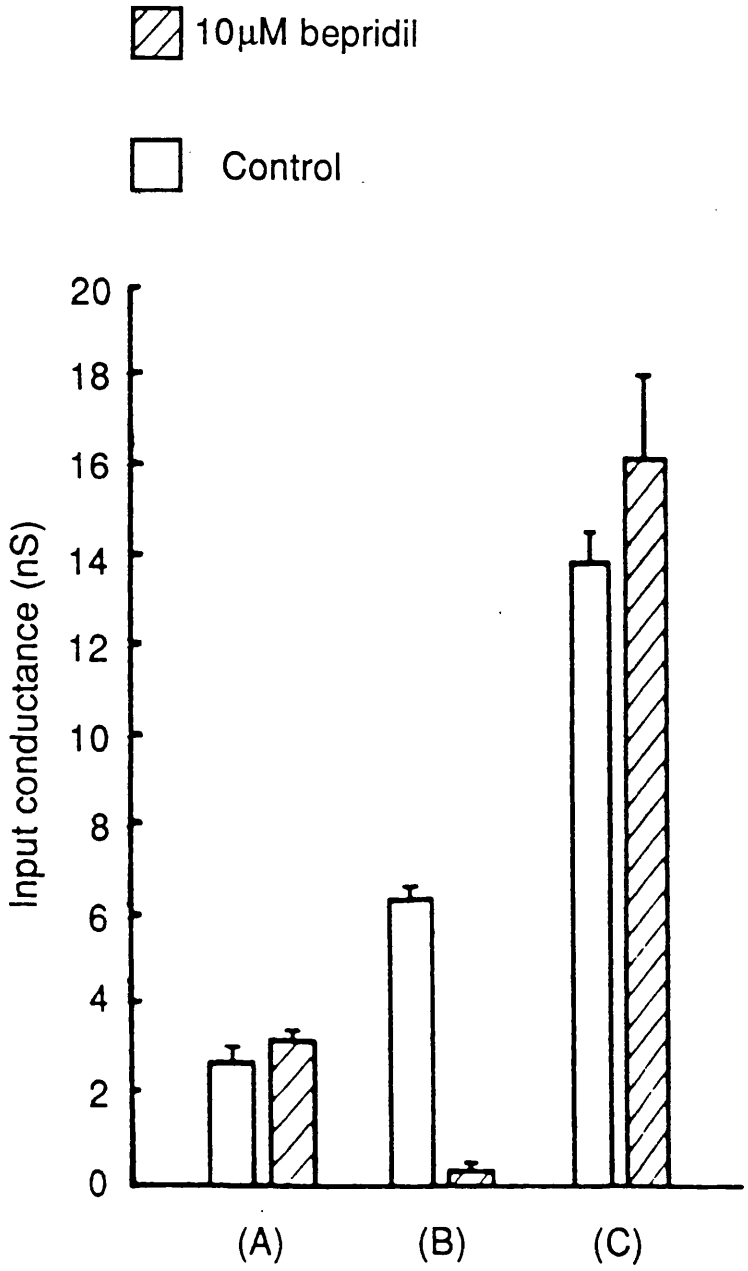


Fig. 3.7 Comparison of the effects of bepridil (10μM) on the resting conductance (A), the volume-activated K^+ conductance (B) and Ca^{2+}-activated K^+ conductance evoked by ATP (C). A and B cells bathed in normal bathing fluid and C chloride-free. Results are means ± s.e.m. of 3-4 experiments.

On the other hand bepridil (another antisickling agent) and adiphenine, which are both structurally similar to cetiedil, (see Fig. 3.5) blocked the volume-sensitive K^+ conductance, though each with very different potencies. Fig. 3.6 illustrates the dose-inhibition relationship of the blocking action of bepridil. Unfortunately insufficient data were collected to allow a reliable estimate of the IC_{50} for bepridil to be derived with any certainty from a logistic fit with equation 3.1, but the raw data suggest a value of approximately 3μM (see Fig. 3.6) comparable to cetiedil. Bepridil at 10μM, a concentration that completely reversed the slow hyperpolarization, had little if any effect on either the resting K^+ conductance or the ATP-induced increase in $P_{K(Ca)}$ (see Fig. 3.7). Adiphenine was much less active than bepridil and cetiedil and even at 30μM, the highest concentration tested, the conductance increase associated with the slow hyperpolarization was reduced by only 37$\pm$3.5% (n=4).

3.2. WHOLE CELL VOLTAGE CLAMP RECORDINGS FROM ISOLATED HEPATOCYTES AT 37°C.

If the additional K^+ conductance described in the last section is indeed attributable to a consequence of cell swelling, it should be possible to activate it more directly. This possibility was explored using the whole cell patch clamp technique. High resistance (5-10GΩ) seals between patch pipette and cell membrane were obtained relatively easily by applying suction to the back of the pipette, and occasionally giga seals formed spontaneously on apposition of the pipette tip to the membrane. Seal formation was seldom achieved unless the pipette tip was lowered directly onto the central region of the cell surface. The success with which the whole cell configuration was subsequently established depended chiefly on the quality of the hepatocyte

preparation. Following a poor liver perfusion, the cytoplasm of the isolated hepatocytes had a "grainy" appearance and attempts to obtain whole cell recordings were unsuccessful in all but a few cases. The whole cell configuration was most readily achieved when cells possessing a distinct nucleus and a clear cytoplasm showing no vacuolation or "blebs", were selected. Though whole cell recordings were noticeably less stable at 37°C than at 20°C, it was nevertheless possible to work at the higher temperature.

3.2.1 Passive electrical properties of guinea-pig hepatocytes under whole cell patch clamp conditions

The passive electrical properties of single guinea-pig hepatocytes in the whole cell recording mode have been examined at 30°C by Capiod & Ogden (1989*a*). The electrical characteristics of cells used in the present work conducted at 37°C were similar to those reported in this study. Thus the resting conductance and membrane potential averaged 1.4±0.1nS (s.e.m; n=10) and -28.3± 2.3mV (s.e.m; n=6) in the current experiments as compared with 1.8nS and -25mV as found by Capiod & Ogden (1989*a*). The mean cell membrane capacitance in the present study was 28.4±1.4pF (n=14) and based on the assumption the specific mean capacitance is 1μF/cm^2 surface membrane area, the specific membrane resistance was 18.9KΩcm^2 which compares with a value of 17KΩcm^2 reported by Capiod & Ogden (1989*a*).

The current-voltage relationship of the cells were linear over the range -100 to +40mV again in keeping with the earlier work of Capiod & Ogden (1989*a*) and with the findings of Sawanobori, Takanashi, Hiraoka, Iida, Kamisaka & Maezawa (1989) and of Henderson, Graf & Boyer (1989) using rat hepatocytes. The latter workers did, however, observe and analyse a departure from linearity at more extreme potentials (from -200

to +175mV) than explored in this study. Marchetti *et al.* (1988) have also described marked rectification of whole cell currents in avian hepatocytes held at positive holding potentials. This discrepancy could reflect either a species difference between guinea-pig and chicken liver cells or differences in the experimental conditions. Marchetti *et al.* (1988) recorded from cells in primary culture at periods more than 24 hours after isolation, whereas in the present study the cells were used within 10 hours of dispersal.

3.2.2 Slow hyperpolarization of hepatocytes under current clamp

Following the establishment of the whole cell recording configuration, on some occasions an outward current associated with an increase in current noise slowly developed over several minutes. In current clamp mode, the corresponding changes were hyperpolarization and a rise in conductance. In keeping with this, Capiod & Ogden (1989*a*) have described a proportion of guinea-pig hepatocytes with a more negative resting potential (-60 to -75mV) than the usual value of ≃ -25mV) and a greater conductance (~5nS as compared with 1.8nS). Application of quinine (1mM) to such cells caused the conductance to fall and the membrane potential to become less negative. This led Capiod and Ogden to suggest that the quinine-sensitive K^+ conductance associated with cell swelling (Bakker-Grunwald, 1983; Kristensen & Folke, 1984; Field & Jenkinson, 1987) had been triggered. This would appear a reasonable conclusion in view of the considerable inflow of fluid possible from a patch pipette with a diameter of approximately 0.5μM and the hydrostatic pressure provided by the column of fluid in the pipette.

To determine whether or not a volume-sensitive P_K underlies the slow hyperpolarization, the effects of deliberately increasing cell volume during whole cell recordings were examined. Application of 15cm

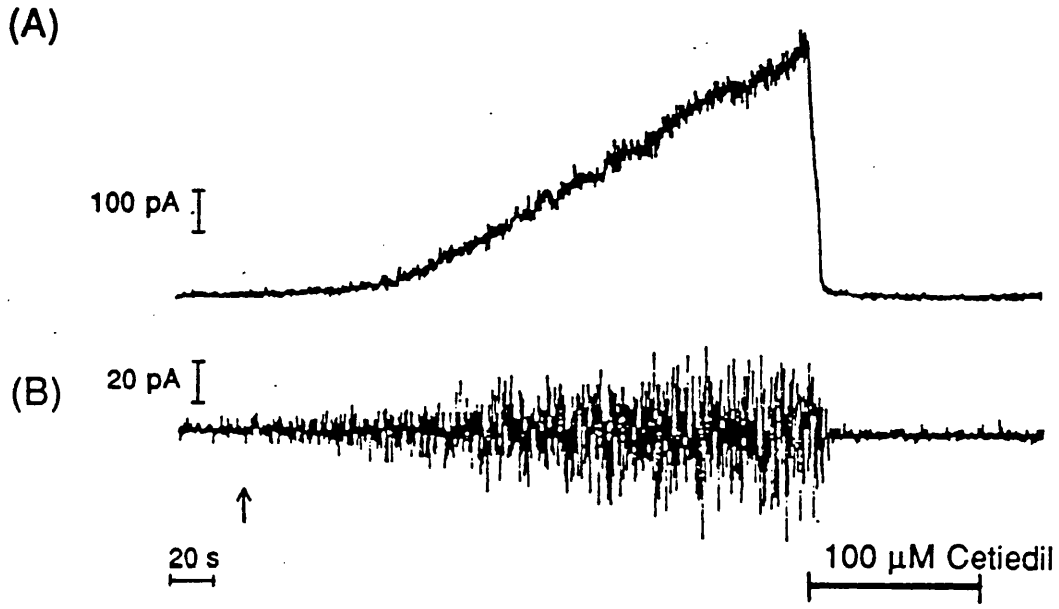


Fig. 3.8A Outward current evoked in cell held at 0mV by 15cm hydrostatic pressure applied to the interior of the patch pipette. Pressure application of cetiedil (100μM) abolished over 90% of the current. B. Membrane current noise trace corresponding to the mean current record in A (band pass filtered between 0.5Hz and 1kHz, -3dB). This cell had to be abandoned before it had recovered from the cetiedil application.

hydrostatic pressure to the suction port of the pipette holder resulted in rupture of the giga seal or cell lysis in seven out of a total of twenty six successful whole cell recordings. In the remaining cells the pressure step initiated the onset of a slow hyperpolarization under current clamp conditions or an outward current under voltage clamp conditions (see Fig. 3.8A). In six cells held at 0mV a pressure step of 15cm H_2O produced a peak outward current of 602±80 pA. As may be seen in Fig. 3.8A, the application of cetiedil at the relatively high concentration of 100μM produced an immediate and rapid reduction in the pressure-induced current. In the five cells tested, 100μM cetiedil abolished 96.7±1.5% of the outward current. Recovery following the pulse of cetiedil at this high concentration was very slow. A complete reversal of the effects of cetiedil was observed in only one of the cells, before recording had to be abandoned.

The current-voltage relationship determined during the pressure-induced current was linear between -100 to +20mV (Fig. 3.9) and reversed at -85.8±4.2mV (n=3), near E_K (-93mV). These results clearly demonstrate that cetiedil blocks a current which is carried mainly, if not exclusively, by K^+ and which can be activated under conditions of cell swelling.

The mean conductance increase evoked in response to the pressure step was 3.9±0.7nS (n=3). This is somewhat smaller than the conductance increase (9.7±0.7nS) associated with the slow hyperpolarization recorded in cells impaled with microelectrodes. However, since smaller cells were generally selected for whole cell patch clamp recording, the amplitude of the whole cell currents would be expected to be correspondingly less (by a factor of approximately 2, as judged by the indirect estimates of surface area already discussed). In any case, the increase in conductance may not have been maximal.

In lymphocytes (Grinstein, Dupre & Rothstein, 1982), Ehrlich ascites cells (Hoffmann, Simonesen & Lambert, 1984) and a variety of epithelia (eg. Hazama & Okada, 1988; Christensen, 1987) evidence has been presented that the K^+ effluxes involved in the regulatory volume decrease seen after cell swelling in hypoosmotic media occur via a Ca^{2+}-activated pathway. In preliminary experiments (n=10) conducted in the present study the addition of 10mM BAPTA to a nominally Ca^{2+} free pipette filling solution did not affect the current associated with cell swelling. Intracellular dialysis of four cells with the same solution abolished the Ca^{2+}-activated K^+ and Cl^- currents evoked by maximal concentrations of noradrenaline.

3.2.3 Noise analysis of volume-activated K^+ conductance

The activation of the volume sensitive K^+ conductance was associated with an increase in membrane current noise. Fig. 3.8B shows a high gain trace of the current response of Fig. 3.8A, band pass filtered between 0.5Hz and 1kHz. Spectral analysis of the current variance increase can provide information on the gating of the ion channels and the elementary conductance. A reliable estimate of the single channel conductance, however, is obtained only if the open probability, p_o, is small (see Eqn. 2.4). A plot of current variance (var(I)) against the mean membrane current (I) at different levels of p_o should follow a parabolic curve with an initial slope equal to the unitary current and a maximum at p_o = 0.5 (Anderson & Stevens, 1973). With the limited time for which whole cell recordings could be maintained and the slow time course of the response, it was not practicable to vary the level of p_o in a given cell by varying the pressure applied to the interior of the patch pipette. However, the degree of channel activation changes during the onset of the response. Fig. 3.10 illustrates the

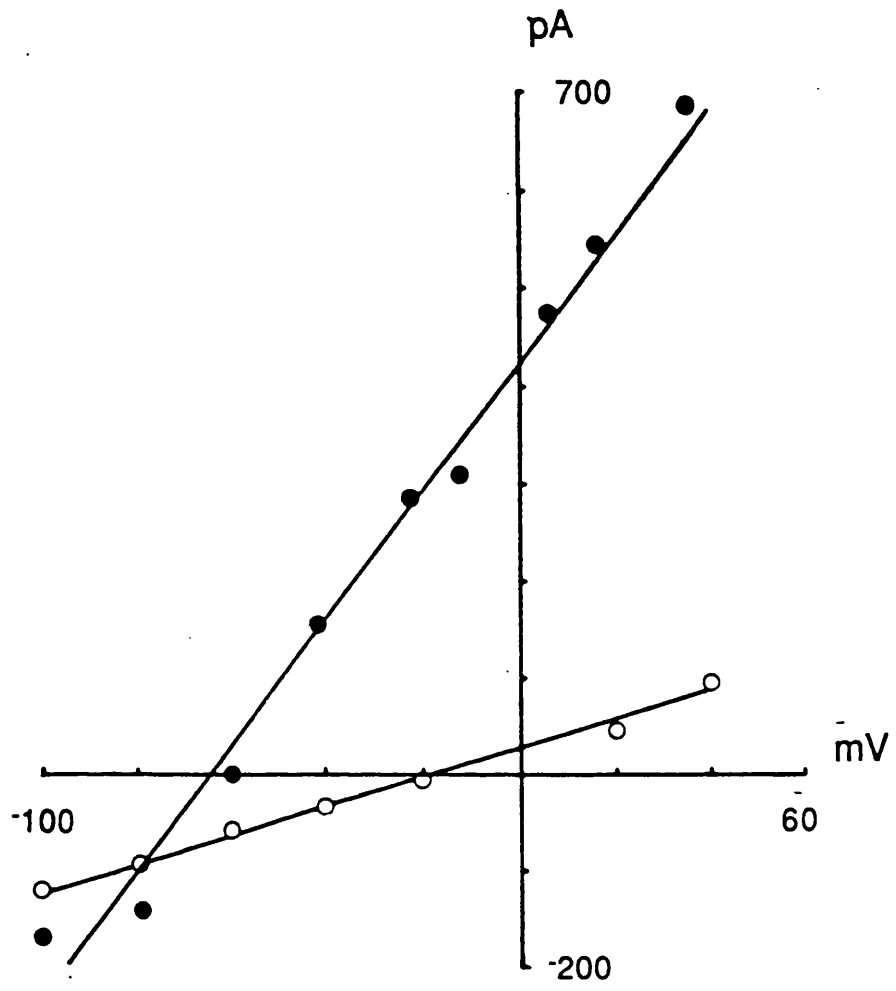


Fig. 3.9 Current-voltage (I-V) relationships obtained before (o) and during (●) current evoked by 15cm hydrostatic pressure applied to the interior of the patch pipette. The holding potential was 0mV and the membrane potential was jumped for 40ms to test potentials of -100, -80, -60, -40, -20 and +20mV. The regression lines cross at ~ -80mV close to E_K (-93mV).

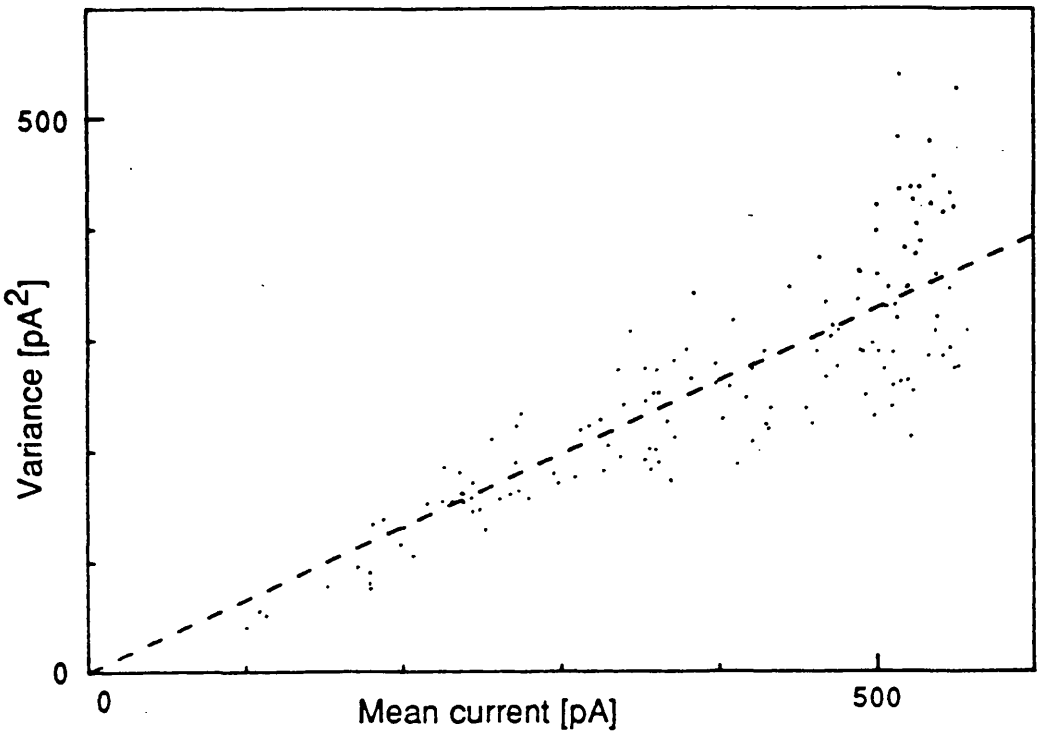


Fig. 3.10 Relationship between the mean current amplitude and current noise variance for a single hepatocyte during the pressure induced response. Experimental conditions were as in Fig. 3.7. Data points were obtained from 300 1 sec samples of current trace, digitized at 1024Hz.

relationship between var(I) and I obtained during this phase of the response for one cell, after the subtraction of a background current and noise variance recorded in the presence of 100μM cetiedil. Although the scatter of data points was often large, there was no clear indication in any of the responses analysed that the values passed through a maximum, suggesting $p_o < 0.5$. For the purpose of unitary current estimation p_o was thus assumed to be small.

Spectral analysis of the variance increase during the plateau of the response yielded spectral density curves which were fitted by the sum of two Lorentzian functions with half power frequencies of 7.2$\pm$0.8 and 152$\pm$12 Hz (n=4). Approximately 70% of the variance was contained in the low frequency component. Fig. 3.11 illustrates the spectral density curve obtained in one such experiment. The ratio of the total variance calculated from the fitted function (after correction for frequencies outside the recording bandwidth) to mean current provided an estimate for the unitary current of 0.65$\pm$0.02pA which corresponds to a single K^+ channel conductance of 6.9$\pm$0.2pS (n=4). It should be emphasised though that this may well be an underestimate, because of the tacit assumption in the foregoing analysis that the open probability of the channel is low. A value of 7pS would imply that approximately 600 channels have to open in individual guinea-pig hepatocytes to account for the conductance increase (4nS) which developed as a consequence of the pressure step applied to the shank of the patch pipette.

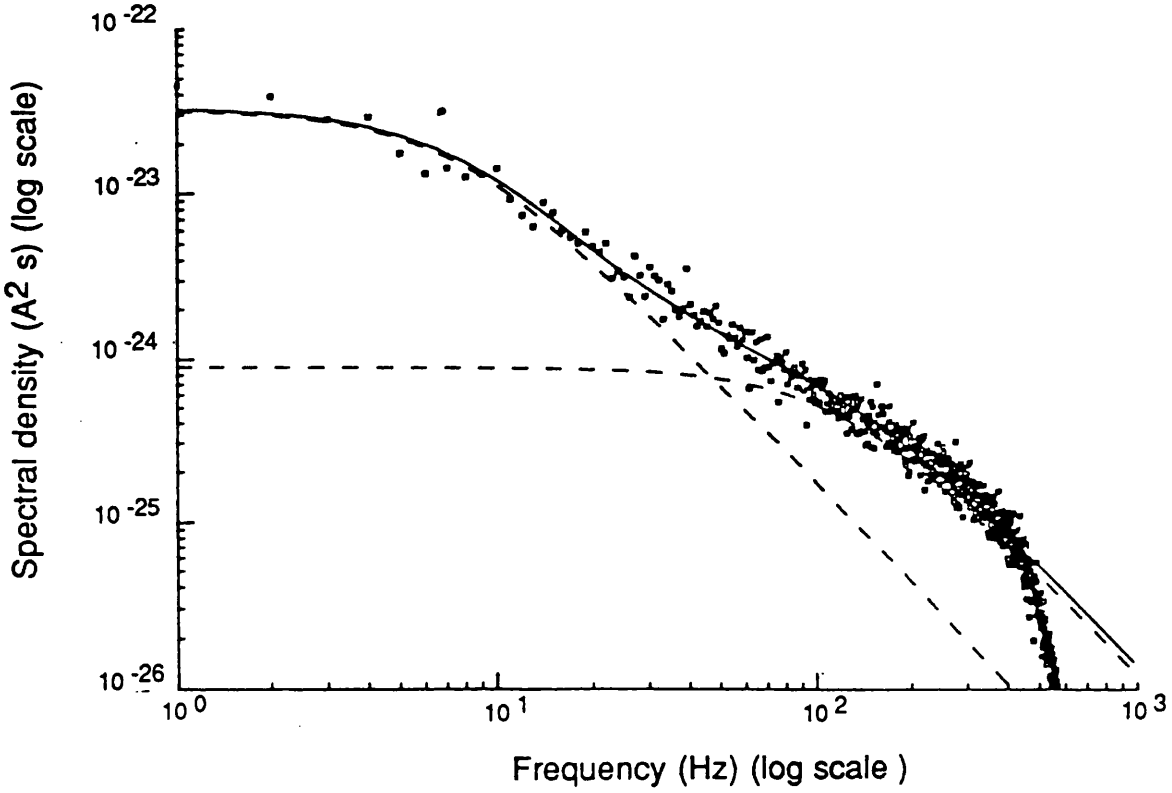


Fig. 3.11 Power spectrum of the increase in current noise induced by 15cm hydrostatic pressure applied to the interior of the pipette. The resting noise current spectrum (taken as the spectrum in the presence of 100μM cetiedil) has been subtracted. Other experimental details as for Figs. 3.6 and 3.8.

3.3 DISCUSSION

3.3.1 The characterization of the volume-sensitive increase in P_K

The results of the present study add to the evidence that the K^+ permeability of guinea-pig hepatocytes increases under certain conditions that would be expected to induce cell swelling. In voltage-clamped cells, this response is seen as a large outward current. Spectral analysis of the spontaneous fluctuations in this current suggests that the K^+ channel involved has a conductance of approximately 7pS.

A variety of blocking agents allow this K^+ channel to be distinguished from the small conductance, Ca^{2+}-activated K^+ channel expressed in these cells (Capiod and Ogden, 1989*b*). For example, cetiedil inhibits the volume-activated K^+ conductance with an IC_{50} of 2.4μM, approximately 500 fold less than is required to depress the net loss of K^+ through Ca^{2+}-activated K^+ channels opened by the addition of angiotensin II to dispersed hepatocytes (Sandford, Sweiry & Jenkinson, 1992). Conversely, the latter are blocked by nanomolar concentrations of apamin which is without effect on the volume-activated K^+ conductance (Kristensen & Folke, 1984; Field & Jenkinson, 1987).

Cetiedil is chemically related to the antisickling agent bepridil and when IC_{50}'s are compared, the two compounds are roughly equiactive against the volume-activated K^+ conductance. The dose-inhibition relation for bepridil, however, is much steeper than for cetiedil (compare Fig. 3.3B ands 3.5B). This suggests that bepridil's mechanism of action may be more complex. It is certainly less selective than cetiedil. There is only approximately a 30-fold difference in the concentration of bepridil required to block the volume- and Ca^{2+}-activated K^+ conductance in these cells (Sandford, Sweiry & Jenkinson, 1992). Cetiedil is thus likely to be the more useful of the two as a reference

compound.

A substantial body of information is already available on the effects of cetiedil on potassium fluxes in erythrocytes and lymphocytes. The inhibitory effects of cetiedil on volume regulation in lymphocytes has been examined in detail by Sarkadi *et al.* (1985) who reported an IC_{50} of approximately 2μM. While this is very close to the value (2.4μM) obtained for cetiedil acting against the slow hyperpolarization of the hepatocytes, it is unlikely that the channels involved are the same in the two cell types. Firstly, the evidence so far available (reviewed by Grinstein & Foskett, 1990) suggests that in lymphocytes, cetiedil probably acts at a K^+ channel that is voltage-sensitive, while the cetiedil-sensitive K^+ conductance identified in liver cells shows a linear I-V relation. Secondly, the regulatory decrease which is observed in lymphocytes subjected to hypo-osmotic stress and which is at least partly attributable to the activation of a K^+ channel, is potently inhibited by oligomycin A (IC_{50}, 0.5μM, Sarkadi *et al.*, 1985) as well as by cetiedil, but oligomycin A proved ineffective here in liver cells even at 15μM.

The action of cetiedil has also been studied in red blood cells where it has been demonstrated to block the increase in K^+ permeability that follows a rise in cytosolic Ca^{2+}, the so-called Gardos effect. However, in the present study, oxypentifylline, another inhibitor of the Gardos effect was found to have no effect on the slow hyperpolarization of the liver cell. Taken together the current results suggest then that volume-sensitive K^+ channels present in the liver cell are pharmacologically distinct from the cetiedil-sensitive channels in both lymphocytes and erythrocytes.

3.3.2. The nature of the mechanism underlying the volume-sensitive increase in P_K

Just how the cetiedil-sensitive K^+ channel is gated in the liver cell has yet to be established. It is unlikely that it is activated by a rise in cytosolic Ca^{2+}; firstly because intracellular dialysis of the cell with high concentrations of the Ca^{2+} chelator BAPTA did not prevent the spontaneous development of the outward current during whole cell recording, but more persuasively, because the current is seen just as readily in rat as in guinea-pig hepatocytes (Field & Jenkinson, 1987). It is generally accepted though that the former lack Ca^{2+}-activated K^+ channels (Burgess *et al.,* 1981; Sawanobori, Takanashi, Hiraoka, Iida, Kamisaka, Maezawa, 1984). Corasanti, Gleeson & Boyer (1990) have also excluded a central role for Ca^{2+} based on their finding that dispersed rat hepatocytes subjected to Ca^{2+} deprivation can still maintain their volume in the face of hypo-osmotic stress. The omission of Ca^{2+} from the external medium bathing mouse liver slices, however, has since been reported to blunt the hyperpolarization caused by hypoosmolarity (Khalbuss & Wondergem, 1991). Whether this discrepancy reflects a species difference or a difference in the response of hepatocytes *in situ* to osmotic stress compared with those of isolated cells in suspensions remains to be seen.

One possibility is that a change in intracellular pH provides the link between cell swelling and the rise in P_K. Recent studies on volume regulation in rabbit proximal convoluted tubules, for instance, have indicated that intracellular alkalization subserves the hyperpolarization and the increase in P_K of the basolateral membrane that follows anisotonic exposure (Beck, Breton, Giebisch & Laprade, 1992). Indeed an increase in pH has previously been demonstrated to elicit K^+ fluxes in rat liver cells, (Fitz, Trouillot & Scharschmidt, 1989) but the present

finding that the cetiedil-sensitive current could be recorded in voltage clamped hepatocytes using patch pipettes filled with a HEPES buffered solution argues against a role for a change in pH in the gating of this current.

The "volume-activated" K^+ channel may instead be opened in direct response to an increase in membrane tension. Of interest in this context is the recent report of a stretch-activated 16pS channel in rat liver cells subject to osmotic stress (Bear, 1990). However, this channel differs from that described here in one important respect in that it discriminates poorly between Na^+, K^+ and Ca^{2+}. So far only in amphibian proximal tubule have osmotically and mechanically gated K^+ selective channels been shown to coincide (Sackin, 1989; Filipovic and Sackin, 1992).

3.3.3 A permeability increase to ions other than K^+?

The regulatory volume decrease that follows cell swelling in animal cells commonly proceeds by a net loss of KCl. In the liver, the relatively large resting Cl^- conductance of the cell membrane could in itself be sufficient to support passive Cl^- exit in parallel to increased K^+ conductance during RVD, particularly if RVD is accompanied by a substantial cell membrane hyperpolarization. However a recent analysis of the dependence of the membrane potential of rat liver on external Cl^- concentration (Haddad, Beck, Boyer and Graf, 1991) suggests that hepatocellular Cl^- permeability does in fact increase during hypotonic stress and to a greater extent than P_K. The finding in the present study that the conductance increase evoked by the application of hydrostatic pressure to the shank of the recording pipette reverses at -85mV (8mV less negative than E_K (-93mV)) is consistent with Cl^- playing some part in RVD. However, if Cl^- is the only ion other than K^+

involved, ΔG_{Cl} would appear to represent less than 10% of the total conductance increase underlying the slow hyperpolarization in guinea-pig hepatocytes.

CHAPTER 4

THE EFFECT OF ATP AND OTHER AGONISTS ON THE ION PERMEABILITY OF ISOLATED GUINEA-PIG HEPATOCYTES

4.1 THE RESPONSE TO ATP

4.1.1 Responses to ATP in chloride containing medium

The first part of this thesis was concerned with the ionic permeability of "resting" hepatocytes. In the remainder, the membrane responses to a range of agonists, and in particular ATP, are considered.

Figure 4.1 illustrates the response of a single guinea-pig hepatocyte to a relatively low concentration of ATP (0.5μM) recorded with an intracellular microelectrode. After a delay of ~ 20s the membrane potential and input conductance rose rapidly, albeit transiently. In a sample of eight such recordings in which the resting membrane potentials were particularly stable (see Section 3.1.1), the membrane hyperpolarized by 10.8$\pm$2.9mV (measured when the conductance was at its peak) from an initial value of -19.0$\pm$1.1mV. At the same time ATP increased the input conductance by 31.7$\pm$5.0nS from a resting level of 4.3$\pm$0.3nS. The response in these cells showed a latency ranging from 9 to 24s and a rise time of 3.2$\pm$0.4s. With 0.5μM ATP only a proportion of cells responded (52%).

If it is assumed that ATP simply introduces a large additional conductance (ΔG) in parallel with the resting membrane conductance (G), then the reversal potential (V_{eq}) for the action of ATP can be estimated using the following relationship (see e.g. Ginsborg, 1967):

$$\Delta V = \frac{\Delta G}{G+\Delta G} \quad (V - V_{eq}) \qquad \text{eq. 4.1}$$

where ΔV is the change in membrane potential evoked by ATP and V is the resting membrane potential. Substituting the values given above for ΔV, ΔG, G and V provides an estimate of -31.9$\pm$3.4mV for V_{eq}, which is of the same order as the value (-37mV) of the reversal potential for the

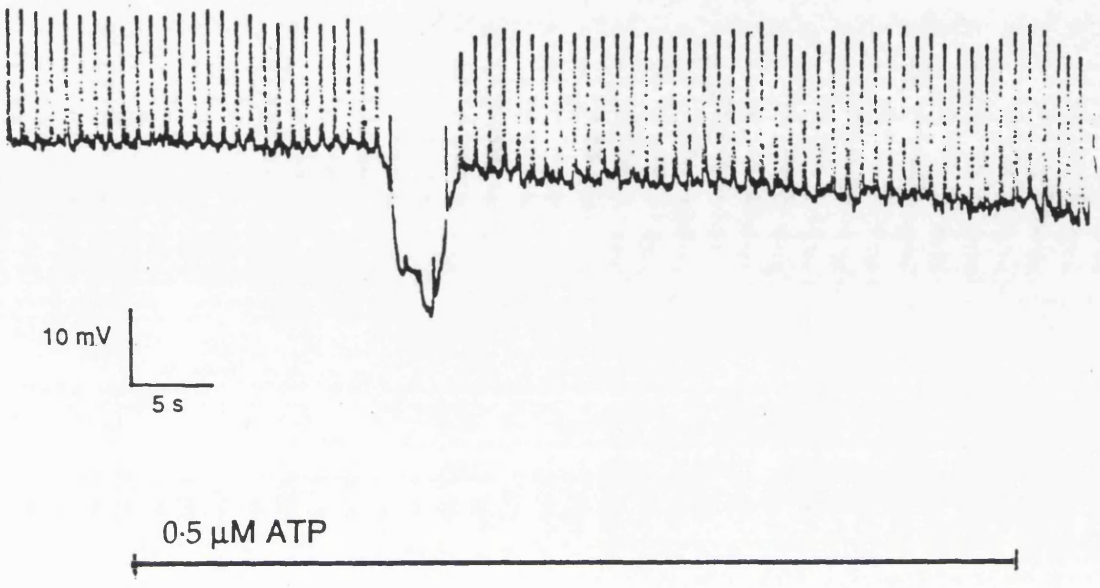


Fig. 4.1 The effect of ATP (0.5µM, during horizontal bar) on the membrane potential and resistance of a single guinea-pig hepatocyte. The vertical deflections show the electrotonic potentials set up by the passage of current pulses (60pA) through the recording microelectrode. The resting potential of the cell was -15mV.

hyperpolarization in these cells to noradrenaline (Field and Jenkinson, 1987). By analogy this would suggest that the stimulation of guinea-pig hepatocytes with low concentrations of ATP produces an increase in membrane permeability to both K^+ and Cl^- (see also section 4.1.3).

4.1.2 Response to high concentrations of ATP

The response to higher agonist concentrations was more complex. With 10μM ATP a transient depolarization, accompanied by a small decrease in input resistance, preceded the hyperpolarization (Fig. 4.2). The latency of the response to ATP was now less than 1s and all the cells responded. In a sample of five cells the membrane potential fell initially by 6.0±2.0mV from a resting level of -21.8±1.3mV, while the input conductance during this phase increased from 4.6±0.4ns by 1.3±0.3nS. Using equation 4.1, these figures provide an estimate for the reversal potential of the depolarization evoked by ATP of -1.7±0.9mV (Table 4.1). Increasing the concentration of ATP to 10μM, however, had no effect on the reversal potential of the hyperpolarization or on the time for the increase in conductance, once initiated, to peak. Interestingly, even with concentrations as high as 200μM the peak conductance increase during the hyperpolarization came to 30.1±2.2nS (n=5), only 60% of that observed with maximal concentrations of noradrenaline (Field & Jenkinson, 1987).

With the equilibrium potential for K^+ at -89mV (see De Witt & Putney, 1984) and for Cl^- at -28mV (see Bear *et al.*, 1985), changes in P_K and P_{Cl} alone cannot explain the sequence of potential changes seen in response to high concentrations of ATP. The two other major ions present, Na^+ and Ca^{2+}, both have equilibrium potentials which are considerably more positive than the resting membrane potential and an increase in either P_{Na} or P_{Ca} would depolarize the cell membrane. To

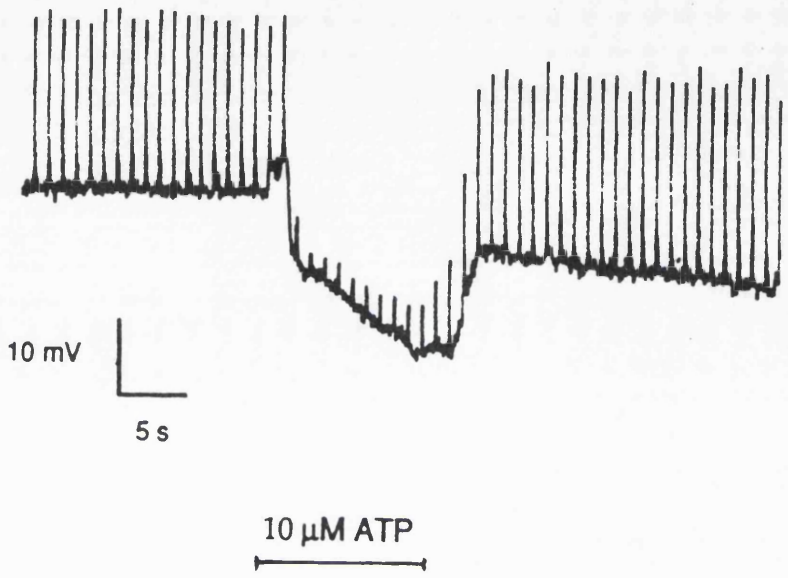


Fig. 4.2 As Fig. 4.1, but illustrating the more complex response of single guinea-pig hepatocytes (resting potential, -19mV) to 10μM ATP. Two phases are distinguishable: a rapid depolarization accompanied by a fall in membrane resistance; followed by a sharp hyperpolarization associated with a further decrease in resistance. Eventually both membrane potential and resistance return to the predrug values.

account for the reversal potential of the depolarizing current though, it is necessary to assume that either K^+ or Cl^- ions are also involved. In the next experiments to be described the role of Cl^- was examined indirectly by replacing Cl^- in the bathing fluid by larger and presumably less permeant anions.

4.1.3 The response to ATP in the absence of chloride

Hepatocytes show an appreciable permeability to Cl^- which is passively distributed across their plasma membrane (see e.g. Claret & Mazet, 1972; Fitz & Scharschmidt, 1987). The loss of chloride from isolated hepatocytes bathed in Cl^--free solution would be expected to be fairly rapid and should be reflected in an increase in membrane input resistance, as assessed by intracellular recording, as the component of the conductance attributable to Cl^- falls. In the present study the cells were equilibrated in Cl^- free solutions for at least 30 mins, at which point the membrane potential and input resistance were -17.4±1.4mV and 358±37MΩ (n = 14) respectively.

Under these conditions 10 μM ATP evoked a depolarization with an equilibrium potential (0.5±1.3mV (n=4)), close to the value of -1.7mV in Cl^--containing solution (see Table 4.1). The conductance increase associated with this phase was also little changed. This is clearly consistent with ATP activating a cation-selective conductance, though a small additional increase in P_{Cl} cannot be ruled out on the basis of the present experiments.

In the absence of Cl^- the initial depolarization was followed by a much larger increase in membrane potential (51.3±2.2mV (n=12) cf 8.9±1.3mV (n=5) in normal Cl^- solutions in the same series of experiments) despite the conductance rising to only a quarter of that in the presence of Cl^-. This is in keeping with the suggestion made

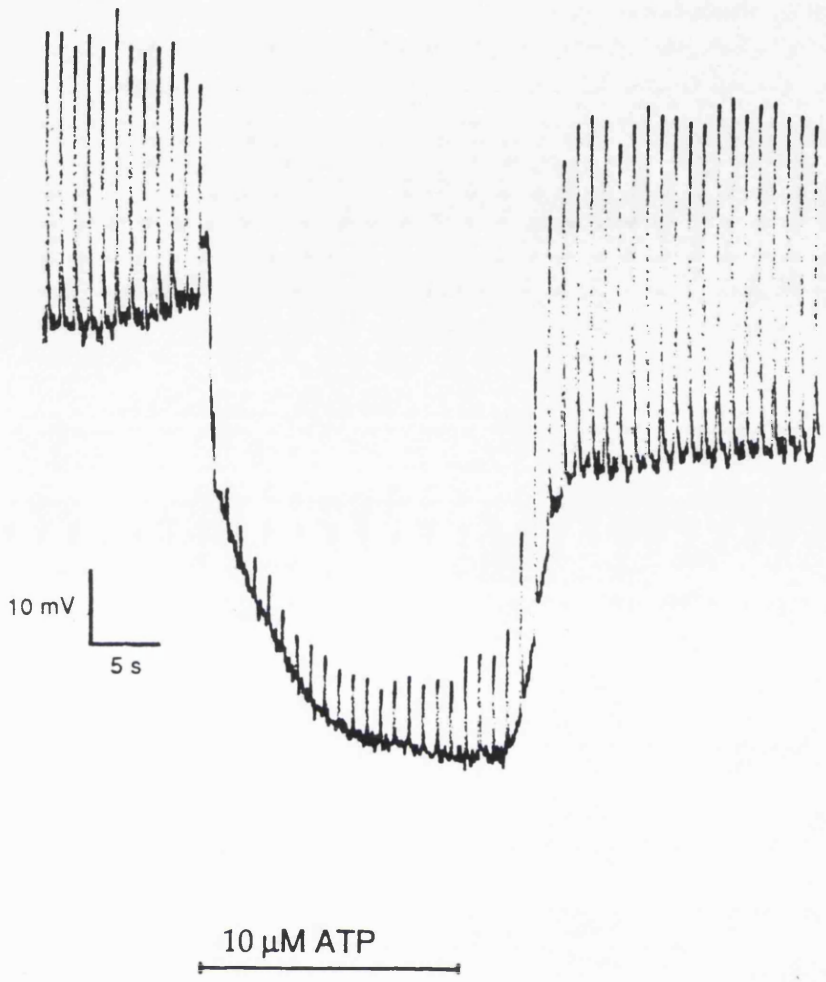


Fig. 4.3 The response to ATP (10µM, during horizontal bar) of a single guinea-pig hepatocye equilibrated in Cl^--free bathing medium (solution B in Table 2.2). The resting potential of the cell was -20mV. The initial depolarization is unaffected by Cl^- substitution, but the hyperpolarization is much larger than in the presence of Cl^-, though the reduction in the electrotonic potentials set up by 100pA current pulses is smaller (cf. Fig. 4.2).

earlier that in normal Cl^- solution an increase in permeability to Cl^- as well as K^+ underlies the hyperpolarization produced by ATP. Consistently two phases could be distinguished in the hyperpolarization induced by ATP (see Fig. 4.3): a rapid initial rise in membrane potential and conductance was followed by a further but slower hyperpolarization, in the face of a reduction in input conductance. The noradrenaline-induced hyperpolarization shows similar characteristics (Field & Jenkinson, 1987). Application of eq. (4.1) to the data obtained in the current experiments provided an estimate of -68.4±2.2mV for the reversal potential of the conductance increase involved in the rapid hyperpolarization (12.7±1.3nS, n=12) as compared with -93.2±3.7mV for the conductance increase (9.0±1.9nS) persisting at the time of the peak hyperpolarization. The observed pattern of potential changes could therefore be explained if along with a rise in K^+ conductance, ATP activated two separate cation-selective conductances, both with reversal potentials close to zero but differing in amplitude and kinetics. One is an early, transient conductance, 1.3nS in size, which gives rise to the initial depolarization. The second is a longer lasting conductance of 4nS which activates only after a delay but soon begins to decay, resulting in the slow phase of the hyperpolarization as P_K becomes dominant. However, it is worth noting that other factors could contribute to the slow phase of the hyperpolarization. For example, the loss of cellular K^+ induced by ATP (and the rise in Na^+ influx due to the higher membrane potential) could increase electrogenic ion transport. This requires further study.

Table 4.1 Summary of data obtained from guinea-pig liver cells bathed in Cl^--containing and Cl^--free solutions before and during the initial depolarizing action of 10μM ATP.

	Bathing Solution		
	Control	Chloride-free	Chloride-free + apamin (50nM)
a) Resting cell			
membrane potential (mV)	-21.8±1.3 (5)	-19.0±1.7 (4)	-17.8±2.6 (5)
membrane conductance (nS)	4.6±0.4 (5)	3.2±0.2 (4)	2.9±0.4 (5)
b) during the initial action of ATP (10μM)			
Evoked conductance increase (nS)	1.3±0.3 (5)	1.2±1.5 (4)	1.8±0.3 (5)
Reversal potential (mV)	-1.7±0.9 (5)	0.5±1.3 (4)	0.9±1.6 (5)

Membrane potentials and the corresponding input conductances (mean ± s.e. mean, with number of observations in parentheses) were determined according to the criteria discussed in section 3. Cells were allowed approximately 30 mins to equilibrate in Cl^--free solutions before measurement were taken.

4.1.4 The effect of apamin

In suspensions of guinea-pig hepatocytes Burgess *et al.* (1981) have shown that apamin (10nM) reduces the increase in K^+ flux triggered by purinoceptor activation to 10% or less. The effects of this toxin were tested on the depolarization produced by ATP in five cells bathed in Cl^-- free solution. The values listed in Table 4.1 show that the addition of 50 nM apamin did not alter either the resting properties of these cells or the reversal potential of the depolarization to ATP. This rules out the unlikely possibility that inhibition of an apamin-sensitive P_K contributes to the depolarization induced by ATP.

To summarize, the results so far suggest that stimulation of guinea-pig hepatocytes with low concentrations of ATP normally initiates a large increase in membrane conductance predominantly to K^+ and Cl^-. At higher concentrations, however, ATP also activates at least one, if not two, small transient cation-selective conductances. The mechanisms of these were next examined further.

4.1.5 The calcium dependence of the ATP responses

ATP increases the concentration of Ca^{2+} in the cytosol of hepatocytes (see e.g. Berthon, Binet, Mauger and Claret, 1984). A large body of evidence indicates that the rise in $[Ca^{2+}]_i$ is directly responsible for activating the K^+ and Cl^- conductances (Haylett, 1976; Burgess *et al.*, 1981; Field and Jenkinson, 1987; Capiod *et al.*, 1987; Capiod and Ogden, 1989*a*,*b*). To assess the importance or otherwise of Ca^{2+} in the changes in membrane permeability underlying the depolarizing response to ATP, the whole cell variant of the patch clamp technique was used in the following series of experiments to introduce Ca^{2+} chelators into the liver cells.

Fig. 4.4A illustrates the whole cell currents evoked by 10μM ATP

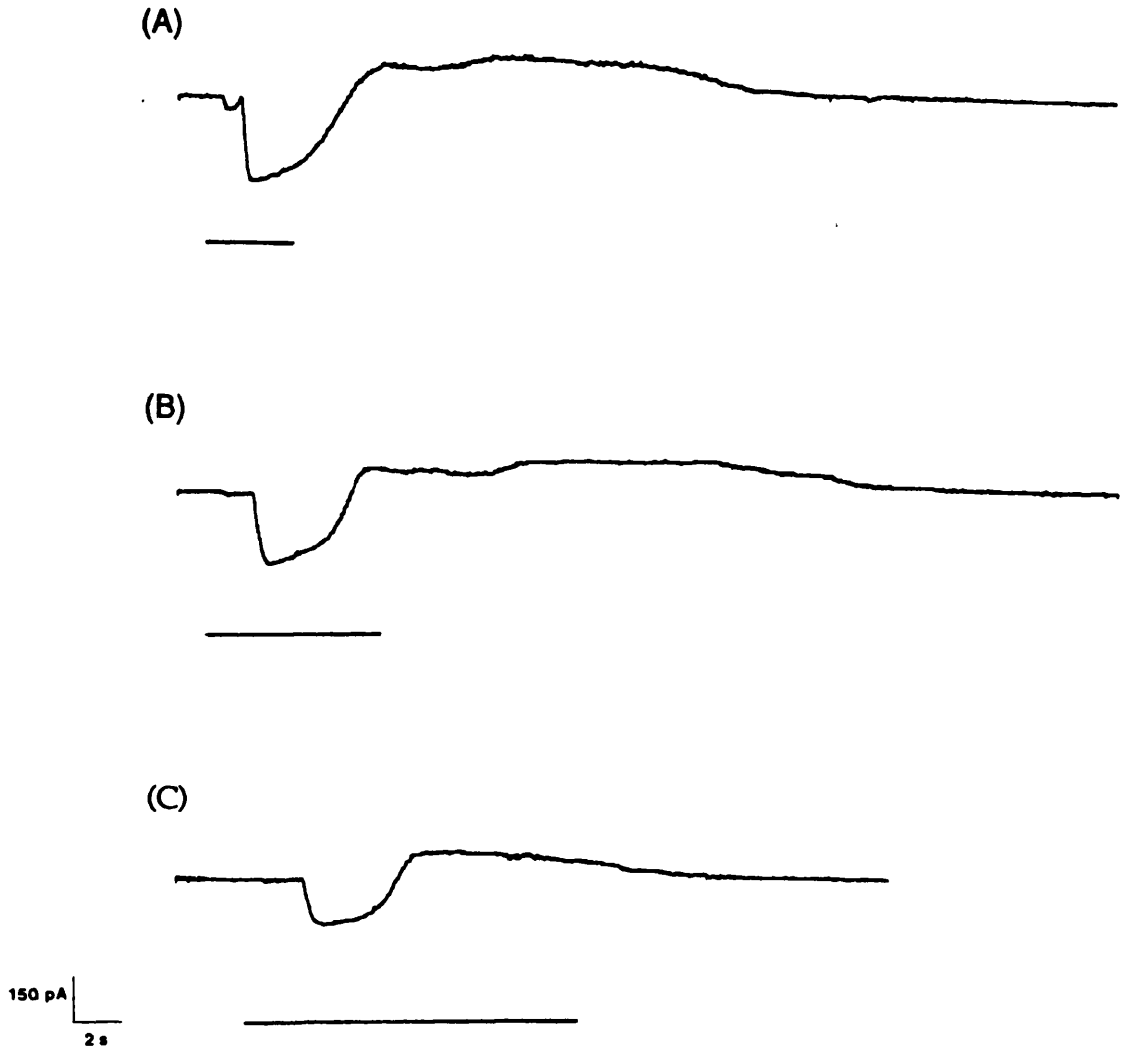


Fig. 4.4 Current response of a single voltage clamped guinea-pig hepatocyte to three successive applications of 10μM ATP (during horizontal bars) separated by ≈60s intervals. The cell was dialysed with an internal solution containing 50μM EGTA and no added Ca^{2+}. Holding potential -40mV. The early, transient inward current seen during the first application of ATP was absent in the second and third trials.

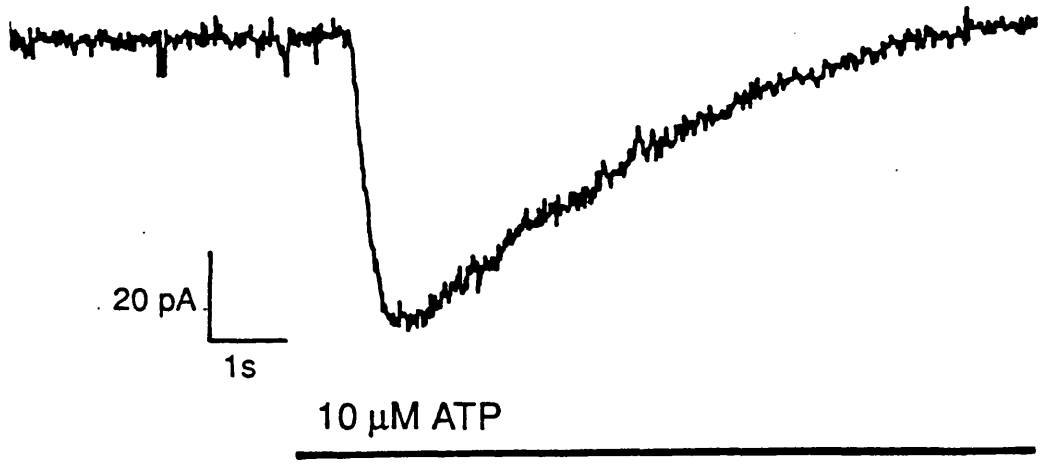


Fig. 4.5 Current response of a single voltage clamped guinea-pig hepatocyte to ATP (10μM, during horizontal bar) recorded with a pipette containing 10mM BAPTA. Holding potential -40mV.

applied at a holding potential of -40mV. The response was recorded with a patch pipette containing a solution with a low Ca^{2+} buffering capacity (50μM EGTA and no added Ca^{2+}). A small, transient, inward current was followed by a second much larger inward current which reversed to give an outward current before finally declining to the control level shortly after the termination of the pressure pulse. A second application of ATP seconds later failed to elicit the early inward current and with further challenges the amplitude of the remaining components of the response also declined (Fig. 4.4 B & C).

The absence of the transient inward current in the second and third traces suggests that this current is in some way selectively desensitized or inactivated during the initial application of ATP, and that it does not have time to recover before the next two challenges. Another possibility that cannot currently be excluded is that the gating of this current depends on the presence of some soluble factor which is eluted from the cell before the second application of ATP.

With 10mM BAPTA included in the pipette ATP simply produced a small, inward current at negative holding potentials which declined spontaneously in the presence of the drug (see Fig 4.5). Over 75% of the cells loaded with 10mM BAPTA responded to a single application of ATP, but, as before, all the cells then failed to respond to a second challenge, even though long rest intervals of several minutes were allowed between drug applications. In the nine cells that responded, 10μM ATP produced a peak inward current of 48$\pm$7pA at a holding potential of -40mV. This current had a rise time of 609$\pm$43ms and decayed over a period of 6.4$\pm$0.6s. The minimum recorded latency of the response was 500ms and the average 672$\pm$59ms. This is of the same order of magnitude as the delay associated with the puffer technique (see methods) and this may mean that the actual latency of the response

is beyond the resolution of this particular method of drug application.

The suppression of the late inward and outward currents seen in the experiments of Fig. 4.4 by the inclusion of 10mM BAPTA in the pipette solution suggests that they represent Ca^{2+}-activated conductances. In keeping with this notion, the Ca^{2+}-activated K^+ and Cl^- currents evoked by noradrenaline (2μM) were completely abolished under the same conditions. In the present experiments the equilibrium potentials for K^+ and Cl^- were -93mV and 0mV respectively. This means that increases in $P_{K(Ca)}$ and $P_{Cl(Ca)}$ following receptor activation would cause K^+ and Cl^- currents to flow in opposite directions. If $P_{Cl(Ca)}$ needs a higher concentration of intracellular Ca^{2+} for activation than $P_{K(Ca)}$ (see Field and Jenkinson, 1987; Capiod *et al.*, 1987), then ATP could induce the sequential activation of inward and outward currents actually observed. In the response illustrated in Fig. 4.4A, the dominant inward current could be the result of the simultaneous activation of both K^+ and Cl^- currents, provided that the Cl^- conductance was substantially larger than the K^+ conductance, as suggested in section 4.1.3. The small late outward current must be carried by K^+ and would only become evident following the earlier decline of $P_{Cl(Ca)}$ as $[Ca^{2+}]_i$ fell.

The influence of clamp potential on the transient current induced by ATP was examined in four cells dialysed with 10 mM BAPTA. Fig. 4.6 illustrates the outcome in two of these cells in which the holding potential was jumped from -40mV for 50ms to test potentials of -60, -20, 0 and +20 mV. In these experiments the current-voltage relationship was linear between -60 mV and +20 mV. The reversal potential averaged 0.4$\pm$3.7 mV and the slope conductance 1.3$\pm$0.1 nS, values close to those inferred for the depolarizing action of ATP from intracellular recordings. It can be safely concluded that this current forms the

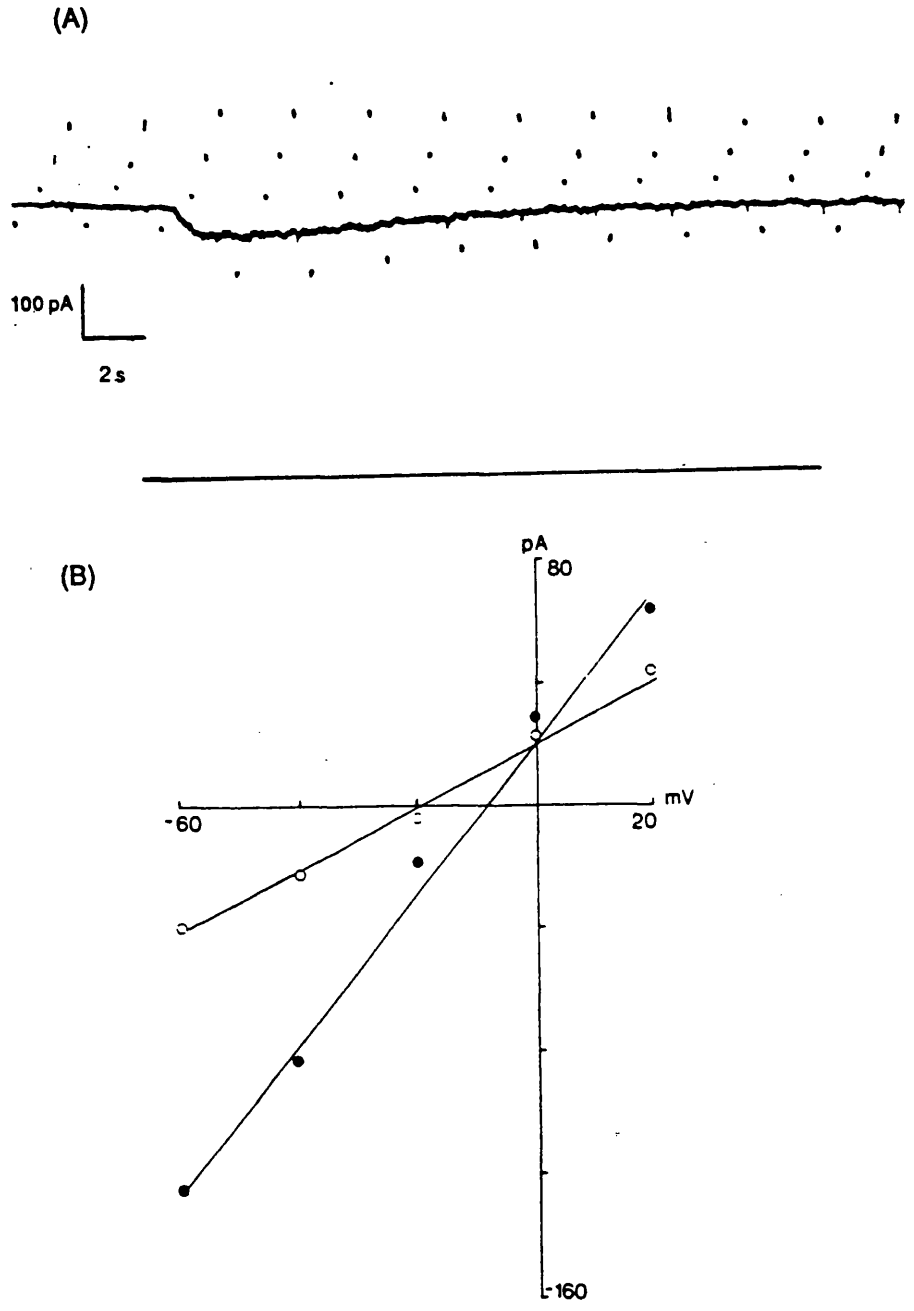


Fig. 4.6 Relationship between amplitude of current response to ATP (10μM, during horizontal bar) and clamp potential in single liver cells loaded with 10mM BAPTA. A. Holding potential was -40mV and the test potentials were -60, -20, +0 and +20mV. Duration of test pulse was 50ms and trains were applied every 2s. B. Amplitude of current before (○) and during (●) ATP response plotted against clamp potential.

basis of the depolarizing response to ATP already described.

4.1.6 The concentration dependence of the time course of the response to ATP and ADP

Prolonged applications of ATP revealed further differences in the response pattern as the agonist concentration was increased. Fig. 4.7 compares responses recorded intracellularly from single hepatocytes exposed to 0.5, 2 and 20μM ATP. In the eight out of fifteen cells that responded to 0.5μM ATP, the input conductance rose only transiently and returned abruptly to resting levels, despite the continued presence of the agonist. When the concentration of ATP was increased to 2μM, the membrane response was still short-lived in one cell out of the sixteen cells tested, but in the remaining cells the conductance increase fell slowly over the first few seconds before breaking into a series of cyclical fluctuations with a period which ranged from 7-15s. By contrast, in all the cells tested (n=9) with 20μM ATP there was a smooth, sustained rise in conductance.

ADP induced responses of an identical pattern to those seen with ATP, though far higher concentrations of ADP were required to produce effects comparable to those of ATP. With both ATP and ADP the effects appear to be graded more by the time for which the conductance was elevated than by the size of the peak conductance over the concentration ranges examined (compare Fig. 4.7 and Fig. 4.8).

Since purinoceptor activation causes a conductance increase in the liver cell which is predominantly Ca^{2+} activated, it seems reasonable to assume that the cyclical changes in membrane potential seen in e.g. Fig. 4.7B follow corresponding changes in cytosolic [Ca^{2+}]. Support for this notion has recently been provided by the experiments of Dixon, Wood, Cuthbertson and Cobbold (1990), who have described rapid sinusoidal

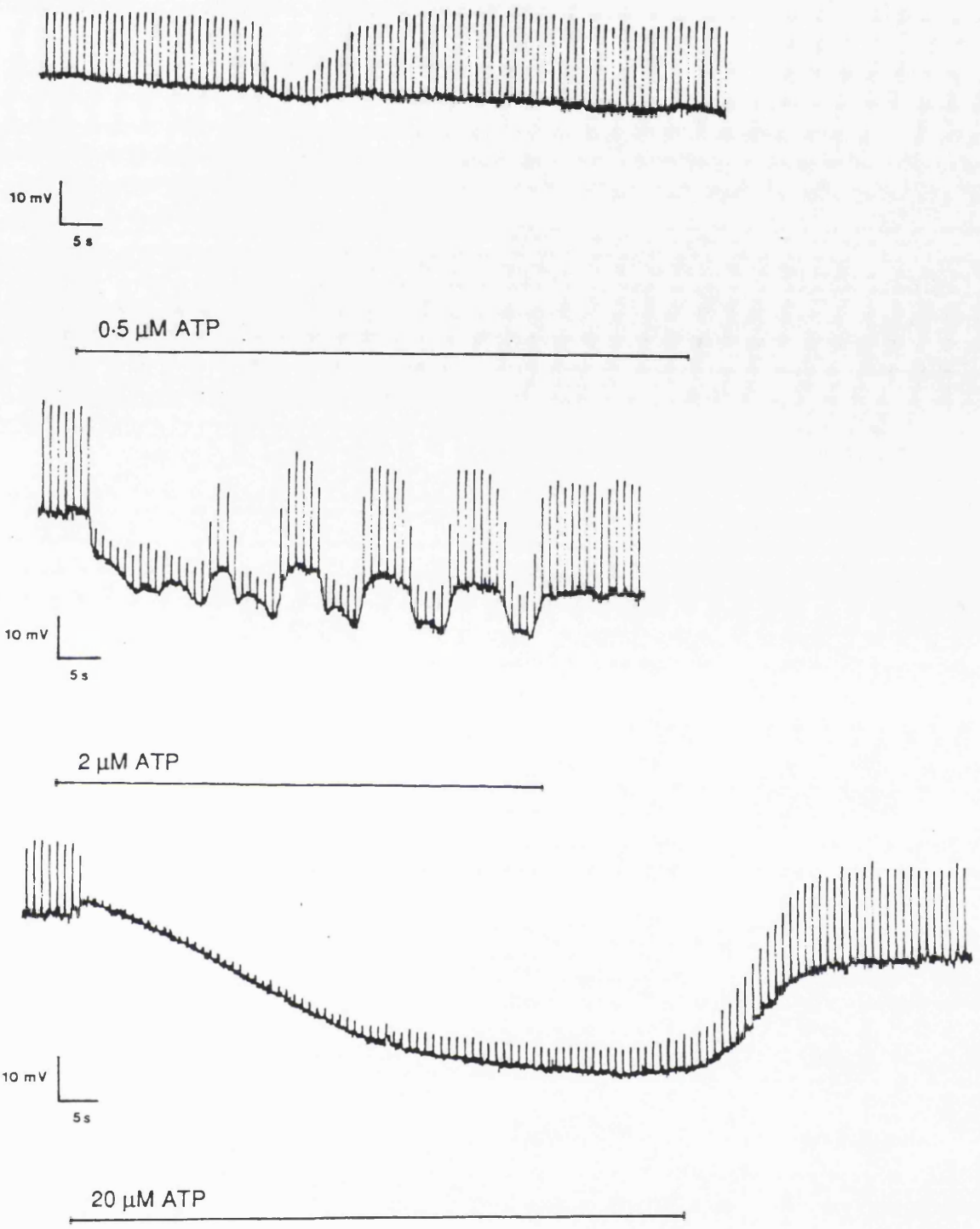


Fig. 4.7 A-C Effect of a prolonged application (during bars) of ATP (0.5-20µM) on the membrane potential and resistance of single hepatocytes. Intracellular recording. The current pulses were 100pA in A and B and 60pA in C. Resting potentials were -13, -27 and -27mV for A, B and C respectively.

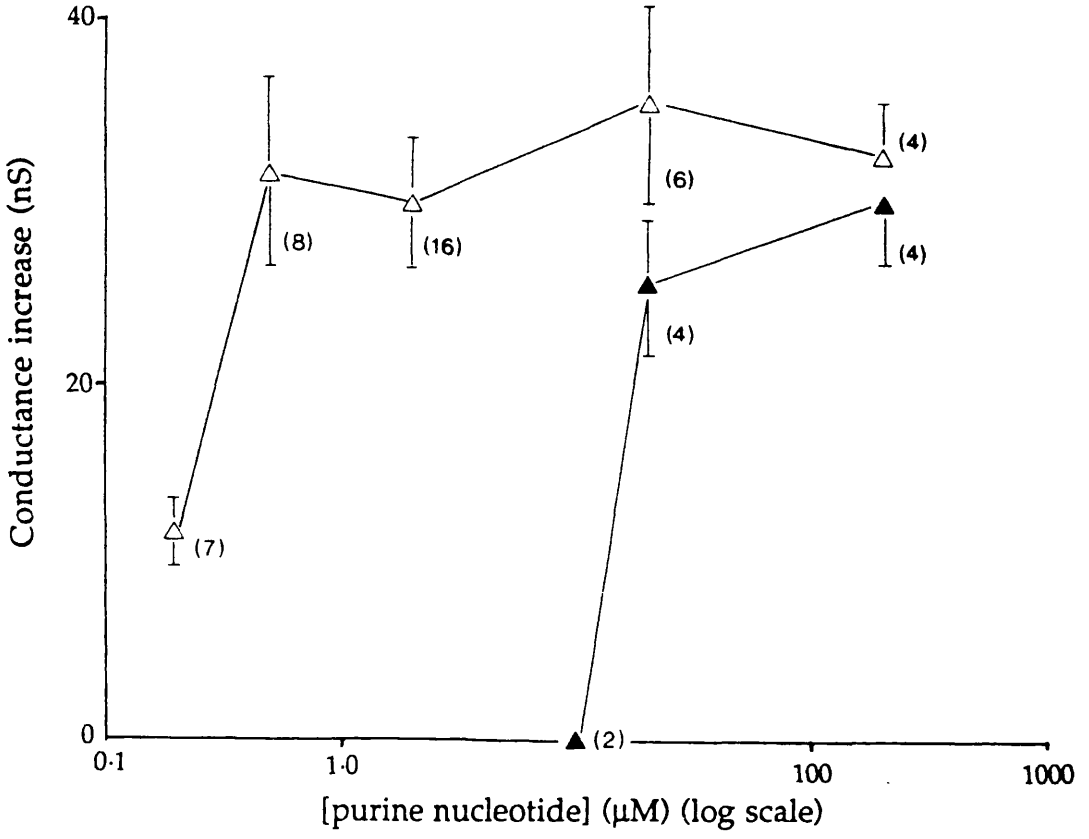


Fig. 4.8 Concentration-response curves for the peak conductance increase evoked by ATP (△) and ADP (▲). Points are means of the number of experiments shown in brackets with s.e.s indicated by bars.

oscillations in Ca^{2+} superimposed on the declining phase of the Ca^{2+} transient triggered by ATP in single aequorin loaded rat hepatocytes.

4.1.7 The response to intracellular dialysis with IP_3

Cobbold and his colleagues tentatively proposed that the pulsatile release of Ca^{2+} in response to agonists is a consequence of cyclical changes in receptor activation leading in turn to IP_3 spikes. This hypothesis was tested in the following experiments using the whole cell variant of the patch-clamp technique to introduce a constant concentration of IP_3 directly into the cell.

Fig. 4.9A illustrates the membrane current during the establishment of the whole cell configuration, using patch pipettes filled with the standard intracellular medium, without IP_3. There was no marked change in pipette current when the membrane patch beneath the pipette was ruptured. The input conductance of the cell was low and remained so throughout the recording.

Fig. 4.9C compares the whole cell currents recorded with a pipette containing IP_3 added to the standard intracellular filling solution at a concentration (10μM) which is sufficient to produce a maximal Ca^{2+} release from the endoplasmic reticulum (Burgess *et al.*, 1984*b*). A net outward current and conductance increase developed rapidly following the rupture of the membrane patch in response to IP_3. As may be seen the current declined typically in a series of sinusoidal oscillations (see also Capiod *et al.*, 1987). Even at concentrations as high as 40μM, IP_3 triggered an oscillating response in three out of four successful whole cell recordings. In the remaining cell the pipette tip became partially blocked as the initial net outward current began to decline. The range of the frequencies with which the conductance fluctuated in response to IP_3 (period 9-15 sec) overlapped with that observed with ATP under

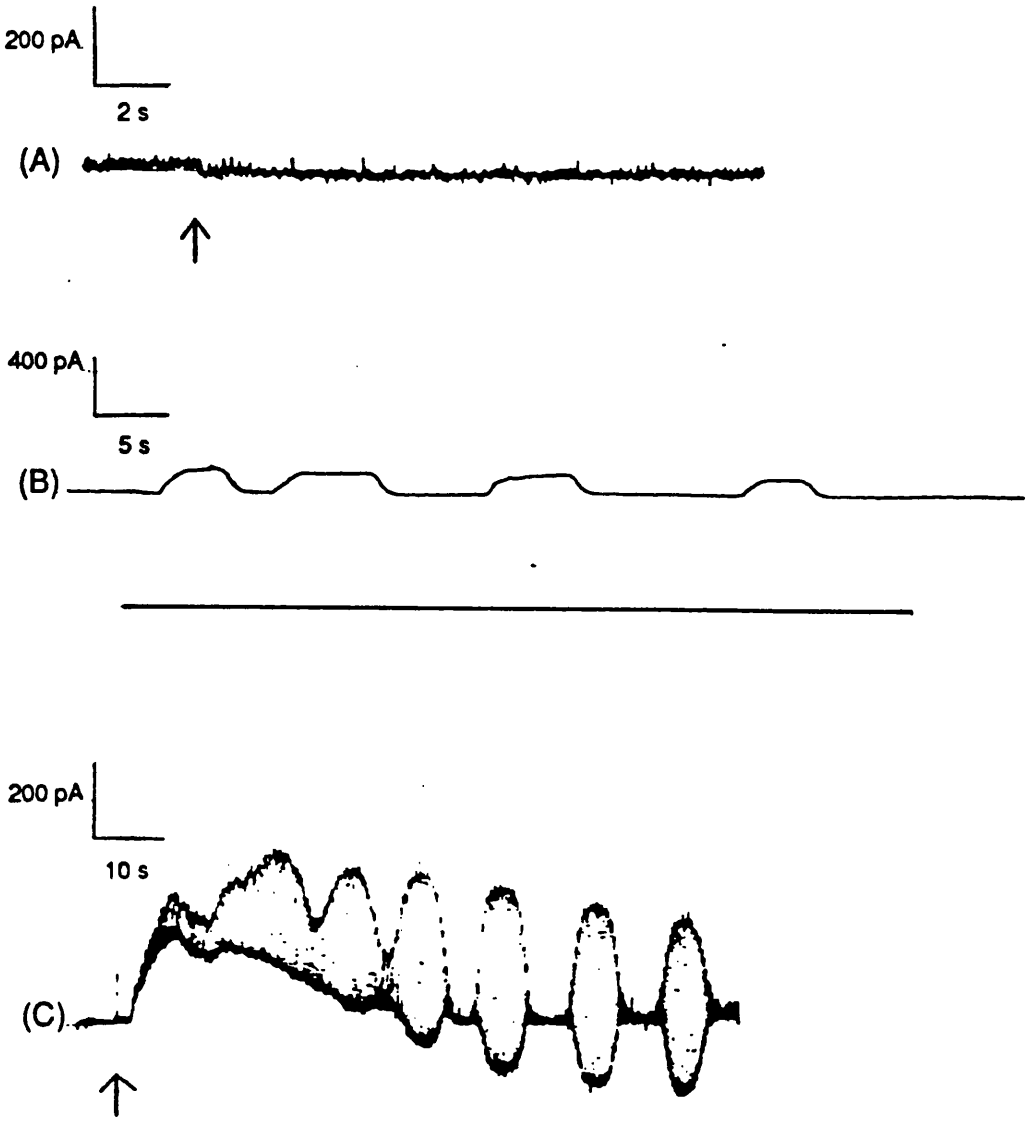


Fig. 4.9 Whole cell recordings from guinea-pig hepatocytes illustrating the cyclical activation of K^+ and Cl^- conductances by internally applied IP_3 and externally applied ATP. A, Control: a patch pipette containing the standard filling solution (section 4.2.2) was brought up against a cell and suction applied to form a high resistance seal. Short pulses of suction ruptured the membrane under the patch. An increase of capacitance and current noise (marked by arrow) indicated continuity between the pipette and cell interior. Holding potential -20mV. Capacity compensations adjusted during record. C, Same conditions as in A, but this time the pipette solution included 10μM IP_3 and 10mV voltage pulses were applied to the pipette to measure input conductance. B, conditions as above, ATP (10μM) applied during horizontal bar.

whole cell and intracellular recording conditions (period 7-15 sec), (cf. Fig. 4.9B and Fig. 4.9C). These data suggests that the origins of the high frequency oscillations in conductance (and by inference $[Ca^{2+}]_i$) observed in response to ATP lie at a stage distal to the receptor. The cellular mechanisms underlying this periodicity are discussed further in section 4.4.4.

4.1.8 The nature of the receptors involved in the ATP response

The Ca^{2+} response of rat hepatocytes to ATP and ADP is mediated by a P_2 purinoceptor (Charest, Blackmore & Exton, 1985). P_2 purinoceptors have recently been subdivided into P_{2x} and P_{2y} receptors on the basis of relative agonist potencies of structural analogues of ATP (Burnstock & Kennedy, 1985). The most potent agonists at the P_{2x} subtypes are the phosphate-modified analogues of ATP, such as α,β-methylene ATP, while the 2-substituted analogues, such as 2-methylthio ATP, are the most potent at the P_{2y} subtype. A brief attempt was made here to subclassify the P_2 receptor in the guinea-pig hepatocyte using α,β-methylene ATP and 2-methylthio ATP.

Both compounds evoked a rapid hyperpolarization of the cell membrane associated with a rise in input conductance. The peak increase in conductance induced by maximal concentrations of 2-methylthio ATP and α,β-methylene ATP were of the same order of magnitude (~30pS) as that observed with ATP (see Fig. 4.10). However, α,β-methylene ATP was consistently less potent than ATP with a minimum effective concentration in the order of around 200μM (cf. in the order of 0.2μM with ATP). The response to 2-methylthio ATP varied markedly from preparation to preparation of hepatocytes, but even in the most sensitive preparations 2-methylthio ATP was only as effective as ATP at

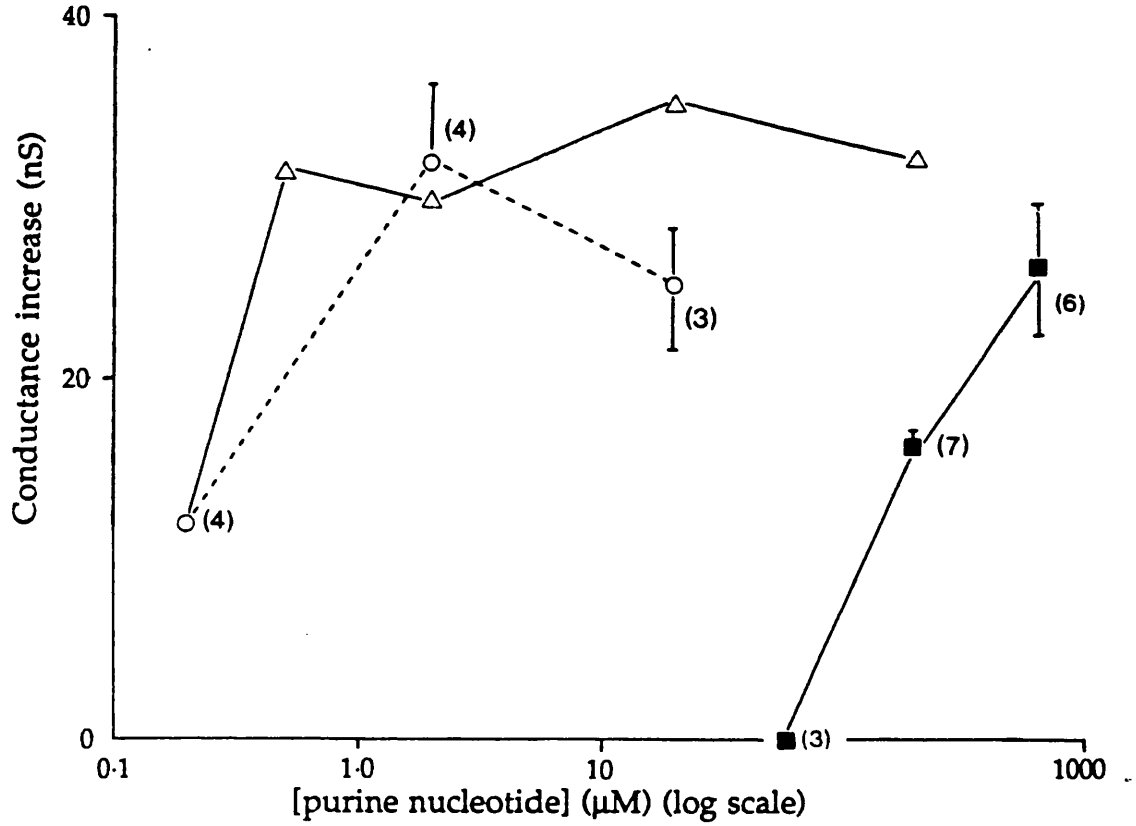


Fig. 4.10 Concentration-response curves for the peak conductance increase evoked by ATP (△ ;values reproduced from Fig. 4.8), α,β-methylene ATP (■) and 2-methylthio ATP (O). Points are means of the numbers of experiments shown in brackets with s.e.s indicated by bars.

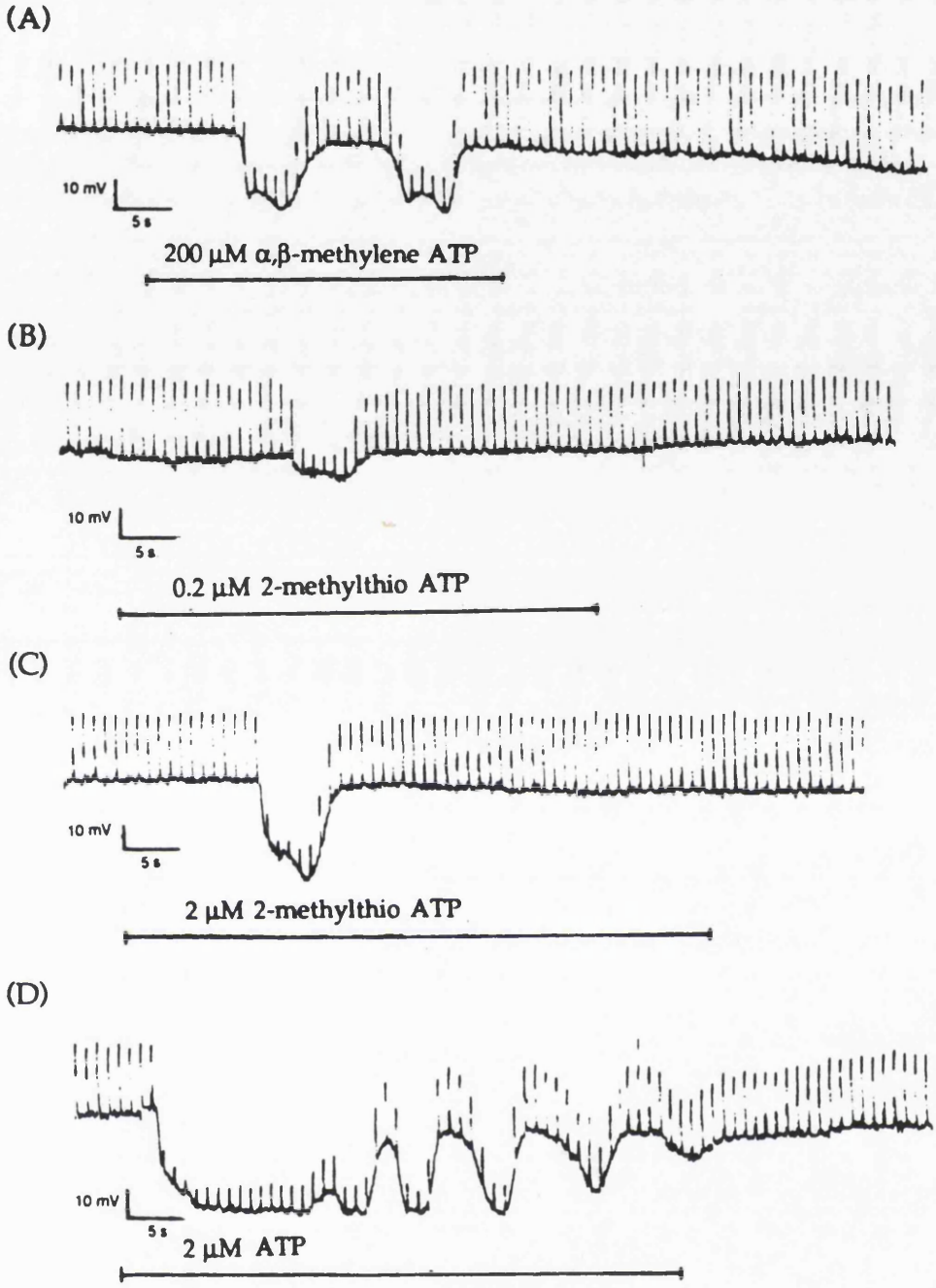


Fig. 4.11 The effect on intracellularly recorded membrane potential and input resistance of prolonged applications (during horizontal bars) of 200μM α,β-methylene ATP (A) 0.2μM, 2μM 2-methylthio ATP, (B & C respectively) and 2μM ATP (D). Resting potentials were -19, -25, -24 and -32mV in A, B, C and D respectively. Amplitude of constant current pulses was 100pA in each case.

increasing the input conductance at low concentrations. Thus 2-methylthio ATP, like α,β-methylene ATP, produced a transient response even at high concentrations (see Fig. 4.11). At high concentrations, however, both 2-methylthio ATP (20μM) and α,β-methylene ATP (650μM) could cause a transient depolarization similar to that seen with ATP. The P_2 purinoceptor controlling the effects of ATP on membrane permeability in the liver cell would not then appear to belong to either of the subclasses described.

4.2 THE RESPONSE TO SALBUTAMOL

β_2 agonists such as isoprenaline and salbutamol may also mobilise intracellular Ca^{2+} stores in rat liver cells (Combettes *et al.*, 1986) and increase K^+ permeability in a proportion of guinea-pig liver preparations (Cocks, Jenkinson & Koller, 1984). In the present study it was found that even in responsive preparations the sensitivity of the cells to salbutamol (but not to ATP) tended to decline in short term culture after a relatively brief period of time, in the range of 6-10 hours. Fig. 4.12 shows the variety of responses recorded in isolated guinea-pig hepatocytes during puffer application of salbutamol (0.5μM). After a delay ranging from 4 to over 30 secs the membrane potential and input conductance rose rapidly. Although the underlying ionic mechanisms were not explored in the current study, the reversal potentials of the hyperpolarization to salbutamol (-32.3$\pm$2.0mV; n=13) and noradrenaline (-35.2$\pm$0.7mV; n=3) were so close that it seems highly likely that the same conductive pathways (ie. $P_{K(Ca)}$ and $P_{Cl(Ca)}$) are involved. The hyperpolarization evoked by salbutamol usually inactivated quickly (e.g. Fig. 4.12B) but in one cell in five the increase in conductance decayed in a series of transients with a period of 8-11 secs (e.g. Fig. 4.12A).

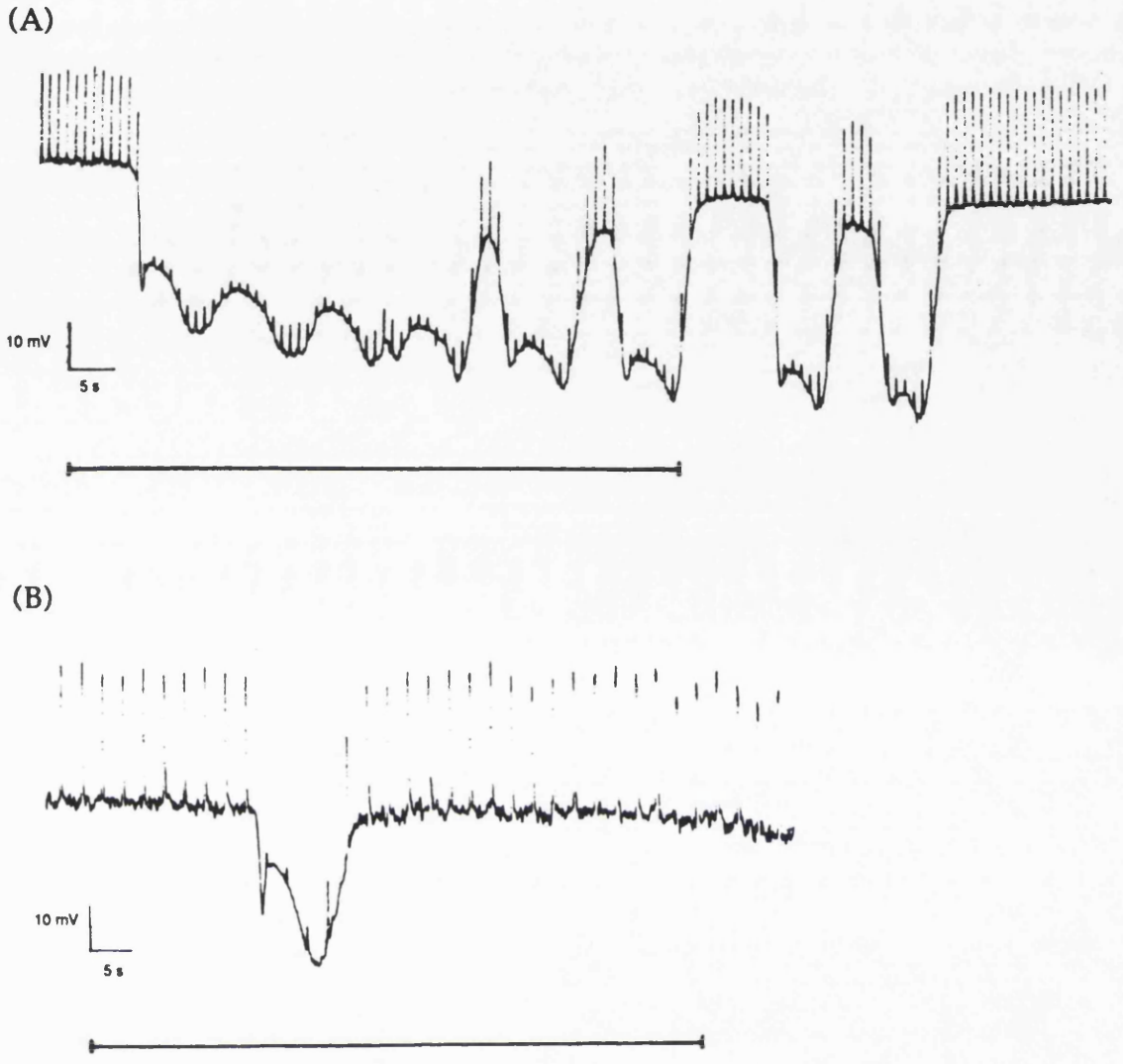


Fig. 4.12 illustrates the range of response patterns recorded intracellularly in single hepatocytes during a prolonged application of 0.5μM salbutamol. Resting potentials were -16 and - 35mV in A and B respectively. The calibration bars apply to both records. Horizontal bars show drug application. Amplitude of constant-current pulses was 60pA in A and 40pA in B.

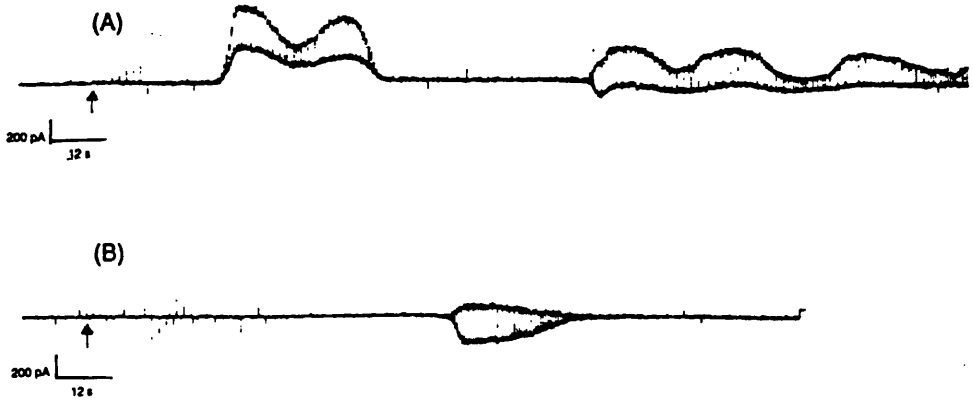


Fig. 4.13 Whole-cell current recordings illustrating the variable response of single hepatocytes to internal cAMP (50μM). The arrow at the beginning of each trace indicates the point of membrane rupture on going from cell-attached to whole-cell recording condition. Holding potential -20mV; 10mV pulses were applied to the pipette to monitor cell conductance. Capacity compensation adjusted during record.

4.2.1 The response to intracellular dialysis with cAMP

The aim of the next experiment was to see if cAMP introduced into the cell via the patch pipette produced conductance changes similar to those observed with salbutamol. Two examples of the responses evoked by cAMP (50μM) are shown in Fig. 4.13. In two of the five cells that responded cAMP simply produced a short-lived inward current and conductance increase (e.g. Fig. 4.13B). In the remaining three cells the conductance increased slightly during a brief period of net outward current, followed by short pulses of inward current of intervals of 7-12 secs (e.g. Fig. 4.13A). Interestingly, the slow decline in sensitivity of cells in primary culture to salbutamol was mirrored by a decline in sensitivity to internal cAMP.

4.3 INTERACTIONS BETWEEN β_2 AGONISTS AND Ca^{2+} MOBILIZING HORMONES

When small doses of a Ca^{2+} mobilizing hormone and a β_2 agonist are applied simultaneously to liver slices and dispersed hepatocytes the effects on membrane permeability and Ca^{2+} mobilization are much greater than the sum of that to each agent alone. This interaction was the subject of the following experiments.

Fig. 4.14 illustrates the effects of a near threshold concentration of ATP (0.5μM) on the response of single hepatocytes to salbutamol (0.25μM). In this particular preparation, the cells (3 in each instance) failed to respond within 30s to either agonist alone. But salbutamol applied simultaneously with ATP initiated in two out of three cells a rise in membrane potential and conductance which followed a delay of a few seconds and declined in a series of transients of increasing amplitude. Doubling the concentration of either ATP or salbutamol alone

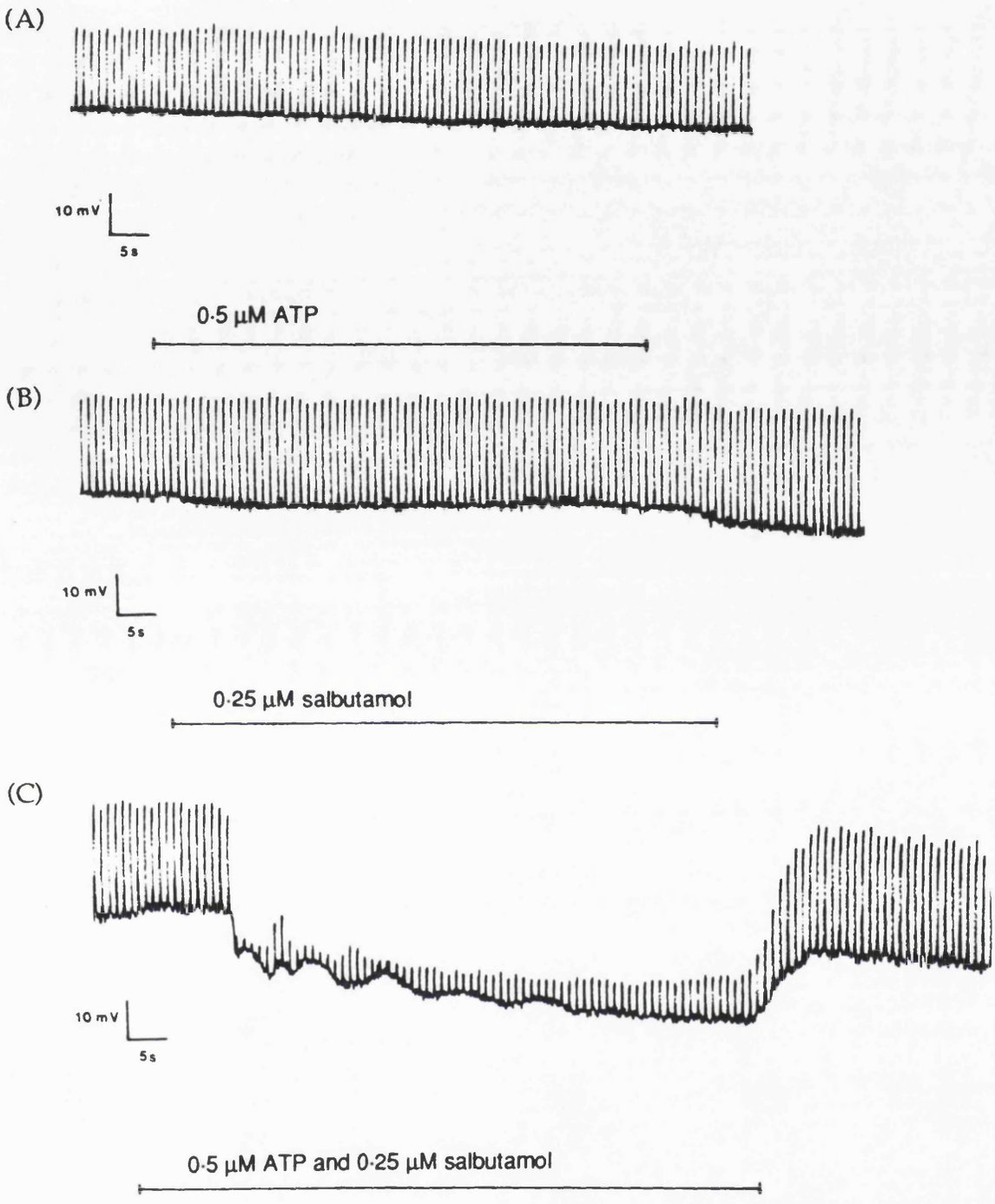


Fig 4.14 Responses of single hepatocytes to 0.5µM ATP and 0.25µM salbutamol applied together and to twice the concentration of both salbutamol (0.5µM) and ATP (1µM) applied separately. Current pulses were 100pA in A, B, and E and 60pA in C and D.

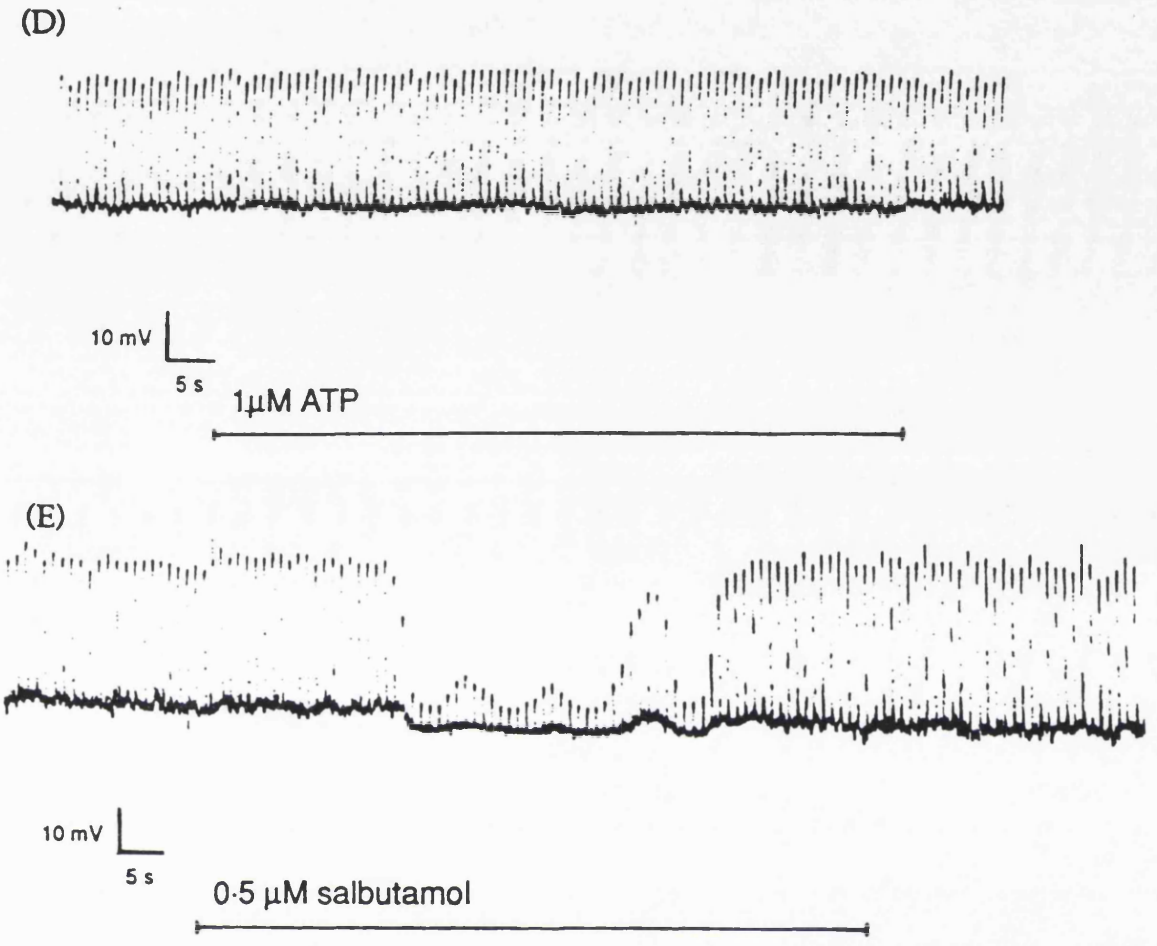


Fig. 4.14 (continued)

failed to reproduce the response patterns seen with 0.5μM ATP and 0.25μM salbutamol applied together. Thus in the same preparation the three cells tested failed to respond to 1μM ATP and in the cell (n=2) that did respond to 0.5μM salbutamol the conductance returned to resting levels after 20sec despite the continued presence of the agonist.

In a larger population of cells a short lived increase in input conductance was seen in the four out of nine cells that responded to 0.5μM ATP (mean duration of response = 8.8$\pm$1.2 sec) and in the one out of twelve cells that responded to 0.25μM salbutamol (duration of response = 20 sec). Of the eleven out of twelve cells that responded when salbutamol (0.25μM) and ATP (0.5μM) were applied together, one cell still showed a transient increase in input conductance (duration of response = 7s), but seven cells now gave an oscillatory increase in conductance and three cells a sustained rise in conductance. In these ten cells the conductance only returned and remained at pre-drug levels after approximately 60 seconds, when the agonist was removed. By contrast in the one cell out of six that responded to 1μM ATP the conductance increase lasted only 7 secs and in the three out of six cells that responded to 0.5μM salbutamol the mean life time of the conductance increase was 16.7$\pm$2.4s. Notably, there was no difference in peak conductance produced by 0.5μM ATP with or without 0.25μM salbutamol (26.4$\pm$3.4nS (n=11) and 28.5$\pm$3.4nS (n=4)).

In a preliminary experiment a similar interaction was seen between threshold concentrations of the α_1 agonist amidephrine (1μM) and salbutamol (0.25μM) (see Fig. 4.15). In this particular preparation none of the cells tested (n=3) responded to salbutamol (0.25μM) and in the two out of three cells that responded to 1μM amidephrine the mean lifetime of the conductance increase was 15 sec. When the agonists were applied together all the cells (n=4) responded, two with a sustained response

and two with an oscillatory response. Again the input conductance only returned to stay at predrug levels when the drug application was terminated after approximately 60 seconds.

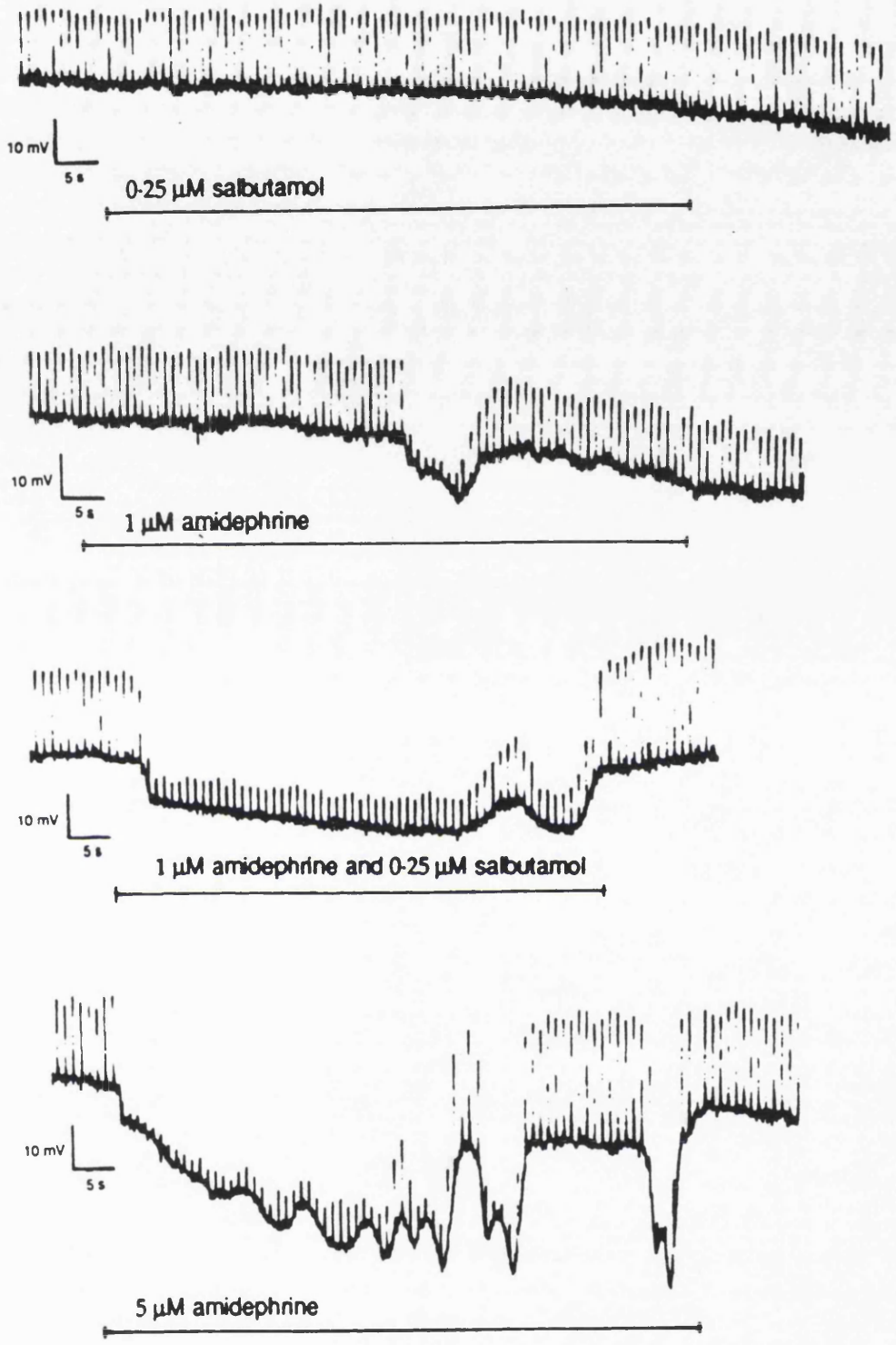


Fig. 4.15 Membrane potential responses of single hepatocytes to low doses of (±) amidephrine (1µM) and salbutamol (0.25µM) applied separately and in combination (during bar). For comparison, the response to 5µM amidephrine is included. The current pulses were 100pA in each record.

4.4 DISCUSSION

4.4.1 The membrane responses to salbutamol and low concentrations of ATP

The electrophysiological responses of hepatocytes to salbutamol and low concentrations of ATP are qualitatively similar to that evoked by noradrenaline working through α_1 adrenoceptors (Field & Jenkinson, 1987). Thus after a delay of seconds all three agonists rapidly hyperpolarise the cell membrane by producing a substantial increase in $P_{K(Ca)}$ and $P_{Cl(Ca)}$. The kinetics of the agonist-induced rise in membrane potential are consistent with a small increase in the concentration of the relevant second messenger, be it IP_3 or cAMP, leading to a large increase in the cytosolic level of Ca^{2+}. This would occur if, for instance, the release of Ca^{2+} sequestered in the ER were mediated by a highly co-operative mechanism. Indeed in a recent study of the fast kinetics of the mobilization of internal Ca^{2+} stores in peremeabilized basophilic leukemia cells Meyer, Holowka and Stryer (1988) concluded that at least three molecules of IP_3 must be bound to open a Ca^{2+} channel. A somewhat lower cooperativity (Hill coefficient 1.6), however, has since been reported in broadly similar experiments conducted on rat hepatocytes by Champeil, Combettes, Berthon, Doucet, Orlowski and Claret (1989). It should also be pointed out that neither of these groups took into account the reaccumulation of Ca^{2+} by the ER which might be expected to reduce the rise in $[Ca^{2+}]_i$ caused by IP_3 induced Ca^{2+} efflux to a greater extent for lower concentrations of IP_3 than for higher concentrations. In view of this, the extent of cooperativity should be regarded as uncertain, for the time being.

The rapid amplification of the Ca^{2+} signal may instead be provided through a self perpetuating process of Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983). In keeping with this notion fura II imaging techniques

have revealed that in hepatocytes the Ca^{2+} signal starts at a few discrete subplasmalemmal sites but then quickly spreads as a wave in a non decremental fashion throughout the cell (Saez, Connor, Spray and Bennett, 1989).

Surprisingly, while P_2 purinoceptor, β adrenoceptor and α_1 adrenoceptor agonists each give comparable maximal Ca^{2+} signals in cell populations loaded with quin 2 and in single hepatocytes injected with aequorin (Charest, Blackmore and Exton, 1985; Charest, Blackmore, Berthon and Exton, 1983; Combettes, Berthon, Binet and Claret, 1986; Woods, Cuthbertson and Cobbold, 1987; Cobbold, Woods, Wainwright and Cuthbertson, 1988), these classes of stimuli show very different abilities when it comes to activating $P_{K(Ca)}$ and $P_{Cl(Ca)}$. A parallel situation exists in the chromaffin cell where muscarine and nicotine can produce similar changes in the average $[Ca^{2+}]_i$ in a cell, but only nicotine is an effective secretagogue. In the case of the chromaffin cell the explanation for this apparent anomaly has recently been shown to lie in a difference in the spatial organization of the Ca^{2+} responses to these stimuli (Cheek, Jackson, O'Sullivan, Moreton, Berridge and Burgoyne, 1990). Whether or not the magnitude of the membrane response of single liver cells is similarly related to the cellular distribution of Ca^{2+} during stimulation clearly warrants further investigation.

4.4.2 An additional ATP-sensitive conductance

Under certain conditions the hyperpolarization induced by ATP is preceded by a small, faster depolarization. This is mediated by a transient, inward current (i_{cat}) which apparently flows through cation-selective channels. i_{cat} is not gated by noradrenaline which implies that the stimulation of purinoceptors recruits receptor specific mechanism(s) in addition to PI turnover. Just what the nature of the

mechanism underlying i_{cat} is remains to be determined. Clearly the latency of this current requires verification with a more rapid means of agonist application than the puffer technique used in this study. If it is indeed of the order of magnitude seen here (0.5-0.9s), this would argue against the direct coupling between nucleotide binding and cation channel opening described in guinea pig cochlear cells (Nakagawa, Akaike, Kimitsuki, Komune & Arina, 1990), mouse lacrimal acinar cells (Gallacher, Sasaki & Smith, 1990) and arterial smooth muscle cells (Benham, Bolton, Byrne & Large, 1987; Benham & Tsien, 1987) and might point instead to the involvement of a second messenger operated ion channel. In keeping with such a suggestion Bear & Li (1991) have recently described a cation-selective channel in rat hepatoma cells that can be stimulated during cell attached patch recordings by the addition of ATP to the cell bath.

The entry of Ca^{2+} through second messenger operated cation channels has previously been invoked to explain how a liver cell manages to maintain an elevated [Ca^{2+}] and membrane response during stimulation in the face of a heightened Ca^{2+} extrusion (Duddy, Kass & Orrenius, 1989). Even though the present study made no attempt to identify the particular charge carriers involved in i_{cat}, it can safely be concluded that this current is not the principal mechanism of receptor mediated Ca^{2+} uptake firstly because it decays with a half life of seconds, yet the Ca^{2+} pools that are emptied by receptor activation in the absence of extracellular Ca^{2+} refill even when Ca^{2+} is restored minutes after the addition of the agonist (DeWitt & Putney, 1984) and more persuasively, because it represents a conductance pathway not open to other Ca^{2+} mobilizing agonists. Perhaps i_{cat} serves instead as a supplementary mechanism for Ca^{2+} influx or as a further means for modulation of the phosphatidyl inositol pathway.

4.4.3 The nature of the receptors involved in the membrane response to ATP

The rank order of glycogenolytic potency of ATP analogues in rat liver previously reported by Gordon (1986) and Keppens & De Wulf (1986, 1991) is entirely consistent with ATP activating glycogen phosphorylase through a P_2Y purinoceptor coupled to Ca^{2+} mobilization. The weak activity of 2-methylthio ATP seen in the present study, however, argues against the involvement of this receptor subclass in the gating of the Ca^{2+}-activated conductances in the guinea-pig liver. A potential reconciliation of these conflicting results may be provided by the experiments of Keppens and De Wulf (1991). These workers have demonstrated that the good correlation that exists for ATP, ADP and α,β-methylene ATP between the nucleotide's glycogenolytic activity, its affinity for the P_2Y receptor as characterized using ATP α [^{35}S], and its ability to stimulate phosphatidyl inositol turnover (Keppens and De Wulf, 1986), breaks down with 2-methylthio ATP (Keppens and De Wulf, 1991). This has lead to the idea that ATP and 2-methylthio ATP interact with two different subspecies of P_2 purinoceptor in the liver and it is tempting to speculate that the difference in the rank order of potency of these agonists in mobilizing Ca^{2+} reported here and in earlier studies in the rat hepatocyte reflects a species dependent variation in either the relative densities or the coupling affinities of these two purinoceptor subpopulations. Proof of this hypothesis, however, will require the development of more selective purinoceptor antagonists.

4.4.4 Conductance fluctuations evoked by prolonged exposure to hormones or by internal application of second messengers

The rapid oscillations in Ca^{2+}-activated conductances reported in the current study in hepatocytes responding to ATP presumably reflect

similar changes in $[Ca^{2+}]_i$. Correspondingly high frequency fluctuations are often found superimposed on the falling phase of the individual calcium spikes produced in aequorin loaded rat hepatocytes treated with ATP (Dixon, Woods, Cuthbertson, Cobbold, 1990).

The time course of membrane response evoked by ATP was mimicked in the present experiments by internally applied IP_3. Rapid cyclical changes in Ca^{2+}-activated conductances have previously been described in a variety of other non-excitable cells including pancreatic acinar cells (Wakui, Potter & Petersen, 1989), photoreceptors (Payne, Walz, Levy & Fein, 1988) and Xenopus oocytes (Berridge, 1988) in response to the intracellular application of IP_3 and its non-metabolizable analogue inositol (1,4,5)trisphosphorothioate.

From their work on IP_3-induced oscillations in Xenopus oocytes Berridge & Gallione (1988) have developed a model to account for this oscillatory activity, based on the Ca^{2+}-induced Ca^{2+}-release mechanism (CICR) proposed by Fabiato (1983), to explain the repetitive transient increases in $[Ca^{2+}]_i$ and tension seen in skinned cardiac and skeletal muscle cells. On Berridge's scheme the oscillations described in nonmuscle cells are a property of the IP_3-insensitive compartment of the ER which is induced to oscillate through the extra load imposed upon it by the Ca^{2+} released from the IP_3-sensitive compartment and/or the influx of external Ca^{2+}. Clearly in this two pool model any stimulus that tends to raise cytosolic free Ca^{2+} should lead to oscillatory release and reuptake of Ca^{2+} by the IP_3-insensitive pool, as stated by Berridge & Irvine (1989). In keeping with this prediction both the cAMP dependent hormone, salbutamol, and cAMP itself were also shown here to induce liver cells to oscillate. Interestingly periodic responses were only seen in a small proportion of salbutamol-sensitive cells, perhaps those in which the IP_3-insensitive stores were unusually full or labile. IP_3

independent oscillations have also since been seen in hepatocytes treated with the ER Ca^{2+} pump inhibitor TBHP (Thomas, Renard & Rooney, 1991) and bile acids (Capiod, Combettes, Noel & Claret, 1991).

Just how the sustained rise in $[Ca^{2+}]_i$ observed in liver cells exposed to ethanol (Cobbold, Woods, Dixon, Sanchez-Bueno & Cuthbertson, 1990) can be reconciled with the two-pool model is less obvious, if ethanol does simply work through IP_3 production as has been suggested (Hoek, Thomas, Rubin & Rubin, 1987; Rubin, Thomas & Hoek, 1987). Equally perplexing is the insensitivity of the ATP induced falling phase oscillations to ryanodine, a marker for CICR (Cobbold, Sanchez-Buenos & Dixon, 1991).

Of interest in the present context are recent reports that Ca^{2+} can directly modify the activity of the IP_3-gated Ca^{2+} channel in such a way that channel opening is promoted by increasing $[Ca^{2+}]_i$ from 100 to 300nM but declines with a further increase in free $[Ca^{2+}]$ (Bezprozvanny, Watras & Ehrlich, 1991). This raises the possibility that CICR may precede by the Ca^{2+} activation of the IP_3 gated Ca^{2+} channel as well as by the Ca^{2+} activation of the IP_3 insensitive channels, and that negative feedback may contribute to Ca^{2+} spiking. In the liver, Ca^{2+} has been claimed to modulate the affinity of the IP_3 receptor and/or the gating of the Ca^{2+} channel, but this idea rests on binding data collected at 4°C that could not be reproduced at 37°C (Pietri, Hilly, & Mauger, 1990).

4.4.5 Receptor interactions in liver cells

In the presence of salbutamol the dose-response relationship of single cells to ATP is shifted to the left so that near threshold concentrations of this agonist become sufficient in some cells to set in motion the series of cyclical fluctuations in permeability, and (by

inference) $[Ca^{2+}]_i$, normally associated with more intense stimulation. These results agree with those obtained by Cobbold, Sanchez-Bueno & Dixon (1991) who showed that dibutyryl cAMP increased the life-time of the Ca^{2+} spike in rat liver cells stimulated with either vasopressin or phenylephrine. Together they may explain, at least in part, the super-additive effects of small doses of cAMP- and IP_3-dependent hormones on P_K and $[Ca^{2+}]_i$ previously described in liver slices (Jenkinson & Koller, 1977; Cocks, Jenkinson & Koller, 1984) and cell suspensions (Cocks *et al.,* 1984; Poggioli, Mauger & Claret, 1986; Burgess, Dooley, McKinney, Nånberg & Putney, 1986).

The definition of the cellular basis of this interaction clearly depends on a more precise knowledge of the mechanism(s) involved in the generation of Ca^{2+} oscillations. One intriguing, though admittedly, highly speculative, possibility is that β agonists may act by facilitating CICR as suggested in myocytes (Pott, 1988). This could be achieved in the context of Berridge's two pool model in a number of ways. cAMP could increase the affinity of the Ins $(1,4,5)P_3$ binding sites or modify the gating of the associated Ca^{2+} channel. Indeed, the modulation of the binding properties of the Ins$(1,4,5)P_3$ receptor by dibutryl cAMP has been demonstrated in rat liver membranes but only at an unphysiological temperature of 4°C (Mauger, Claret, Pietri & Hilly, 1989). A second possibility is that cAMP could short circuit the Ca^{2+} transport between the IP_3-sensitive and IP_3-insensitive pools, perhaps by forming a link analogous to a gap junction as postulated for GTP (Chueh *et al.,* 1987) and Ins$(1,3,4,5)$ P_4 (Merrit & Rink, 1987) or by lowering the set point for Ca^{2+} uptake into the IP_3-insensitive pool. Support for the latter idea is provided by studies showing that cAMP analogues enhance Ca^{2+} uptake by microsomal fractions or enriched reticulum fractions from pretreated rat liver (Andia-Waltenbaugh & Friedman, 1978; Taylor *et al.,*

1980). Alternatively the positive influence of cAMP of the Ca^{2+} entry process (Poggioli *et al.*, 1986; Burgess *et al.*, 1986) could provide the stimulus for CICR. All but the last possibility could explain the dramatic reduction produced by cAMP dependent kinase in the EC_{50} value for IP_3 mediated Ca^{2+} release seen by Burgess, Bird, Obie & Putney (1991) in permeabilized hepatocytes.

4.5 CONCLUDING REMARKS

4.5.1 The physiological significance of the Ca^{2+}-activated and volume-sensitive mechanisms

The present study while settling several issues leaves open a number of questions relating to the steps linking receptor activation to net ion movements in liver cells, not least the origin of the Ca^{2+} oscillations and the convergence point for the interaction between cAMP dependent and IP_3 dependent hormones. One of the most perplexing aspects of the hormonal control of the permeability of liver cells is the marked species variation that exists. The membrane response described here in guinea-pig hepatocytes treated with a Ca^{2+} mobilizing hormone is common to all the animals investigated to date with one notable exception, the rat. It's not that the rat hepatocyte doesn't respond to noradrenaline or ATP with an increase in $[Ca^{2+}]_i$ (Charest, Blackmore, Berthon & Exton, 1983; Charest, Blackmore & Exton, 1985). It simply seems that these cells lack $P_{K(Ca)}$ and $P_{Cl(Ca)}$ (Field & Jenkinson, 1987), at least under physiological conditions. In keeping with this idea, rat hepatocytes bind an insignificant amount of ^{125}I labelled apamin (Cook & Haylett, 1985). Rat liver cells in fact respond initially to Ca^{2+} mobilizing hormones by taking up rather than losing K^+ (Burgess, Claret & Jenkinson, 1981; Coats, 1985). This response has been shown to be a consequence of an increase in the activity of Na^+, K^+ ATPase (Berthon,

Burgess, Capiod, Claret & Poggioli, 1983).

With the complete absence of the rapid K^+ loss response in such a "successful" animal as the rat it is difficult to ascribe a physiological role to the permeability change in other species. However, it should be remembered that the Ca^{2+} mobilizing hormones do cause rat liver to lose K^+ by another much slower process of unknown mechanism (Coats, 1985). In view of the rapid changes in the cell contents of osmotically active solutes brought about by the concurrent stimulation of gluconeogenesis and amino acid uptake, it is not unreasonable to suggest that the volume-sensitive P_K mechanism may in fact subserve the later hyperkaelemia seen in intact rats in response to a bolus injection of adrenaline – a postulate which should now be easy to test using cetiedil as a probe.

It is unclear whether the activation of the volume-sensitive K^+ permeability would be necessary to obtain appropriate cell volume regulation in the liver of species other than the rat following a challenge with a glycogenolytic hormone; after all the substantial release of ions produced by the activation of $P_{K(Ca)}$ and $P_{Cl(Ca)}$ would itself be expected to guard against cell swelling in this setting.

4.5.2 Directions for future research

There is still some ground to cover before the mechanisms regulating the resting membrane potential of the liver cell are fully understood and in this vein the volume-activated P_K mechanism warrants more detailed investigation. Preliminary estimates of the unitary conductance of the volume-activated potassium channel derived from noise analysis suggests that it should be possible to determine the selectivity and gating of this channel in single channel recordings. Single channel recordings may also produce an insight into the nature

of the cetiedil block.

In addition the origins of the rapid fluctuations in cytosolic calcium seen in liver cells responding to hormones still requires clarification. Bearing in mind the central role that calcium-activated calcium release (CICR) from the IP_3 insensitive pool plays in Berridge's two pool model, the importance of CICR in the periodicity seen in liver cells needs to be determined with some urgency. The most direct approach to this problem would be to study the effects of perfusing the tip of the patch pipette with Ca^{2+} or EGTA containing solutions during whole cell recordings and during simultaneous microfluorimetrical Ca^{2+} measurements (see Osipchuk *et al.*, 1990). This technique could also be used to investigate the interactions reported to occur between cAMP and IP_3 dependent pathways at the level of the second messenger.

Lastly, the spatial organisation of the Ca^{2+} oscillation deserves consideration. Imaging data has been presented on the spread of the Ca^{2+} signal in single cells and clusters of coupled cells responding to submaximal concentrations of glycogenolytic hormones (Nathanson & Murgstahler, 1992), but so far the concentration dependence of the Ca^{2+} response to these agonists has not been examined in detail in either setting. The influence of cAMP-dependent agonists on the spread of an IP_3-dependent Ca^{2+} signal throughout the cells of its origin and neighbouring cells also remains to be investigated, and such measurements could provide important clues as to the mechanism of the interactions described in this chapter.

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Oscillations of Ca^{2+}-dependent K^+ permeability evoked by β-adrenoceptor agonists and cAMP in isolated guinea-pig liver cells

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Cyclic AMP and the β-adrenoceptor agonists salbutamol (SALB) and isoprenaline (ISO) elicit an apamin-sensitive increase in K^+ permeability in a proportion of guinea-pig hepatocyte preparations (Cocks *et al.* 1984). It has also been shown that dibutyryl cAMP, and agonists acting through cAMP, can release Ca^{2+} sequestered in an intracellular pool (Combettes *et al.* 1986) and it seems likely therefore that the resulting increase in cytosolic Ca^{2+} concentration induces the opening of Ca^{2+}-dependent K^+ channels in the plasma membrane. We have now investigated the time-course of the changes in permeability, and the mechanisms involved, by means of whole-cell patch-clamp and intracellular recording from single hepatocytes in short-term culture (31–37 °C) in normal and Cl^--free solution.

In responsive preparations (~ 40% of these tested) the external application of ISO (1–5 μM) or SALB (0·5 μM) produced a large (10·3 ± 0·9 nS S.E.M., $n = 13$) rise in K^+ conductance. In 2 in 5 cells, however, the response consisted of cyclical fluctuations in conductance which continued for the duration of the application and which occurred with a frequency (period 8–12 s) comparable to that observed (Field & Jenkinson, 1987) with noradrenaline acting through α_1-adrenoceptors, which stimulate the production of inositol 1,4,5-trisphosphate (InsP3). On some occasions, the frequency was much lower (0·5–1 min^{-1}). Intracellular application of cAMP (50–100 μM) via the patch pipette reproduced both types of response to β-adrenoceptor stimulation. The ISO response was not altered by internal perfusion with heparin (150 μg ml^{-1}), which abolished the responses to angiotensin II (which acts through InsP3 production). This result suggests that the activation of Ca^{2+}-dependent K^+ channels by β-agonists in guinea-pig hepatocytes occurs independently of the InsP3 pathway.

The similarities between the response patterns evoked in the present experiments by externally applied β-adrenoceptor agonists and internally applied cAMP indicate that the oscillatory activity seen following receptor stimulation is not primarily due to events at the receptor level. It may instead reflect an intrinsic property of intracellular Ca^{2+} regulation.

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Increase of potassium and chloride conductance with internal perfusion of inositol trisphosphate or calcium ions in guinea-pig isolated hepatocytes

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Noradrenaline (NA) acting through α-adrenoceptors is known to increase cytosolic Ca levels by stimulating the formation of inositol phosphates in liver cells (Burgess, Godfrey, McKinney, Berridge, Irvine & Putney, 1984). Ion flux and electrophysiological studies have shown a concomitant increase in potassium and chloride ion permeability (Burgess, Claret & Jenkinson, 1981; Capiod, Field, Jenkinson & Ogden, 1985), thought to be activated by Ca ions released from endoplasmic reticulum by inositol trisphosphate (IP_3). Experiments to see if IP_3 or high [Ca] applied internally produce conductance changes similar to α receptor stimulation were made with the whole cell patch clamp method (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). IP_3 containing (20 μM), or Ca solutions of 10 nM or 0·7–1·2 μM free [Ca] buffered with BAPTA were applied in the patch pipette, either with 150 mM [Cl] solutions or in Cl-free conditions (anion gluconate or aspartate). Both elevated [Ca] and IP_3 evoked conductance increases comparable to those observed with NA (Table 1). Solutions with [Ca] buffered to 10 nM had no effect. Noise analysis of the apamin sensitive K-conductance gave mean single channel conductances of 1·7 pS at 30 °C in each case. These results support the idea that IP_3 production and the resulting increased free Ca levels following α receptor stimulation underlie the increase of Cl and apamin sensitive K-conductance observed in guinea-pig hepatocytes.

TABLE 1. Conductance increase (nS, mean ± S.E. of mean) of hepatocytes evoked by external NA (1 μM with 2 μM propranolol) and by increases in either internal IP_3 (20 μM) or internal Ca (0·7–2 μM/10 mM BAPTA). Temperature 30 °C or *22 °C.

	Normal Cl	Cl-free
NA	51·2 ± 4·6	3·5 ± 0·8
IP_3	42·9 ± 2·5	3·4 ± 0·4
0·7–2 μM Ca	46·6 ± 16*	2·2 ± 0·6

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Properties of a volume-activated potassium conductance in isolated guinea-pig hepatocytes

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Hepatocytes possess a quinine-sensitive K^+ conductance which becomes activated under conditions associated with cell swelling (for references see Kristensen, 1986). The characteristics of this conductance have now been studied in isolated guinea-pig hepatocytes in short-term (2–10 h) culture at 35–37 °C, using intracellular and whole-cell patch-recording techniques. The earlier finding (Field & Jenkinson, 1987) that the conductance could develop as a consequence of electrolyte leakage from even fine-tipped intracellular electrodes was confirmed; under these conditions, the conductance of the cell (initially $4 \pm 0{\cdot}3$ nS, S.E.M., $n = 20$) rose by $6{\cdot}3 \pm 0{\cdot}7$ nS ($n = 20$). At the same time, the membrane potential increased from its resting value of ~ -20 mV (uncorrected for the leak conductance introduced by the microelectrode) to -60 to -80 mV. The conductance increase was blocked by quinine (Field & Jenkinson, 1987) and more potently and selectively by cetiedil which caused half-maximal inhibition at $2{\cdot}7 \pm 0{\cdot}3$ (S.D.) μM. Though cetiedil (see e.g. Sarkadi *et al.* 1984) also blocked the Ca^{2+}-activated K^+ conductance that these cells possess (e.g. Field & Jenkinson, 1987; Capiod & Ogden, 1989), a 1000-fold greater concentration was needed.

The conductance could also be evoked by the application of gentle hydrostatic pressure (15 cmH_2O) to the shaft of a patch electrode (whole-cell mode, membrane potential clamped at 0 mV; pipette filling solution 145 mM-KCl, 8 mM-HEPES, 50 μM-EGTA). Under these conditions an outward cetiedil-sensitive current developed, corresponding to a K^+ conductance increase of $3{\cdot}9 \pm 0{\cdot}7$ nS (S.E.M., $n = 3$). Spectral analysis of the current noise revealed two components which could be fitted by the sum of two Lorentzian functions with half-power frequencies of $7{\cdot}2 \pm 0{\cdot}8$ and 156 ± 12 Hz ($n = 4$). Approximately 70% of the variance was associated with the low-frequency component. A single channel conductance of $7{\cdot}5 \pm 0{\cdot}2$ pS ($n = 4$) was estimated.

In preliminary experiments inclusion of the Ca^{2+} buffer BAPTA (10 mM) in the patch electrode filling solution did not affect the current associated with cell swelling so that it seems unlikely to be attributable to a rise in cytosolic Ca^{2+}.

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Internal perfusion of guinea-pig hepatocytes with buffered Ca^{2+} or inositol 1,4,5-trisphosphate mimics noradrenaline activation of K^+ and Cl^- conductances

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External application of noradrenaline to voltage-clamped guinea-pig isolated hepatocytes evoked membrane conductance increases to K^+ and Cl^-. This effect was reproduced by internal perfusion of the cells with 2 μM buffered Ca^{2+} and with 20 μM inositol 1,4,5-trisphosphate (IP_3). The kinetic properties of the K^+ conductance and its selective block by the toxin apamin were the same in each case. Cyclical fluctuations of conductance observed with noradrenaline were reproduced by internal IP_3 but not by Ca^{2+} perfusion, indicating that oscillations of intracellular free Ca^{2+} may arise from properties of the Ca^{2+} sequestration mechanism at constant IP_3 concentration.

Hepatocyte; Noradrenaline; Apamin; Ca^{2+} activation; K^+ conductance; Cl^- conductance; Inositol 1,4,5-trisphosphate

1. INTRODUCTION

Noradrenaline (NA) acting through α-adrenoceptors produces a large increase in the membrane permeability of guinea-pig hepatocytes to K^+ and Cl^- [1-3]. This is thought to result from activation of K^+ and Cl^- conductances by the rise of cytoplasmic free Ca^{2+} concentration which α-adrenoceptor activation causes [4]. The source of Ca^{2+} is probably the endoplasmic reticulum, since inositol 1,4,5-trisphosphate (IP_3), which appears rapidly after α-receptor stimulation as a result of the hydrolysis of phosphatidylinositol bisphosphate [5,6], can release Ca^{2+} from this organelle [7]. We have now applied the whole-cell patch-clamp technique [8] to compare the properties of the conductance increase produced by external NA with those observed when hepatocytes are perfused internally either with BAPTA-buffered Ca^{2+} solutions or with IP_3. The similarities found strongly suggest that IP_3 production and a rise of internal free Ca^{2+} concentration alone can account for the membrane permeability increase evoked by NA. Moreover, the finding that IP_3 can elicit cyclical changes in conductance throws fresh light on the observation by Woods et al. [9] that the elevation of internal calcium occurs in a series of transients.

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Abbreviation: BAPTA, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid

2. EXPERIMENTAL

Hepatocytes were isolated from the livers of

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guinea-pigs or rabbits by perfusion with collagenase followed by mechanical disruption [2]. Cells in suspension were plated onto 35 mm Falcon petri dishes and whole-cell voltage-clamp recordings [8] made after 1–8 h. Patch pipettes of 2–6 MΩ resistance contained internal solutions given below. Cell conductance was monitored with small voltage pulses applied to the pipette. In experiments with Cl^- present, the external solution contained (mM) 142 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 8 Hepes, 11 glucose (pH 7.3), and the internal solution 150 KCl, 8 Hepes, 0.5 EGTA (pH 7.3). Cl^--free solutions contained: external: 137 Na gluconate, 5.4 K gluconate, 5 $CaSO_4$, 8 Hepes, 11 glucose (pH 7.3); internal: 150 K gluconate, 8 Hepes (pH 7.3). Internal solutions contained IP_3 or BAPTA/Ca where indicated. A dissociation constant of 0.1 μM was assumed for BAPTA/$CaCl_2$ or $CaSO_4$ solutions of specified free Ca^{2+}. Propranolol (2 or 5 μM) was present throughout to block β-adrenoceptors. IP_3 was purchased from Amersham and BAPTA from BDH. Inositol bisphosphate was a generous gift from Dr R.F. Irvine.

3. RESULTS

Fig.1 shows the main characteristics of the conductance increase evoked by NA in hepatocytes voltage-clamped with the whole-cell patch-clamp technique. The response at −20 mV membrane potential, with 5 mM [K] externally and 150 mM internally (equilibrium potential $E_K = -85$ mV) and 150 mM [Cl] in both external and internal solutions ($E_{Cl} = 0$ mV), comprised 3 phases. A transient net outward current was followed by a large inward current which reversed to an outward current before finally declining to control level on washout of NA (upper trace). This sequence of currents suggests that conductance changes to both K^+ (outward) and Cl^- (inward current) occurred. Responses obtained when internal and external Cl^- were replaced with the impermeant anion gluconate showed only outward current at potentials positive to −80 mV. Current/voltage relations in both Cl^- and Cl^--free conditions were linear from −75 to 40 mV with mean conductance increases of 51 nS in Cl^--containing and 3.5 nS in Cl^--free conditions (row 1, table 1). Reversal potentials of −19 and −80 mV were obtained in

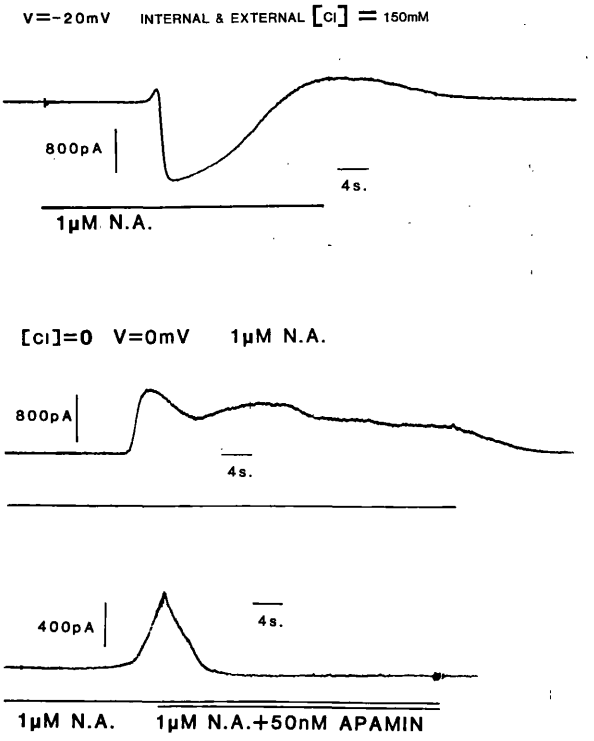

Fig.1. Membrane currents evoked by NA in isolated guinea-pig hepatocytes. (Upper trace) Cl^--containing solutions; potential −20 mV. (Middle trace) Cl^--free gluconate solutions; potential 0 mV. (Lower trace) Cl^--free solutions; apamin (50 nM) was applied during initial part of outward current. Potential 0 mV. Temperature 30°C. 5 μM propranolol present.

Cl^- and Cl^--free conditions, respectively, the latter value being close to the equilibrium potential for K^+. The response obtained at 0 mV in Cl^--free solution, a net outward K^+ current, is shown in the

Table 1

Conductance increase (nS, mean ± SE) of hepatocytes evoked by external NA (1 μM with 2 μM propranolol) and either internal IP_3 (20 μM) or internal Ca^{2+} (0.7–2.0 μM/10 mM BAPTA)

	Normal Cl^-	Cl^--free
NA	51.2 ± 4.6	3.5 ± 0.8
IP_3	42.9 ± 2.5	3.4 ± 0.4
0.7–2.0 μM Ca^{2+}	46.6 ± 16.0[a]	2.2 ± 0.6

Temperature, 30°C or 22°C[a]. Values were corrected for the effects of series resistance between pipette and cell interior

middle trace and the lower trace shows suppression of this K^+ current by apamin, a peptide toxin from bee venom which selectively blocks K^+ flux through a Ca^{2+}-activated K^+ conductance in hepatocytes [2,10].

A further characteristic of the outward K^+ current evoked by NA in Cl^--free solution was an increase in current noise, shown in fig.3a. This had a minimum variance at about -70 mV, near E_K, and was blocked by apamin. Results of spectral analysis of the noise increase will be given below.

Prolonged application of NA in Cl^- or Cl^--free conditions often resulted in a response which consisted of slow cyclical fluctuations of conductance which lasted several minutes when measured with microelectrode [3] or patch-clamp methods [11].

The results of introducing a high level of internal free $[Ca^{2+}]$ by means of pipette solutions buffered with 10 mM BAPTA [12] are shown in fig.2a–c. The procedure was as follows. A gigaohm seal was obtained and the effects of rupturing the membrane under the pipette tip were recorded while ap-

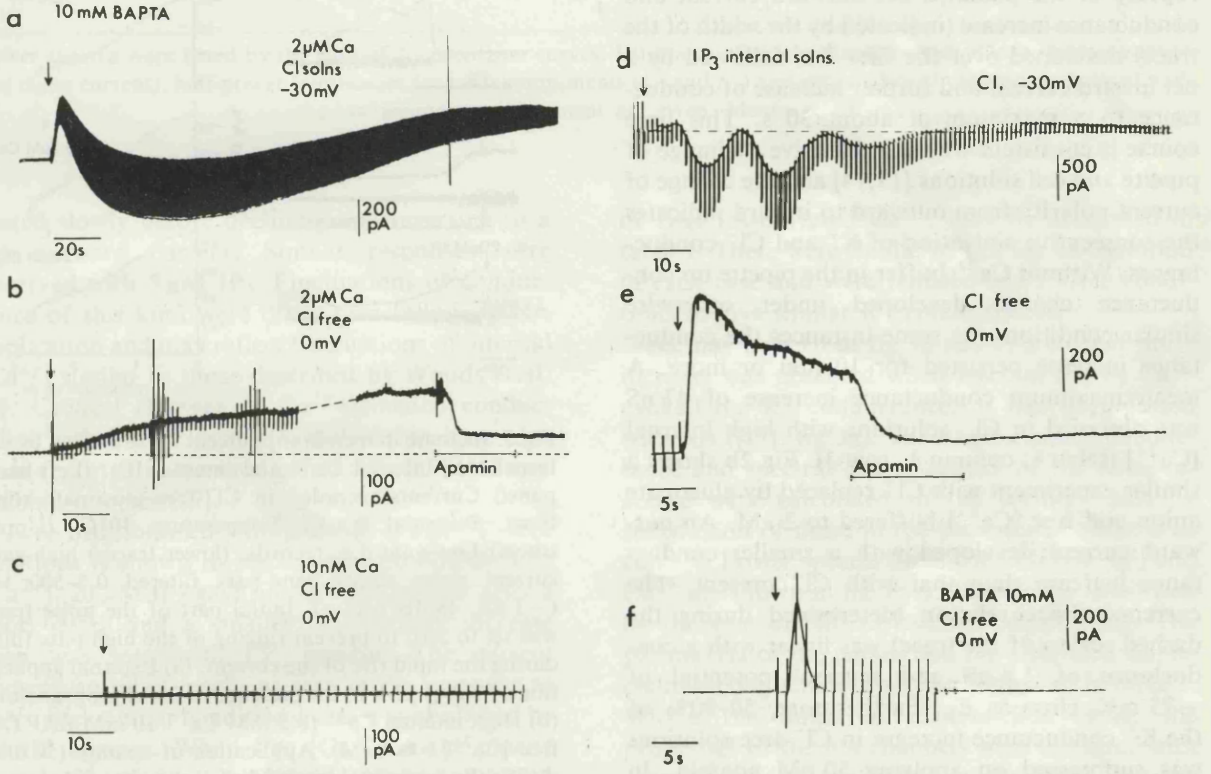

Fig.2. Membrane conductance changes evoked by high free Ca^{2+} (2 μM) or IP_3 (20 μM) in the patch pipette solution. The arrow at the beginning of each trace indicates the point of membrane rupture on going from cell-attached to whole-cell recording condition [8]. Capacity transients partly subtracted. (Left-hand panel) (a) Cl^--containing solutions. Potential -30 mV. Temperature 22°C. 9.4 mM Ca^{2+}/10 mM BAPTA (2 μM free Ca^{2+}) in the pipette. 8 mV potential pulses applied to pipette at 5 Hz to measure membrane conductance. (b) Cl^--free gluconate solutions. Potential 0 mV. Temperature 30°C. 2 μM free $[Ca^{2+}]$ in pipette. Capacity compensation adjusted during record (at approx. 20–30 s after membrane rupture), producing large transients on the trace. 20 mV pulses at 1 Hz applied to monitor cell conductance. Current/voltage relation determined during dashed portion (30 s duration). Apamin (50 nM) reduced the conductance by 70%. (c) Same conditions as above with free $[Ca^{2+}]$ buffered to 10 nM (0.3 mM Ca^{2+}/10 mM BAPTA). No capacitance compensation. (Right-hand panel) (d) Cl^--containing solutions. Pipette contained 20 μM IP_3. Potential -20 mV. Temperature 30°C. (e) Cl^--free solutions. Pipette contained 20 μM IP_3. Potential 0 mV. Apamin (50 nM) applied during the horizontal bar. (f) Conditions as in (e) with the addition of 10 mM BAPTA to the pipette solution to buffer cytosolic $[Ca^{2+}]$.

plying small voltage pulses to the pipette. An increase of capacitance and current noise (arrows in fig.2) indicated continuity between the pipette and cell interior. The capacity transient was incompletely subtracted and appears in the first few seconds of these records. Changes in membrane conductance resulting from diffusion of components of the pipette solution into the cell were recorded.

Fig.2a shows the result obtained when pipette and external solutions contained Cl^- and free $[Ca^{2+}]$ in the pipette was buffered to 2 μM. After rupture of the patch, a net outward current and conductance increase (indicated by the width of the trace) developed over the first 7 s, followed by a net inward current and further increase of conductance to a maximum at about 30 s. This time course is consistent with the diffusive exchange of pipette and cell solutions [13,14] and the change of current polarity from outward to inward indicates the consecutive activation of K^+ and Cl^- conductances. Without Ca^{2+} buffer in the pipette no conductance change developed under otherwise similar conditions. In some instances the conductance increase persisted for 10 min or more. A mean maximum conductance increase of 47 nS was observed in Cl^- solutions with high internal $[Ca^{2+}]$ (table 1, column 1, row 3). Fig.2b shows a similar experiment with Cl^- replaced by gluconate anion and free $[Ca^{2+}]$ buffered to 2 μM. An outward current developed with a smaller conductance increase than that with Cl^- present. The current/voltage relation (determined during the dashed region of the trace) was linear with a conductance of 1.8 nS and reversal potential of -75 mV, close to E_K. Furthermore, 50–90% of the K^+ conductance increase in Cl^--free solutions was suppressed on applying 50 nM apamin. In several experiments a smaller component of K^+ conductance resistant to apamin also developed. This was suppressed by 1 mM quinine and showed different spectral (noise) characteristics. In fig.2c the experiment was repeated with free Ca^{2+} in the pipette solution buffered to a low level. No conductance increase was seen at the known resting Ca^{2+} level of rat hepatocytes, 200 nM [4], or at a lower level of 10 nM.

A series of experiments was made to see if IP_3 introduced into the cell via the patch pipette produced conductance changes similar to those with NA and Ca^{2+}. Fig.2d–f shows the effect of 20 μM IP_3 in the pipette, a concentration producing maximal Ca^{2+} release from permeabilized hepatocytes [7]. Fig.2d was obtained with Cl^- in both internal and external solutions with no Ca^{2+} buffer present in the pipette. After a delay of about 3 s, the conductance increased slightly during a brief period of net outward current, followed by a large conductance increase associated with a substantial inward current. As may be seen, the conductance fluc-

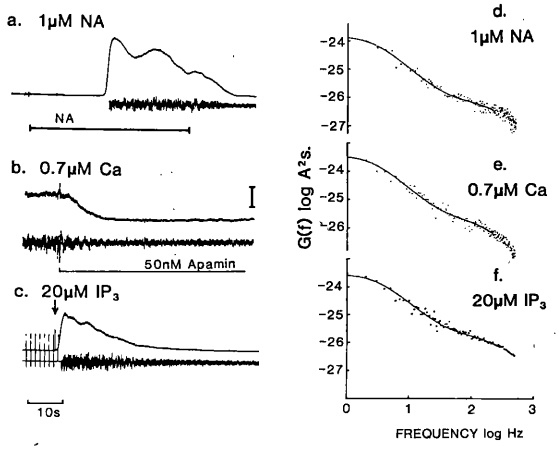

Fig.3. Increase in membrane current noise evoked by external NA, internal Ca^{2+} and internal IP_3. (Left-hand panel) Currents recorded in Cl^--free gluconate solutions. Potential 0 mV. Temperature 30°C. (Upper traces) Low-gain d.c. records, (lower traces) high-gain current noise traces band-pass filtered 0.5–500 Hz (-3 dB, 48 dB/octave). Initial part of the noise trace was set to zero to prevent ringing of the high-pass filter during the rapid rise of the current. (a) External application of NA (1 μM, in the presence of 2 μM propranolol). (b) High internal Ca^{2+} (8.5 mM Ca^{2+}/10 mM BAPTA; free $[Ca^{2+}] = 0.7$ μM). Application of apamin (50 nM) during the horizontal bar abolished the outward current and reduced the current noise. (c) Internal IP_3 (20 μM) applied via the pipette solution; the arrow indicates rupture of the membrane patch. Calibration: upper traces 400 pA, lower 40 pA. (Right-hand panel) Power spectra of current noise variance evoked by NA, Ca^{2+} and IP_3 (traces shown in the left-hand panel a–c). Average of 10–30 1 s samples digitised at 1024 Hz. Spectra fitted with the sum of 2 Lorentzian functions by least squares. For internal Ca^{2+} or IP_3, noise recorded in the presence of 50 nM apamin was subtracted as control. Estimates of single-channel conductance (γ) were calculated from the mean current (I), the variance of the fitted spectral density functions (var) and $V - E_K$: $\gamma = \text{var}/I(V - E_K)$

Table 2

Results of noise analysis of NA-evoked conductance increase in Cl^--free (aspartate or gluconate) solutions, compared with noise associated with conductances activated by internal IP_3 (20 μM) or high internal Ca^{2+} (free Ca^{2+} 0.7–2 μM/10 mM BAPTA)

	Gluconate, 30°C			Aspartate, 22°C	
	NA	IP_3	Ca^{2+}	NA	Ca^{2+}
Single-channel conductance (pS)	1.69 ± 0.21	1.65 ± 0.27	1.74 ± 0.52	1.04 ± 0.51	0.75 ± 0.21
f_{c1} (Hz)	2.13 ± 0.33	2.60 ± 0.17	4.27 ± 1.64	1.82 ± 0.48	4.72 ± 0.86
f_{c2} (Hz)	189 ± 11	192 ± 9.0	179 ± 39	138 ± 34	233 ± 35
Low-frequency variance (%) of total	71 ± 2	69 ± 3	69 ± 9	64 ± 11	47 ± 9
n	7	4	4	3	6

Power spectra were fitted by the sum of 2 Lorentzian curves. Estimates of single-channel conductance (from variance and mean current), half power frequencies for both components (f_{c1} and f_{c2}) and proportion of variance associated with the low-frequency component are given. Means ± SE

tuated slowly before declining and reversing to a net outward current. Similar responses were observed with 5 μM IP_3. Fluctuations of conductance of this kind were often seen following NA application and may reflect fluctuations of internal $[Ca^{2+}]$ similar to those described by Woods et al. [9]. Cyclical changes of Ca^{2+}-activated conductance with intracellular IP_3 application have also been reported in lacrimal gland cells [15] and amphibian oocytes [17].

A record obtained with internal IP_3 in Cl^--free solutions is shown in fig.2e. As with NA or high $[Ca^{2+}]$, 20 μM IP_3 gave an outward current after a short delay and a smaller conductance increase than was seen with Cl^- present. The current showed an initial peak, as seen in the response to NA under similar conditions, and was abolished by 50 nM apamin. When free $[Ca^{2+}]$ in the pipette was buffered to 10 nM with 10 mM BAPTA (fig.2f), 20 μM IP_3 produced a transient outward current which rapidly declined to zero, suggesting that Ca^{2+} released by the high IP_3 concentration was buffered as the concentration of BAPTA in the cell rose.

Inositol 1,4-bisphosphate (IP_2) is formed in hepatocytes following breakdown of IP_3 by its 5′-phosphomonoesterase. However, when IP_2 (20 μM) was introduced into hepatocytes via the patch pipette, the cell conductance remained low.

The peak conductances recorded in Cl^- and Cl^--free conditions with external NA, internal IP_3 or BAPTA-buffered Ca^{2+}, may be compared in table 1. These were similar in size for Cl^- solution in each case and were reduced in Cl^--free conditions to give similar K^+ conductances.

As may be seen in fig.3b and c, a current noise increase was observed when internal IP_3 or Ca^{2+} evoked the K^+ conductance, as had been found with NA ([17]; fig.3a). The single-channel conductance and spectral characteristics of the noise increase were calculated in the former cases by subtraction of noise in the presence of apamin as control. Power spectra obtained with NA, IP_3 and Ca^{2+} are shown in fig.3d–f. These were fitted well by the sum of two Lorentzian components. The parameters of the curves and the estimated single-channel conductances may be compared in table 2. Within the limits of experimental error, the properties of the ion channels were the same with regard both to conductances estimated for the open channel (1.7 pS at 30°C, 1.0 pS at 22°C) and to the half power frequencies and amplitudes of the spectral components.

4. DISCUSSION

The membrane conductance increases evoked by external NA, by intracellular application of IP_3 or by raised internal $[Ca^{2+}]$ show marked similarities in respect of the ion selectivity for K^+ and Cl^-, the sequential activation and magnitude of K^+ and Cl^- components, the suppression of K^+ conductance

by low concentrations of apamin and the spectral characteristics of the apamin-sensitive conductance. This clearly suggests a common pathway for ion channel activation. The present results provide direct evidence of the activation of the K^+ and Cl^- conductances of hepatocytes by raised internal Ca^{2+}, and provide strong support for the chain of events from α-adrenoreceptor stimulation to ion conductance increase via IP_3 production and Ca^{2+} release from internal stores. Whatever role the simultaneously formed diacylglycerol may have [18], it is clearly not essential for the responses we have studied. The observation of cyclical changes of conductance with NA and internal IP_3 (see also [15,17]) but not internal high $[Ca^{2+}]$ is in keeping with the report of cyclical increases in cytosolic $[Ca^{2+}]$ during NA action [9]. The fact that these occurred in the present experiments with a maintained high concentration of IP_3 suggests that when elicited by NA they do not result from fluctuations in the concentration of IP_3. The explanation is more likely to be found in processes regulating Ca^{2+} sequestration and release by intracellular stores.

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PROPERTIES OF A CELL VOLUME-SENSITIVE POTASSIUM CONDUCTANCE IN ISOLATED GUINEA-PIG AND RAT HEPATOCYTES

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SUMMARY

1. Whole-cell voltage clamp and intracellular recording techniques were used to study the increase in K^+ conductance that accompanies swelling in isolated guinea-pig and rat hepatocytes in short-term culture at 37 °C.

2. Swelling was induced (i) by the application of pressure (15 cmH_2O) to the shank of the patch pipette, (ii) by exposing the cells to hypotonic solutions and (iii) as a consequence of leakage of electrolyte from an intracellular microelectrode.

3. Applying pressure to the patch pipette caused a large outward current (~ 600 pA) to develop in guinea-pig hepatocytes voltage clamped to 0 mV. This current reversed direction at −86 mV, close to the reversal potential for K^+, E_K (−93 mV), and is attributable to the activation of a K^+ conductance.

4. Spectral analysis of current noise during this response suggested a single-channel conductance of 7 pS, though this may well be an underestimate. The power spectrum could be fitted by the sum of two Lorentzian components, with half-power frequencies of 7 and 152 Hz. Seventy per cent of the variance was associated with the lower frequency component.

5. The steady-state current–voltage relationship for guinea-pig hepatocytes, as determined by whole-cell recording, was linear over the range −70 to +40 mV both before and during the increase in K^+ conductance induced by swelling.

6. Confirming earlier work, intracellular recording using microelectrodes filled with 1 M-potassium citrate sometimes resulted in a slow hyperpolarization and a large rise in input conductance. These changes are also attributable to an increase in K^+ conductance as the cell swelled because of leakage from the electrode.

7. Application of hypotonic external solutions during intracellular recording caused hyperpolarization and an increase in conductance. Conversely, hypertonic solution evoked depolarization and a fall in conductance in partly swollen cells.

8. The volume-activated K^+ conductance was reversibly blocked by cetiedil, which caused half-maximal inhibition at 2·3 μM. Bepridil, quinine and barium were also effective, with IC_{50}s (concentrations giving 50% maximal inhibition) of 2·7, 12 and 67 μM respectively.

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9. Much greater concentrations of cetiedil and bepridil ($IC_{50} \sim 1$ mM and 77 μM respectively) were required to inhibit the loss of K^+ which follows the application of angiotensin II (100 nM) to guinea-pig hepatocytes, and which occurs via Ca^{2+}-activated K^+ channels. Our evidence suggests that the activation of K^+ channels by cell swelling is Ca^{2+} independent.

10. Oligomycin A (2 and 15 μM) and oxpentifylline (20 and 100 μM) were ineffective, indicating that the cetiedil-sensitive K^+ channels in hepatocytes differ from those in lymphocytes and red blood cells.

11. In summary, swelling causes the opening of low conductance K^+ channels in hepatocytes. The channels are potently blocked by cetiedil and bepridil, and their opening is not mediated by internal Ca^{2+}.

INTRODUCTION

Bakker-Grunwald (1983) and Kristensen & Folke (1984) have shown that the net uptake of alanine via the sodium–alanine co-transport system results in an increase in the K^+ permeability of rat liver cells. This effect on permeability is generally attributed (Kristensen, 1986) to the rise in cell volume caused by the inward movement of solute (though see Cohen & Lechene, 1990). It is an important factor in the 'regulatory volume decrease' which is now known to follow swelling in hepatocytes (Graf, Haddad, Häussginger & Lang, 1988; Haddad & Graf, 1989; Lang, Stehle & Häussginger, 1989; Corasanti, Gleeson & Boyer, 1990) as in many other kinds of cell (Hoffmann & Simonsen, 1989). A similar increase in K^+ permeability can be observed in single guinea-pig, rabbit and rat hepatocytes as a consequence of the leakage of electrolyte from an intracellular microelectrode: it is then evident as an increase in membrane potential and conductance which can be blocked by quinine (Field & Jenkinson, 1987). Cell swelling can also be induced by the application of hypotonic solutions, and Howard & Wondergem (1987) have shown that this results in hyperpolarization and in a rise in the K^+ permeability of hepatocytes in mouse liver slices (see also Graf *et al.* 1988). The aim of the present work was to further characterize this volume-dependent K^+ permeability in terms of its electrical characteristics, and of the pharmacological agents, including cetiedil and bepridil, that inhibit it. Cetiedil was of particular interest since it has been found to block K^+ channels that are concerned in volume regulation by other cells (e.g. lymphocytes: Sarkadi, Cheung, Mack, Grinstein, Gelfand & Rothstein, 1985; DeCoursey, Chandy, Gupta & Cahalan, 1987). Our results show it to be equally effective in liver cells.

A preliminary account of some of this work has been given (Jenkinson & Sandford, 1990).

METHODS

Isolated hepatocytes were prepared by collagenase perfusion of the livers of male Hartley guinea-pigs and Sprague–Dawley rats. The guinea-pigs were anaesthetized with sodium pentobarbitone (Sagatal, 30 mg/kg), fentanyl (Sublimaze, 0·1 mg/kg) and droperidol (Droleptan, 10 mg/kg) given as successive intraperitoneal injections. For rats, sodium pentobarbitone alone (60 mg/kg) was used. Other details of the procedure have been described previously (Burgess, Claret & Jenkinson, 1981; Field & Jenkinson, 1987).

After preparation, the cells were washed twice by gentle centrifugation at 50 *g* in Eagle's Minimum Essential Medium (MEM) and then resuspended in supplemented MEM (SMEM; see

below). After 30 min incubation at 37 °C in a shaking bath the cells were again resuspended in SMEM. Ten minutes later, samples of the suspension were plated out in 35 mm Falcon Petri dishes, at a density of approximately 7×10^5 cells in 2 ml SMEM, and incubated at 37 °C in an atmosphere of 5% CO_2, 95% O_2. An hour was usually sufficient to allow the cells to become attached to the Falcon dishes. The SMEM was then replaced by 6 ml of 'recording medium' containing (mM): NaCl, 137; KCl, 4·4; $CaCl_2$, 2·5; $MgSO_4$, 0·81; HEPES, 8; glucose 5·5 and bovine serum albumin (1%). The pH was adjusted to 7·4 with NaOH. Finally, 1 ml of liquid paraffin (Spectrosol, BDH) was added to the contents of the dish, to form a film that limited evaporation. The dish was then placed in a machined recess in an aluminium block located on the stage of an inverted microscope (Nikon Diaphot) fitted with a long working distance objective (Nikon 40/0·55 Ph3DL). Holes drilled through the block allowed the cells to be examined at up to 600 × magnification, under either bright-field or phase-contrast illumination. The temperature of the block was controlled so that the cells were at 35–37 °C.

Drugs were dissolved in the recording medium and applied to individual cells by pressure ejection from a nearby micropipette ('puffer' technique). In later experiments, two puffer pipettes were used so that the actions of two agents or treatments on individual cells could be compared.

Intracellular recording. The single-microelectrode 'bridge' method was employed to follow changes in the membrane potential and conductance of single hepatocytes, as previously described (Field & Jenkinson, 1987). In most experiments, the electrodes were filled with 1 M-potassium citrate and 10 mM-KCl, adjusted to pH 6 with citric acid. Resistances were in the range 120–200 MΩ. Other filling solutions are mentioned in the text.

Whole-cell patch clamp recording. Guinea-pig hepatocytes were maintained at 35–37 °C in a Falcon dish which for these experiments was superfused at approximately 4 ml/min with pre-warmed recording solution of the same composition as above (but for the omission of albumin). The mechanical and optical arrangements were as before.

Conventional tight seal recordings were made using a List EPC-7 patch clamp amplifier. Patch pipettes were prepared from thin-walled borosilicate capillary tubing (Clark Electromedical GC 150 TF), using a two-stage vertical puller (List LM 3PA). They were coated to within 50 μm of the tip with Sylgard (Dow Corning) which was then heat-cured. The tips were fire-polished, and the electrodes back-filled immediately before use with a solution containing 145 mM-KCl, 8 mM-HEPES and 50 μM-EGTA, titrated to pH 7·1 with KOH and filtered through a 0·22 μm filter (Millipore). Resistances were from 2 to 8 MΩ. For some experiments EGTA was replaced by a more rapidly acting Ca^{2+}-chelating agent, BAPTA. The composition of the filling solution was then 130 mM-KCl, 10 mM-BAPTA and 8 mM-HEPES, titrated to pH 7·1 with KOH.

In several experiments, once whole-cell recording had been achieved, gentle positive pressure (15 cmH_2O) was applied to the interior of the pipette in order to produce a small increase in the volume of the cell. This was done by connecting the 'suction' port of the patch pipette holder (Clark Electromedical, EH-2MS), via a solenoid valve, to a 1 l glass jar in which the air pressure had been raised. A water manometer was placed in parallel.

Spectral analysis of current noise. For this purpose, the output (already filtered at 10 kHz) of the patch clamp amplifier was fed to a Racal Store 4DS FM tape-recorder. A second channel was used to record the noise signal at a higher gain, after bandpass filtering (−3 dB cut-off at 0·5 Hz and 1 kHz). The spectral density of the current noise was analysed using a FORTRAN program written by Professor D. Colquhoun. Prior to the analysis, the bandpass signal from the tape was amplified and filtered again at 500 Hz, to avoid aliasing errors. The signal was then digitized at 1024 Hz, and edited to remove obvious artifacts. The mean current amplitude, variance and spectral densities were computed and averaged for a series of 1 s samples. This was done for noise records both before and after the application of cetiedil (100 μM) which blocks the component of current attributable to increases in cell volume (see later). The values with cetiedil were subtracted from those in its absence, to yield net variances and spectral densities. The latter were fitted by the sum of two Lorentzian functions, using a least-squares procedure (see Colquhoun, Dreyer & Sheridan, 1979).

Measurement of K^+ flux. In a few experiments, the net loss of K^+ which occurs when guinea-pig hepatocytes are exposed to angiotensin II (100 nM) was recorded using a K^+-sensitive electrode placed in the cell suspension, as described by Burgess *et al.* (1981).

Materials and drugs. Materials for the preparation of Eagle's Minimum Essential Medium were obtained initially from Wellcome Reagents and subsequently from GIBCO. The medium was supplemented when required by the addition of 2% bovine serum albumin (BSA Fraction V, Miles

Lab or Sigma), 10% newborn calf serum (GIBCO) and penicillin (100 units/ml) + streptomycin (100 μg/ml) (GIBCO).

Samples of cetiedil citrate were kindly provided by Innothéra (Arcueil, France) and McNeil Pharmaceutical (Pennsylvania). We are also grateful to Hoechst (UK) and to McNeil Pharmaceuticals for gifts of oxpentifylline and bepridil HCl respectively. HEPES, BAPTA, quinine and raffinose were purchased from Sigma, tetraethylammonium chloride (TEA) from Aldrich and collagenase (isolated from *Clostridium histolytica*) from Boehringer.

RESULTS

Whole-cell recordings from guinea-pig hepatocytes

The application of the whole-cell patch clamp technique to the study of isolated guinea-pig and rabbit hepatocytes has been described by Capiod, Field, Ogden & Sandford (1987) and in greater detail by Capiod & Ogden (1989*a*). The electrical characteristics of the guinea-pig cells used in the present work were similar to those previously reported. Thus the resting membrane potential was $-28{\cdot}3 \pm 2{\cdot}3$ mV (S.E.M., $n = 6$), and the conductance $1{\cdot}4 \pm 0{\cdot}1$ nS (S.E.M., $n = 10$), as compared with -25 mV and $1{\cdot}8 \pm 0{\cdot}3$ nS respectively observed by Capiod & Ogden (1989*a*). The current–voltage relationships of the cells were linear over the range -100 to $+40$ mV, again in keeping with the earlier work, and with the findings of Sawanobori, Takanashi, Hiraoka, Iida, Kamisaka & Maezawa (1989), and of Henderson, Graf & Boyer (1989), using rat hepatocytes. The latter workers did, however, observe and analyse a departure from linearity at more extreme potentials (from -200 to $+175$ mV) which we did not explore.

The commencement of whole-cell recording was sometimes followed by the slow development (over several minutes) of an outward current associated with an increase in current noise. This occurred even when the patch electrode contained the Ca^{2+}-chelating agent BAPTA (10 mM). In current clamp mode, the corresponding changes were hyperpolarization, and a rise in conductance. In keeping with this, Capiod & Ogden (1989*a*) have described a proportion of guinea-pig hepatocytes with more negative potentials (-60 to -75 mV) than the usual value (~ -25 mV), and with a greater conductance (~ 5 nS as compared with 1·8 nS). Application of quinine (1 mM) to such cells caused the conductance to fall, and the membrane potential to become less negative. This led Capiod & Ogden (1989*a*) to suggest that the quinine-sensitive K^+ conductance associated with treatments and conditions that cause cell swelling (Bakker-Grunwald, 1983; Kristensen & Folke, 1984; Field & Jenkinson, 1987) had become activated. To study this further, we tested the effect of deliberately inducing cell swelling by the application of gentle pressure (15 cmH_2O) to the shank of the patch pipette, subsequent to the establishment of the whole-cell recording configuration. Only cells with low initial resting potentials were used, and the membrane potential was clamped to 0 mV thereafter. In twenty-seven such recordings, the application of pressure led to the loss of the gigaseal in seven instances. In each of the other cells, the seal was maintained, and an outward current slowly developed (see Fig. 1), associated with a rise in conductance. In the six most satisfactory of these experiments, the peak current averaged 602 ± 80 pA (S.E.M., $n = 6$) at the holding potential of 0 mV. As Fig. 1 also illustrates, the application of cetiedil (100 μM) greatly reduced the current, by $96{\cdot}7 \pm 1{\cdot}5$% ($n = 5$). The actions of cetiedil on guinea-pig and rat hepatocytes are described in greater detail on p. 142.

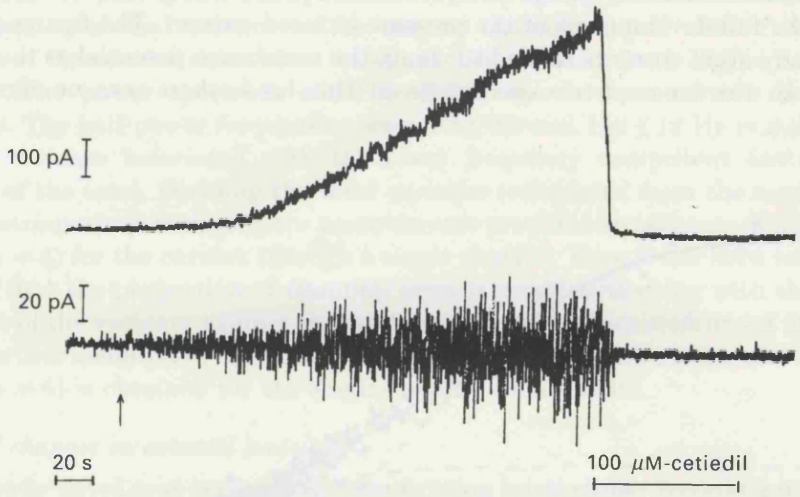

Fig. 1. Upper, current record from a patch-clamped guinea-pig hepatocyte at 37 °C. From the arrow onward, positive pressure (15 cmH_2O) was applied to the shank of the patch pipette, resulting in the slow development of outward current (upward deflection). The current was greatly reduced by the application of cetiedil (100 μM, during the horizontal bar) from a nearby puffer pipette. The membrane potential was clamped to 0 mV throughout, requiring an inward current of ~ 4 pA when whole-cell recording conditions were first established. Lower, high-gain bandpassed record (cut-offs at 0·5 Hz and 1 kHz).

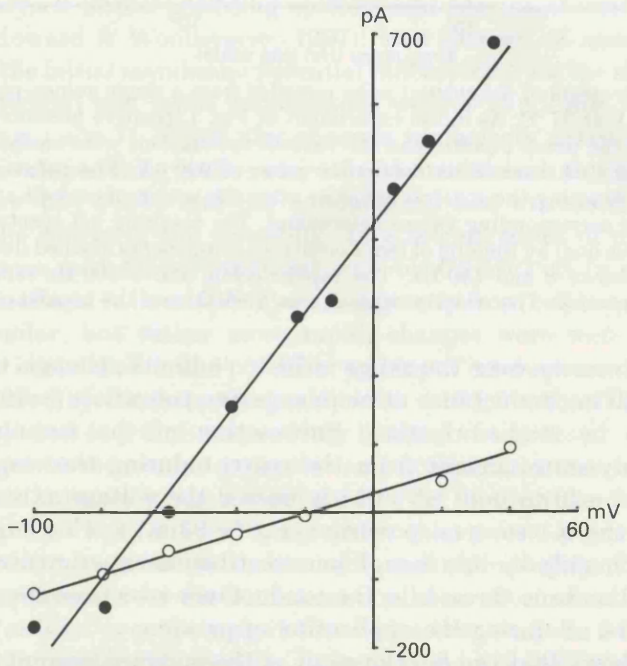

Fig. 2. Steady-state current–voltage relations for a single patch-clamped guinea-pig hepatocyte before (○) and after (●) the application of pressure to the shank of the pipette had resulted in the changes illustrated in Fig. 1.

Figure 2 shows current–voltage (I–V) relationships for a single hepatocyte before and after the full development of the pressure-induced current. The figures plotted are the steady-state currents needed to clamp the membrane potential at the values indicated. In three complete experiments of this kind, there was no significant

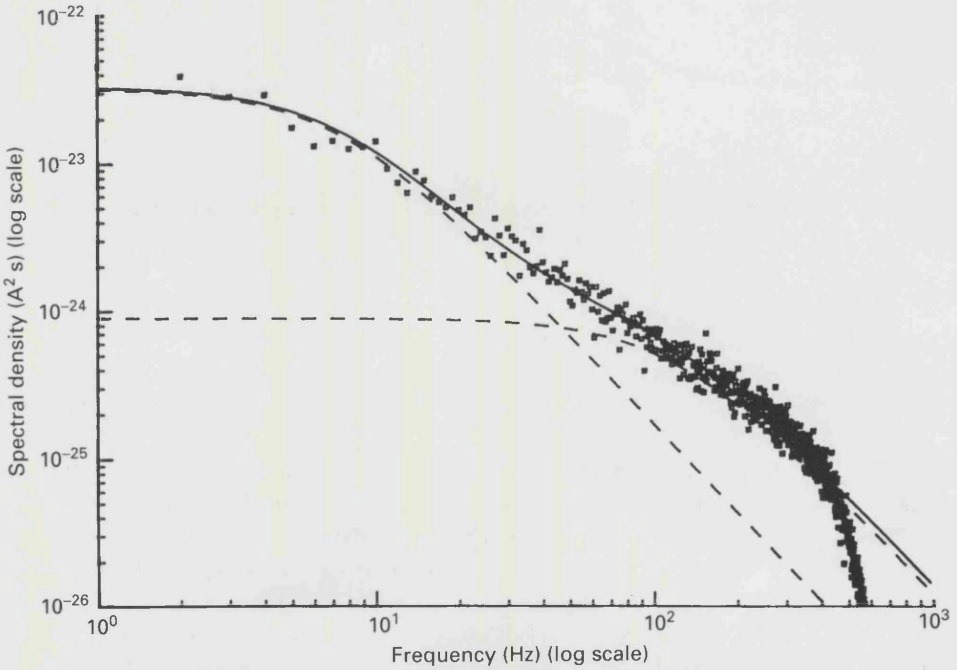

Fig. 3. Power spectrum of the current noise recorded from a single guinea-pig hepatocyte clamped to 0 mV at 37 °C. As in the experiment of Fig. 1, positive pressure was applied to the shank of the patch pipette, and the current fluctuations were analysed when the outward current had reached its maximum value of 900 pA. The points shown were obtained by subtracting the spectral densities after the action of cetiedil at 100 μM (see Fig. 1) from the corresponding values beforehand. The resulting net spectrum could be fitted (continuous line) by the sum of two Lorentzian components (dashed lines) with half-power frequencies of 8 and 140 Hz. The rapid decline above 500 Hz reflects filtering beyond this frequency. The series resistance was 5·3 MΩ, and the capacitance 34 pF.

departure from linearity over the range −70 to +40 mV, though there was some suggestion of a fall in conductance at more negative potentials (−70 to −100 mV). This remains to be studied further. Subtraction of the resting (pre-pressure application) steady-state current from the current during the response provided linear I–V curves (−70 to +40 mV) which crossed the voltage axis at -86 ± 4 mV ($n = 3$), close to the K^+ reversal potential, E_K (−93 mV). This suggests that the current is carried mainly by K^+, though ion substitution experiments are needed for confirmation. In the same three cells, the conductance rose from its resting value of $1{\cdot}3 \pm 0{\cdot}1$ to $6{\cdot}0 \pm 0{\cdot}6$ nS during the application of pressure.

Figure 1 also shows that the development of the outward current was associated with a rise in the 'noisiness' of the record, as would have been expected from an increase in the open probability of a population of ion channels (Katz & Miledi, 1972;

Anderson & Stevens, 1973). The records were analysed (as described in Methods) to give estimates of the variance as the current developed, and also of the power spectrum of the current noise when it had reached a steady level. Figure 3 shows an example. The power spectrum could be fitted by the sum of two Lorentzian functions. The half-power frequencies were $7{\cdot}2 \pm 0{\cdot}8$ and 152 ± 12 Hz (S.E.M., $n = 4$) and the variance associated with the lower frequency component amounted to 69 ± 7 % of the total. Dividing the total variance (calculated from the amplitude of the Lorentzian components) by the mean current provided an estimate of 640 ± 19 fA (S.E.M., $n = 4$) for the current through a single channel. Here it has been tentatively assumed that the proportion of channels open is small, in keeping with the finding that plots of the variance against the mean current showed no evidence of flattening. On the further assumption that all the current is carried by K^+, a value of $6{\cdot}9 \pm 0{\cdot}2$ pS (S.E.M., $n = 4$) is obtained for the single-channel conductance.

Effects of changes in external tonicity

As already noted, our earlier experiments using intracellular recording had shown that, depending on the quality of the hepatocyte preparation, the microelectrode and the impalement, a sequence of events sometimes occurred that could plausibly be attributed to cell swelling as a consequence of leakage of electrolyte from the microelectrode. In outline, the membrane potential became more negative (reaching as much as -60 to -80 mV), and at the same time the conductance increased. Quinine (200 μM) blocked the rise in conductance, and caused depolarization (Field & Jenkinson, 1987). These effects are in keeping with activation of the K^+ conductance analysed in the preceding section, and were confirmed in the present work (see also Howard & Wondergem, 1987). In a first set of measurements with guinea-pig cells, the initial membrane potential (uncorrected for the shunt introduced by the microelectrode) and input conductance were -18 ± 2 mV and $4{\cdot}1 \pm 0{\cdot}3$ nS, respectively (S.E.M., $n = 7$). When the changes tentatively attributed to volume activation had developed fully in the same cells, the membrane potential had become more negative by 40 ± 4 mV, and the conductance had increased by $6{\cdot}8 \pm 0{\cdot}6$ nS ($n = 7$) during the course of the recording. In a second series of experiments, the resting conductance was $4{\cdot}0 \pm 0{\cdot}3$ nS, and rose by $6{\cdot}3 \pm 0{\cdot}7$ nS ($n = 20$). The time required for these effects to develop fully varied greatly from cell to cell, ranging from 3 to 15 min. Similar, but rather more rapid, changes were seen with single rat hepatocytes. The initial membrane potential and input conductance (uncorrected for the shunt introduced by the microelectrode) were -18 ± 2 mV and $5{\cdot}0 \pm 0{\cdot}4$ nS, and rose by 49 ± 4 mV and 11 ± 2 nS respectively (S.E.M., $n = 5$).

If these changes in membrane potential and conductance are indeed linked to alterations in cell volume, it should be possible to mimic them by reducing the external tonicity. This was tested with rat rather than guinea-pig hepatocytes since they displayed 'volume activation' more readily and reproducibly. Preliminary tests showed that a requirement for the demonstration of an effect of external tonicity in either direction (at least over the range of tonicities that we have used) was that the cells should be only moderately swollen, as suggested by stable intermediate values of membrane potential and conductance. This could best be achieved by using a less concentrated microelectrode filling solution (500 mM- rather than the usual 1 M-

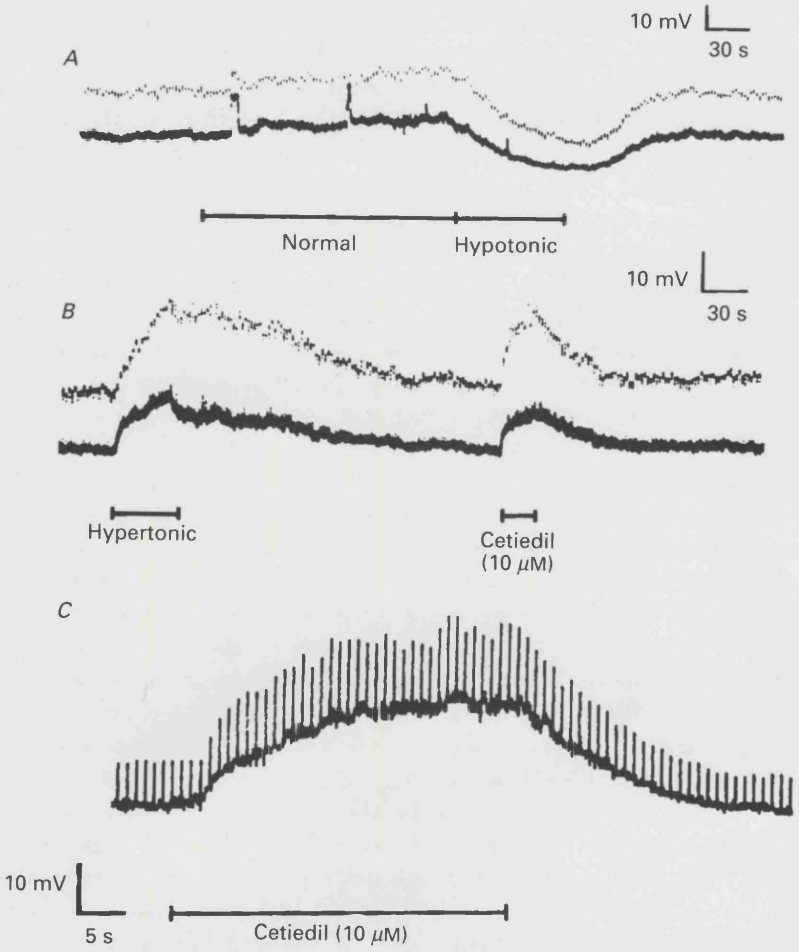

Fig. 4. The effect of changes in external tonicity and of cetiedil on the intracellularly recorded membrane potential and conductance of individual hepatocytes at 37 °C. *A*, during the horizontal bar, a rat hepatocyte was first exposed to the normal bathing solution applied by pressure ejection from a puffer pipette. This was to test for flow artifacts. A second puffer pipette was then used to apply a hypotonic solution, which caused hyperpolarization and an increase in membrane conductance, as assessed by the change in the depolarizing potentials (vertical deflections) elicited by 60 pA current pulses. The microelectrode was filled with 0·5 M-potassium citrate, and the membrane potential was −29 mV at the start of the trace. The record also shows two spontaneous transient depolarizations during the 'normal' period, one of which is clearly associated with a conductance increase. These events were seen in approximately a quarter of intracellular recordings from rat hepatocytes, and their mechanism is unknown. *B*, as *A*, but showing the depolarization and reduction in conductance caused by the application of a hypertonic solution (+80 mM-raffinose) to another hepatocyte which was already partly swollen, as assessed by its high membrane potential (−66 mV as the trace began). The electrode filling solution was 0·75 M-potassium citrate. The response to cetiedil (10 μM) is also shown, for comparison. *C*, the effect of cetiedil (10 μM) on a single guinea-pig hepatocyte with an initial membrane potential of −79 mV. 100 pA current pulses.

potassium citrate). The bathing fluid used in these experiments was also modified, by replacing 40 mM-NaCl by raffinose at 80 mM. A hypotonic bathing solution with the same electrolyte composition as before could then be prepared simply by omitting raffinose. This protocol is based on the work of Lang *et al.* (1989). Figure 4*A* illustrates the effect of applying the hypotonic solution to a single rat hepatocyte during the course of intracellular recording. The membrane potential changed from -23 to -41 mV, and the conductance increased from 3·4 to 5·0 nS. In the four cells (out of eight tested) which responded, the membrane hyperpolarized by $19 \pm 1{\cdot}9$ mV (from an initial value of $16{\cdot}8 \pm 2{\cdot}6$ mV), and the conductance rose from $2{\cdot}8 \pm 0{\cdot}1$ to $4{\cdot}9 \pm 0{\cdot}3$ nS ($P < 0{\cdot}002$, paired t test). The time needed for the response to hypotonicity to develop, and reverse, varied greatly, ranging from a few seconds to 2·5 min. We gained the impression that the relationship between the degree of swelling of the cell (as assessed from the initial potential and conductance) and the ensuing increase in K^+ permeability was highly sigmoidal, so that the failure of some cells to respond to hypotonicity may be at least partly due to variations in the position of a steep volume–conductance relation. Differences in the degree of swelling in individual hepatocytes may also have contributed. Direct measurements of cell volume will be needed to confirm this.

We also tested whether the application of a hypertonic solution (which should reduce cell volume) would reverse the K^+ conductance increase which we have ascribed to swelling as a consequence of leakage from the intracellular microelectrode. The test solution, which contained raffinose (80 mM) in addition to the normal constituents, was applied to individual cells from a puffer pipette, and the microelectrode was filled with potassium citrate at 750 mM (to induce a substantial but not maximal degree of swelling). Figure 4*B* shows an example of the response obtained: the hypertonic solution caused the expected depolarization with a concomitant decrease in conductance. In the four cells tested, each of which responded in this way, the mean depolarization was 14 ± 3 mV, from an average membrane potential of -53 ± 5 mV. The conductance decreased from $12{\cdot}4 \pm 1{\cdot}9$ to $6{\cdot}2 \pm 1{\cdot}0$ nS ($P < 0{\cdot}02$). These changes are consistent with a reduction in K^+ permeability.

Comparable records from single mouse hepatocytes have been presented by Graf *et al.* (1988), who also interpreted them in terms of changes in K^+ conductance.

Blocking agents

Though quinine blocks the volume-activated K^+ conductance in guinea-pig and rat hepatocytes (Field & Jenkinson, 1987; Howard & Wondergem, 1987; Corasanti *et al.* 1990), it lacks potency and selectivity. A more satisfactory agent is cetiedil which, as already mentioned, has been shown to inhibit the K^+ permeability changes that underlie volume regulation by several other types of cell. The actions of cetiedil were studied further by using a puffer pipette to apply known concentrations to individual hepatocytes, the membrane potential and conductance of which were monitored by intracellular recording. The microelectrodes were filled with 1 M-potassium citrate. Under these conditions, the response to cetiedil depended on the extent of volume activation. In six guinea-pig hepatocytes with low membrane potentials (-18 ± 2 mV, S.E.M.) cetiedil (10 μM) caused only a small depolarization

(1·5±0·4 mV) and no significant change in conductance (ΔG = +0·02±0·14 nS). Similar results were obtained with cetiedil at 100 μM (resting conductance before cetiedil, 3·1±0·7 nS, n = 4; in the presence of cetiedil (100 μM), 3·1±0·6 nS, n = 5). In contrast, when volume activation was well developed, as suggested by a strongly

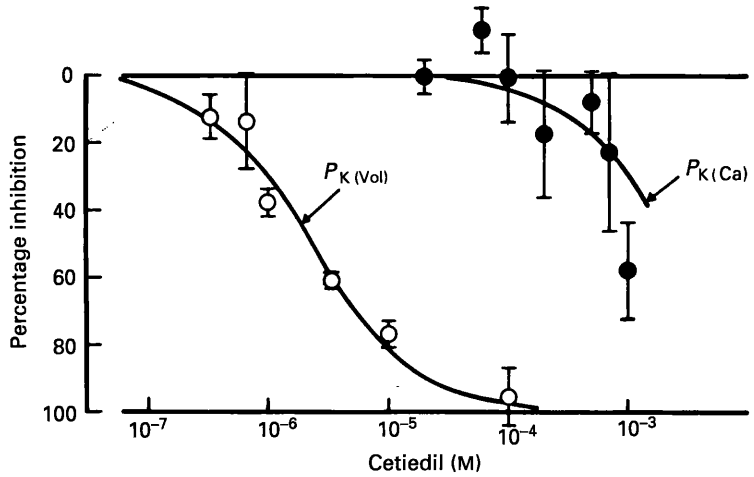

Fig. 5. Guinea-pig hepatocytes. The relationship between the concentration of cetiedil and the inhibition of (1) the additional K^+ conductance ($P_{K(Vol)}$) which we attribute to cell swelling (see text) and (2) the net loss of K^+ from the hepatocytes in response to angiotensin II (100 nM) acting via Ca^{2+}-activated K^+ channels ($P_{K(Ca)}$). Each value is the mean of three to six observations (±S.E.M.). The line drawn through the $P_{K(Vol)}$ points was fitted by a least-squares procedure using the equation

$$\text{percentage inhibition} = 100\frac{[\text{cetiedil}]}{K+[\text{cetiedil}]},$$

where the fitting constant, K (equivalent to an estimate of the concentration giving 50% inhibition) = 2·3 μM.

negative membrane potential (greater than −40 mV) and a large conductance, cetiedil now elicited a substantial depolarization and a fall in conductance (see Fig. 4*B* and *C*). These changes are in keeping with K^+ channel blockade. To examine the dose–response relationship for this action the following procedure was adopted. Guinea-pig hepatocytes were impaled with a microelectrode filled with 1 M-potassium citrate, sufficient to induce swelling in an adequate proportion of cells. If the initial impalement was satisfactory, and if over the next 5–15 min the membrane potential rose to a stable value greater than −60 mV, the actions of blocking agents such as cetiedil were tested, as in Fig. 4*C*. The reduction in conductance (ΔG) caused by the agent was expressed as a percentage of the amount by which the conductance had previously increased (ΔG_{max}) during the course of 'volume activation'. Taking Fig. 4*C* as an example, the initial conductance of the cell, once the electrode had sealed in, was 4·0 nS. This had risen to 11·1 nS by the time that cetiedil was applied, i.e. ΔG was 7·1 nS. Cetiedil reduced the conductance by 5·5 nS (ΔG), corresponding to an inhibition of 100×5·5/7·1, i.e. 77%. The concentration dependence of this action is shown in Fig. 5 which also includes for comparison the results of parallel experiments on the ability of cetiedil to inhibit the net loss of K^+ which results from the application

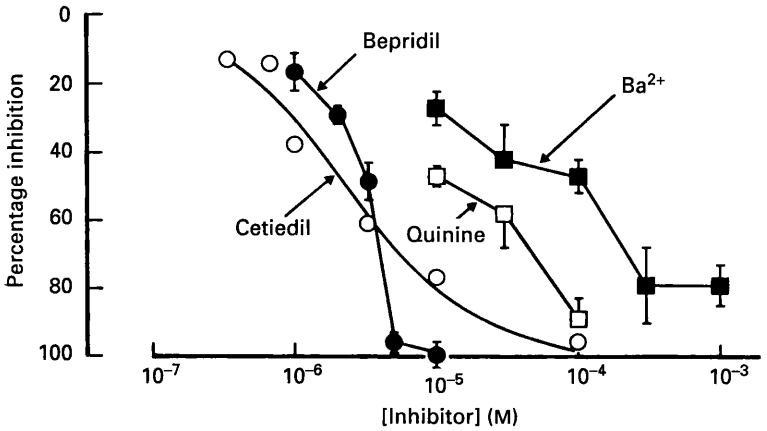

Fig. 6. Inhibition by bepridil (●), quinine (□) and Ba^{2+} (■) of the additional K^+ conductance attributable to cell swelling. The values plotted are the means ± S.E.M. of three to six observations, each on a single guinea-pig hepatocyte. For comparison, the results with cetiedil (○), and the fitted curve from Fig. 5, are also shown. Other details as in Fig. 5.

TABLE 1. The concentrations (μM ± S.D. from a least-squares fitting procedure; see legend to Fig. 5) of blocking agents needed to cause 50% inhibition of (A) the volume-sensitive K^+ conductance and (B) the release of K^+ from guinea-pig hepatocytes in response to angiotensin II

	A	B
Cetiedil	2·3 ± 0·3	~ 1000 (see Fig. 6)
Bepridil	2·7 ± 0·2	77 ± 14
Quinine	~ 12	150*
Ba^{2+}	67 ± 22	Not tested
TEA	24% inhibition at 3 mM	7900*

* From Cook & Haylett (1985).

of angiotensin II to guinea-pig liver cells. This response is the consequence of the opening of Ca^{2+}-activated K^+ channels (Weiss & Putney, 1978; Burgess *et al.* 1981), and it is clear from Fig. 5 that it is much less sensitive to cetiedil. Thus the concentration causing half-maximal inhibition of the volume-activated K^+ conductance was 2·3 ± 0·3 (S.D.) μM, as compared with ~ 1 mM for blockade of the angiotensin II response.

Rat hepatocytes were also sensitive to cetiedil. In these cells, the increase in K^+ conductance which we ascribe to swelling was reduced by 72 ± 5% (S.E.M., $n = 5$) in the presence of cetiedil at 10 μM. This is close to the value (78 ± 6%, $n = 7$) seen with guinea-pig hepatocytes under the same conditions.

We also studied the blocking activity of quinine, Ba^{2+}, TEA and bepridil (which shares structural features with cetiedil, and is also an anti-sickling agent). The results are shown in Fig. 6, and summarized in Table 1. Bepridil was the most active of this group, causing 50% inhibition at ~ 3 μM in guinea-pig hepatocytes. Like cetiedil, it was less effective in blocking the Ca^{2+}-activated K^+ permeability in the same cells.

This was assessed, as before, by measuring the reduction in the net release of K^+ initiated by angiotensin II. Bepridil was tested at concentrations of 20, 50, 100 and 200 μM, which reduced the release by 18 ± 11, 24 ± 17, 60 ± 13 and 84 ± 8% (mean $\pm$ S.E.M., $n = 3$ or 4 for each value), respectively. From these results, 50% inhibition is estimated to occur at a concentration of 77 μM.

Two additional substances tested were oligomycin A, which is as active as cetiedil in blocking the regulatory volume decrease response in human lymphocytes (Sarkadi *et al.* 1985), and oxpentifylline which inhibits the Gardos effect in red blood cells (see Discussion). Neither oxpentifylline (tested at 20 and 100 μM on three cells in each instance) nor oligomycin A at 2 μM (four cells) or 15 μM (five cells) had any effect on the volume-activated K^+ conductance in guinea-pig hepatocytes. TEA was only weakly active (3 mM causing inhibitions of 29, 19 and 25% in the three cells tested).

DISCUSSION

Our results add to the evidence that the K^+ permeability of guinea-pig and rat hepatocytes increases under conditions that induce cell swelling. In voltage-clamped cells, the response is evident as the development of a large outward current. Spectral analysis of the fluctuations in this current suggested that the K^+ channel involved has a conductance of ~ 7 pS, though this may be an underestimate because of the tacit assumption in the analysis that the open probability of the channel is very low. A value of 7 pS would imply that approximately 1000 channels have to open in individual guinea-pig hepatocytes, and 1500 in the rat, to account for the conductance increases (7 and 11 nS respectively) which developed as a consequence of the leakage of electrolyte from intracellular microelectrodes. Applying pressure to the shank of a patch pipette during the course of whole-cell recording from guinea-pig hepatocytes resulted in a comparable increase in K^+ conductance (5 nS).

The conductance does not appear to be voltage activatable, as evidenced by the linear *I–V* relationship observed both before and after volume activation (Fig. 2). This distinguishes it from the strongly voltage-dependent K^+ conductance found in avian hepatocytes maintained in cell culture for over 24 h (see Marchetti, Premont & Brown, 1988). Nor is the conductance activated by a rise in cytosolic Ca^{2+}. The main evidence for this is that the current is as readily seen in rat as in guinea-pig hepatocytes. The former lack Ca^{2+}-activated K^+ channels (Burgess *et al.* 1981; Sawanobori *et al.* 1989, though see also Bear & Petersen, 1987). Thus treatments (e.g. the application of Ca^{2+} ionophores or of Ca^{2+}-mobilizing agonists such as noradrenaline) which raise cytosolic Ca^{2+} in rat as in guinea-pig liver cells cause neither the rapid net loss of K^+ seen with other species, and which is attributable to a Ca^{2+}-activated increase in K^+ permeability (P_K), nor changes in membrane current. Lack of a role for Ca^{2+} in the control of the volume-activated P_K is also suggested by our finding that the inclusion of a high concentration (10 mM) of the rapidly acting Ca^{2+} chelator BAPTA in the pipette-filling solution did not prevent the spontaneous development of outward current during whole-cell recording in a proportion of cells. Corasanti *et al.* (1990) have also concluded that the K^+ conductance involved in volume regulation in rat liver cells is not Ca^{2+} dependent. This contrasts with the suggested role of Ca^{2+} (possibly acting via Ca^{2+}-dependent K^+

channels) in volume regulation by certain other cells e.g. those of the proximal tubules (Filipovic & Sackin, 1991; Kawahara, Ogawa & Suzuki, 1991).

The distinction between the K^+ permeabilities activated by increases in cell volume and cytosolic Ca^{2+} in guinea-pig hepatocytes is underlined by our experiments with blocking agents, in particular cetiedil. This compound inhibited the volume-activated K^+ conductance with an IC_{50} (concentration giving 50% maximal inhibition) of 2·3 μM, approximately 500-fold less than needed to block the net loss of K^+ in response to angiotensin, acting via Ca^{2+}-activated K^+ channels. Conversely, the latter are blocked by apamin at a concentration (50 nM) which is without effect on the volume-activated K^+ conductance (Kristensen & Folke, 1984; Field & Jenkinson, 1987).

Interestingly, cetiedil when applied at concentrations (10 and 100 μM) sufficient to reduce by 75 and 98% respectively the additional conductance we attribute to volume activation, had no effect on the conductance of resting (i.e. non-swollen) hepatocytes. This suggests that these channels do not contribute significantly to the resting permeability under our experimental conditions. However, it is worth noting that were cetiedil to be a state-dependent or open channel blocker (as it appears to be in lymphocytes; see e.g. DeCoursey *et al.* 1987) the amount of inhibition produced by a given concentration would be expected to depend on p_o, the proportion of channels open. (For a recent discussion of this point in relation to K^+ channels, see Davies, Spruce, Standen & Stanfield, 1989). Thus, were p_o to be initially very low (say 0·1), it is readily shown that the concentration of cetiedil needed to reduce p_o by 50% (i.e. to 0·05), would be 10 times greater than that required to cause a 50% reduction had p_o been close to unity. However, the finding that cetiedil was without effect even at a concentration 40-fold greater than its IC_{50} suggests that this was not a complicating factor. It would nevertheless be interesting to test concentrations of this order under the experimental conditions which have led to occasional reports of very negative membrane potentials (−70 to −80 mV) for rat hepatocytes (Petzinger & Bigalke, 1986; Kawanishi, Blank, Harootunian, Smith & Tsien, 1989). It is possible that these values reflect an unusually high K^+ permeability subsequent to cell swelling as a consequence of the use of intracellular microelectrodes filled with 4 M-potassium acetate. This filling solution may also have resulted in some intracellular alkalinization which is now known to increase the K^+ conductance of rat hepatocytes (Fitz, Trouillot & Scharschmidt, 1989). The relationship between this pH-sensitive conductance and the one we have studied is unclear as yet. Our finding that the cetiedil-sensitive outward current can be recorded using the whole-cell patch clamp technique with a HEPES-buffered solution in the pipette (see Fig. 1) strongly suggests that under these conditions this current is not an indirect consequence of a rise in intracellular pH.

Though bepridil is almost as active as cetiedil when IC_{50}s are compared, the relation between its concentration and the inhibition of the K^+ conductance is much steeper than for cetiedil (Fig. 6). This suggests a more complex action. It is also less selective than cetiedil, in terms of the relative concentrations needed to block the volume- and Ca^{2+}-activated K^+ conductances in guinea-pig hepatocytes (see Table 1). Cetiedil is therefore likely to be more useful as a reference compound, and a good deal of information is already available from studies of its effects on K^+ movements

in lymphocytes and red blood cells. The inhibitory effect of cetiedil on volume regulation in human lymphocytes exposed to hypotonic solutions has been studied in detail by Sarkadi *et al.* (1985) who reported an IC_{50} of $\sim 2\ \mu M$. While this is very similar to the value (2·3 μM) in hepatocytes, it would be premature to conclude that the channels involved are the same. In the first place, the evidence so far available (see Grinstein & Foskett, 1990, for a review) suggests that in lymphocytes, cetiedil is likely to act on a K^+ channel that is voltage sensitive, in contrast to that in liver cells. Second, the regulatory decrease in cell volume which follows the application of hypotonic solutions to lymphocytes, and which is at least partly attributable to the opening of a K^+ channel, is strikingly inhibited by oligomycin A (IC_{50}, 0·5 μM; Sarkadi *et al.* 1985) as well as by cetiedil, whereas we found oligomycin A to be ineffective in liver cells.

The action of cetiedil has also been examined in red blood cells, where it blocks the increase in K^+ permeability and the resulting net loss of K^+ that follow a rise in cytosolic Ca^{2+} (the Gardos effect): see e.g. Christophersen & Vestergaard-Bogind (1985). Oxpentifylline is also active in this regard (e.g. Bilto, Player, West, Ellory & Stuart, 1987), which led us to test its effect on the volume-activated K^+ conductance in hepatocytes. However, no block was seen at concentrations that have been reported to substantially inhibit the Gardos effect in red blood cells. The indications are, then, that the volume-activated K^+ channel in liver cells differs from the cetiedil-sensitive channels in lymphocytes and red cells.

Other agents tested included quinine and Ba^{2+}. Both were effective, with IC_{50}s of ~ 12 and 70 μM, respectively. Though quinine also blocks the apamin-sensitive $P_{K(Ca)}$ channels present in the liver cells of most species, much higher concentrations are needed (IC_{50}, 150 μM; Cook & Haylett, 1985). This adds to the evidence that the channels are different.

Several questions remain for further study. Perhaps the most important is the directness of the link between cell swelling and the increase in K^+ permeability that follows. The simplest possibility is that the channel opens as a direct consequence of stretching of the membrane. It is most interesting in this context that Bear (1990) has reported that rat liver cells possess a 16 pS channel which is activated by membrane stretch. However, this channel differs in an important respect from the one we have studied in that it discriminates poorly between Na^+, K^+ and Ca^{2+}, with a reversal potential which would appear to be in the order of 0 mV. Bear has suggested that the influx of Ca^{2+} that will result from the opening of this channel may trigger changes in ion conductance or in cytosolic components involved in cell volume regulation. A stretch-activated cation channel, with a similar conductance, selectivity and possible function, has more recently been described in *Necturus* proximal tubules (Filipovic & Sackin, 1991).

A second type of stretch-activated channel, highly selective for K^+, is also present in *Necturus* proximal tubules (Sackin, 1989; Filipovic & Sackin, 1991). Though its conductance (~ 40 pS) is considerably larger than the value suggested by our spectral analysis, the uncertainties in the latter are probably too great to allow it to be concluded from this evidence alone that the channels are different. Single-channel recordings are needed, and these should also allow us to distinguish between various models for channel activation. Our observation that two Lorentzian functions are

required to fit the power spectrum of whole-cell current fluctuations could be explained if the channel existed in at least one open and two closed states, as found by Sackin for the stretch-activated K^+ channel in *Necturus* kidney and by Capiod & Ogden (1989*b*) for the apamin-sensitive $P_{K(Ca)}$ channel in hepatocytes.

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