CHLORIDE CHANNELS IN EPITHELIA

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ABSTRACT

The outwardly rectifying chloride channel is found in most vertebrate cells but its physiological role is uncertain. Patch clamp, short-circuit current, and electronic cell sizing techniques were used to investigate the role of the outward rectifier in transepithelial chloride secretion and cell volume regulation, the two main functions that have been proposed for this channel in epithelia. Patch clamp studies of the human cell lines PANC-1 and T_{84} showed that the chloride channel blockers DIDS, IAA-94 and NPPB decrease the open probability of the outward rectifier with maximal inhibition at 15 μM and 23 μM, respectively. At these concentrations the blockers did not affect cAMP-induced short-circuit current. They did inhibit the regulatory volume decrease (RVD) which occurs after hypotonic cell swelling, but only at much higher concentrations. Moreover, the commonly-used inhibitor DIDS, which blocks the outward rectifier in the 10-20 μM range, had no effect on the RVD when tested at 100 μM. The results indicate that the outwardly rectifying Cl channel does not mediate a significant fraction of transepithelial Cl secretion across T_{84} cells. Although the data do not exclude a role for the outward rectifier in cell volume regulation, the selectivity and pharmacological properties of the swelling-induced anion conductance in T_{84} cells is more similar to the ClC-2 channel than to the outward rectifier.

RESUME

Le canal chlore rectifiant sortant se trouve dans la plupart des cellules des vertébrés pourtant son rôle est encore imprécis. Les techniques de «patch clamp», de courant de court circuit, et des mesures électroniques du volume cellulaire ont été utilisés pour examiner le rôle de ce canal dans la sécretion de chlore et dans la régulation du volume cellulaire, deux fonctions principales qui sont proposées pour ce canal. Les études de «patch-clamp» des cellules épithéliales humaines PANC-1 et T_{84} indiquent que les inhibiteurs des canaux chlores, IAA-94 et NPPB diminuent la probabilité d'ouverture du canal, avec une inhibition demi-maximale de 15 µM et 23 µM, respectivement. A ces concentrations, les inhibiteurs n'affectent pas le courant de court circuit. Ils bloquent la diminution du volume après un gonflement hypotonique, mais les concentrations requisent sont plus élevées que celles qui affectent le canal. De plus, l'inhibiteur fréquemment utilisé DIDS, qui bloque le canal avec un concentration de 10-20 µM, n'a aucun effet sur la diminution du volume à 100 µM. Ces résultats indiquent que ce canal n'a pas de rôle significatif dans les mécanismes de sécrétions transépithéliales de chlore dans les cellules T_{84}. Bien que les résultats n'excluent pas un rôle du canal chlore rectifiant sortant dans la régulation du volume cellulaire, la sélectivité et les caractéristiques pharmacologiques de la conductance anionique induite par le gonflement des cellules T_{84} ressemblent plus au canal ubiquitaire, ClC-2 qu'au canal rectifant sortant.

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I INTRODUCTION

1 Chloride channels

Chloride conductances are thought to play essential roles in regulating membrane excitability, fluid secretion, cell volume regulation, and other cellular processes. For example, in neurons, the inhibitory transmitters GABA (g-amino-n-butyric acid) and glycine increase Cl conductance, which hyperpolarizes the membrane and inhibits post synaptic potentials (Bormann et al., 1987). In skeletal muscle, Cl conductance helps to restore the resting potential of the T-tubule membrane, thereby allowing the muscle to relax after a train of action potentials. Autosomal recessive generalized myotonia (Becker's disease) and myotonia congenita, a genetic disease in goats and man that causes muscle tetany, stiffness, and hyperexcitability, both result from a defect in this Cl conductance (Lipicky et al., 1971; Koch et al., 1992). In cardiac muscle, Cl conductances activated by calcium and cAMP help determine the shape and duration of the action potential and are important for normal electrical function (Bretag, 1987). They have also been proposed to regulate the frequency of spontaneous beating of Purkinje cells and papillary muscles by carrying part of the depolarizing current during the pacemaker potential. Recently, a cAMP-activated Cl channel has been identified in hamster ventricular cells that is regulated by ß adrenergic agonists and appears to be identical to the cystic fibrosis transmembrane conductance regulator (CFTR; Ehara and Ishihara, 1990; Tareen et al., 1991). A cell volume-sensitive Cl channel in heart cells has also been identified (Tseng, 1992). In epithelia, Cl conductances regulate cell volume and mediate chloride secretion, and thus are essential for the general regulation of ion transport processes and preservation of electrolyte balance (reviewed by Dawson, 1991).

With the exception of GABA and glycine receptors, much less is known about the properties of Cl channels compared to sodium, potassium, and calcium channels.

Nevertheless, several Cl channel types have been studied at the single channel level and can be categorized by their biophysical and pharmacological properties. GABA and glycine receptor channels are members of a superfamily of ligand-gated channels that are present in a wide variety of excitable tissues (Bretag, 1987). The ClC family of Cl channels includes the voltage-gated chloride channel which was first observed in membranes from the electroplax of the electric marine ray *Torpedo californica* (White and Miller, 1981). The primary structure of this channel was recently deduced by expression cloning (Jentsch et al., 1990, Bauer et al., 1991), its cDNA has been used to identify homologous channels in mammalian cells. One of these homologues mediates Cl conductance in mammalian skeletal muscle and has been named ClC-1. The other (ClC-2) is ubiquitously expressed (Thiemann et al., 1992) and is believed to play a role in cell volume regulation (Gründer et al., 1992). The product of the cystic fibrosis gene, named CFTR (cystic fibrosis transmembrane conductance regulator) is a low-conductance chloride channel in epithelial cells which is regulated by phosphorylation and ATP (reviewed by Hanrahan et al., 1993). Other plasma membrane Cl channels which have been studied physiologically in some detail but have not yet been cloned include those activated by calcium in *Xenopus laevis* oocytes (Barish, 1983), rat lacrimal glands (Evans and Marty, 1986) and neurons (Mayer et al., 1990). Finally, the voltage-dependent anion channel (VDAC) from the mitochondrial outer membrane has been studied electrophysiologically and its gene sequence is known (Colombini, 1980; Mihara and Sato, 1985) however it is not discussed further here because it does not function in the plasma membrane. Other Cl channels are present in intracellular membranes and are essential for controlling the pH of lysosomes and endosomes (Bae et al., 1990); however, little is known concerning their single channel properties, and their genes have not yet been identified.

2 Epithelial Cl channels

Many distinct Cl channel types have been described in epithelia where they are implicated in the regulation of cell volume and electrolyte transport. This section reviews the literature concerning the four major Cl channels that have been identified in mammalian intestinal, airway, and kidney epithelial cells, and in model epithelia from other species such as the frog skin and shark rectal gland. However, the specific role each channel plays in regulating ion transport processes in most tissues remains controversial.

Maxi-Cl channels

Maxi-Cl channels are named for their high conductance (>200 pS) when bathed in symmetrical 150 mM NaCl solutions. They were the first Cl selective channels to be described at the single channel level in epithelial cells (Nelson et al., 1984) and have been identified in several systems. These channels are strongly voltage dependent; i.e., they are active at voltages near 0 mV but inactivate at voltages outside of ±20 mV (Blatz and Magleby, 1983; Gray et al., 1984). In most preparations maxi-Cl channels are only active after patches have been excised from the cell and following large positive or negative steps (Blatz and Magleby, 1983; Gray et al., 1984; Hanrahan et al., 1985).

The physiological role of maxi-Cl channels remains obscure although many possibilities have been suggested. It has been proposed that they may be connexins, forming part of the gap junctional complexes that normally mediate cell-to-cell communication (Blatz and Magleby, 1983; Gray et al., 1984; Woll and Neumcke, 1987), or that they may simply be "background channels" responsible for resting Cl conductance in intact cells (Blatz and Magleby, 1983). Maxi-Cl channels have been proposed to mediate cell volume regulation (Falke and Misler, 1989), and to cause the membrane depolarization that accompanies fertilization in *Xenopus* oocytes (Young et al., 1984). As mentioned above, a maxi-Cl channel has been identified in the apical membrane of the A6 epithelial cell line from

Xenopus kidney where it has a unit conductance of 360 ± 45 pS in 105 mM NaCl solution, however its contribution to transepithelial NaCl transport is unknown (Nelson et al., 1984). A similar voltage-sensitive, high-conductance (350-400 pS) anion channel has been identified in alveolar (type II) cells from adult rat lung, where it might play a role in salt absorption by the alveolar epithelium (Schneider et al., 1985). Because of its low selectivity and the fact that it is usually observed in excised patches, it could also be a denatured form of some other, less conspicuous, Cl channel (Hanrahan et al., 1985).

ClC family of Cl channels

This family of recently-cloned Cl channels has three members named ClC-0, ClC-1, and ClC-2. ClC-0 is the voltage-gated Cl channel from *Torpedo* electric organ (Jentsch et al., 1990). The primary structure of this channel was deduced by Jentsch et al. (1990) by injecting mRNA from the *Torpedo* electroplax into *Xenopus* oocytes. The predicted protein has 805 amino acids containing 12 or 13 membrane spanning regions. Expression of this protein in *Xenopus* oocytes showed that this protein is sufficient for channel function (Jentsch et al., 1990), and the channels expressed on oocytes have voltage-dependent gating that is identical to that observed when crude membranes from electroplax cells are fused with planar lipid bilayers (White and Miller, 1981). The biophysical properties of this channel have been characterized extensively using this bilayer preparation (White and Miller, 1981; Miller, 1982; Miller and White, 1984) and using giant liposomes (Tank et al., 1982). It has two types of voltage-dependent gating: one which is slow (time scale of seconds) and the other fast (time scale of milliseconds). The channel might actually be two co-channels because it appears to function as independent subunits once a common gate has opened. It is present at very high density on one side of the electroplax cell, which allows stacks of these cells to act as a "low internal resistance battery" that can be used by the *Torpedo* to stun its prey (Miller and White, 1980; White and Miller, 1979).

The second channel in this family, ClC-1, was cloned by homology to ClC-0 and is present mainly in mammalian skeletal muscle (Steinmeyer et al., 1992). When expressed in oocytes, ClC-1 produces a Cl conductance indistinguishable from that described previously in vertebrate muscle (Bauer et al., 1991). Recessive myotonia in some inbred strains of mice is caused by a large transposon insertion in the ClC-1 channel gene, and in humans recessive myotonia is caused by a point mutation in the eighth predicted transmembrane region (Steinmeyer et al., 1992; Koch et al., 1992). More recently, another member of this family, ClC-2, has been identified in many tissues (rat heart, brain, lung, pancreas, and liver) and in cell lines of fibroblastic, neuronal, and epithelial origin (Thiemann et al., 1992). When overexpressed in *Xenopus* oocytes, ClC-2 was found to be activated when the oocytes were exposed to hypotonic medium. More recently, the volume-sensing region of ClC-2 has been identified near the amino terminus (Gründer et al., 1992). Interestingly, transplantation of the volume sensor to other locations in the protein does not adversely affect the responsiveness of ClC-2 to hypotonic swelling (T. Jentsch, pers. commun.).

Outwardly rectifying Cl channel

This channel has a characteristic rectifying current-voltage (I/V) relationship, with conductance ranging from ~10 pS at -100 mV to 100 pS at +100 mV. The outward rectifier has been studied in intestinal cells (HT_{29}; Hayslett et al., 1987; T_{84}; Tabcharani et al., 1989; Halm et al., 1988, *Necturus* small intestine; Giraldez et al., 1988), pancreatic cells (human fetal pancreas; Gray et al., 1989; PANC-1; Tabcharani et al., 1989; CF-PAC; Schoumacher et al., 1990), airway cells (Frizzell et al., 1986; Welsh and Leidtke, 1986), sweat gland and nasal polyps (Bijman et al., 1988), placental trophoblasts (Davis et al., 1988), and colonic membranes incorporated into planar lipid bilayers (Reinhardt et al., 1987). The outward rectifiers in T_{84} cells are indistinguishable from those in PANC-1 and sweat gland cells with respect to their I/V relationship, selectivity sequence, and gating

behaviour. The outward rectifier was the first channel implicated in cystic fibrosis (CF, Schoumacher et al., 1987; Li et al., 1988), however its role in secretion remains highly controversial (Tabcharani et al., 1991; Egan et al., 1992). There is also evidence that it functions in cell volume regulation (Worrell et al., 1989; Solc and Wine, 1991).

The most commonly observed outward rectifier has a conductance of 46 pS near the reversal potential when recorded at 20°C in symmetrical 154 mM Cl (Hanrahan and Tabcharani, 1990) although reported conductances range from 25 pS (Welsh, 1986) to 50 pS (Frizzell et al., 1986). There is general agreement regarding its single channel characteristics and its activation by non-physiological stimuli, such as depolarizing voltage, elevated temperature, protease, hypotonicity (Schoumacher et al., 1987; Halm et al., 1988; Hwang et al., 1989; Li et al., 1989; Welsh et al., 1989; Worrell et al., 1989; Egan et al., 1992) and chaotropic solutions (Tabcharani and Hanrahan, 1991a) but disagreement concerning its regulation by physiological stimuli. When the groups of Welsh and Frizzell originally identified the outwardly rectifying Cl channel on airway cell primary cultures (Frizzell et al., 1986; Welsh, 1986) they reported that its activity was increased by cAMP. Secretagogues activated outward rectifiers in cell attached patches on normal cells, but failed to activate those on CF cells. Similar results were described by Hwang et al.(1989) and Jetten et al. (1989), but a number of laboratories have been unable to reproduce the results. Nevertheless, recent papers from two laboratories still maintain that the outward rectifier is defectively regulated in CF (Egan et al., 1992; Gabriel et al., 1993).

Worrell et al. (1989) have identified a volume-sensitive Cl conductance in whole cell patch clamp studies of the T_{84} cell line. The swelling activated whole cell currents were outwardly rectified, Cl selective, activated at hyperpolarizing potentials, and showed time- and voltage-dependent inactivation at large depolarizing potentials. These properties parallel those of the outward rectifier, however single channel events that were resolved in

some whole cell recordings had a conductance of ~75 pS, approximately twice that reported for the outward rectifier in excised patches. Outward rectifiers have also been identified in isolated frog skin epithelial cells (Garcia-Diaz, 1991) and human nasal epithelial cells (Grygorczyk, 1991), where they were proposed to be involved in regulating cell volume.

In addition to the swelling-induced conductance, Cliff and Frizzell (1990) have also identified two distinct Cl conductances in T_{84} cells which are activated by second messengers. One of these is activated by Ca and has a linear instantaneous I/V curve, however the steady-state I/V curve rectifies in the outward direction due to activation by positive voltage. The other conductance is activated by cAMP and has an ohmic current-voltage relationship over the range ±100 mV. The cAMP-activated conductance is mediated by the low conductance Cl channel (see below).

Low-conductance Cl channel (CFTR)

Channels with low unitary conductance have been identified in several epithelia and have been implicated in cAMP-stimulated secretion in several tissues. Gray et al. (1989) first described the low conductance channel (4-7 pS in cell-attached patches) on pancreatic duct cells, and found that it was stimulated by secretin, dbcAMP, or forskolin. It was proposed to function in parallel with Cl^-/HCO_3^- exchange, mediating Cl "recycling" at the apical membrane during electrogenic bicarbonate secretion, a model proposed previously for the turtle urinary bladder (Stetson et al., 1985).

A similar low conductance Cl channel has also been described in the porcine thyroid gland (Champigny et al., 1990). The channel in thyroid cells had a linear current-voltage relationship, single channel conductance of 5.5 pS in cell-attached patches, and was stimulated by 8-chloro-cAMP. Its physiological role in thyroid cells is uncertain but might involve thyrotrophin-stimulated fluid transport, which is presumably regulated by cAMP.

Finally, a low-conductance Cl channel has been characterized in the apical membrane of chloride secreting T_{84} cells (Tabcharani et al., 1990). It had an ohmic current-voltage relationship and a conductance of 8.7 pS at 37 ^{o}C. Although activated by cAMP in cell-attached patches, the activity of the low-conductance channel disappeared when patches were excised. The conductance that is generated by expression of the cystic fibrosis gene product, CFTR (cystic fibrosis transmembrane conductance regulator) is mediated by an ohmic, low-conductance Cl channel identical to the low conductance channels described above in human fetal pancreatic duct, thyroid, and T_{84} cells (Kartner et al., 1991), therefore it was proposed that CFTR is itself the low-conductance Cl channel. Recent reconstitution studies have confirmed that CFTR is indeed the low-conductance Cl channel (Bear et al., 1992; Tilly et al., 1992). As shown in this thesis, the pharmacological properties of this low-conductance Cl channel match those of the cAMP stimulated transepithelial current, providing evidence for its role in Cl secretion.

3 Epithelial Cl channels in disease

Abnormal Cl conductance in epithelial cells appears to be the basis of electrolyte transport diseases including cystic fibrosis, cholera, and secretory diarrhea.

Cystic Fibrosis

Cystic fibrosis (CF), the most common fatal genetic disease in the US, affects one out of about every 1800 Caucasians. It is an autosomal recessive disorder characterized by defective ion transport across epithelia, which usually leads to pancreatic and pulmonary insufficiency. CF affects mostly exocrine tissues in the airways, pancreas, sweat glands, liver, intestine, and genital tract (Quinton, 1990). Patients are more susceptible to recurrent pulmonary infections, which in turn cause chronic tissue inflammation and secondary complications. Deterioration of lung function causes most morbidity in CF, however the pancreas also deteriorates markedly in about 85% of all CF patients; the ducts, which are obstructed by mucous, eventually rupture and release their enzymes into the parenchyma, causing atrophy and fibrosis of the pancreas. The sweat gland also malfunctions in CF, and an elevated level of salt in the sweat is the standard diagnostic test of CF. However, malabsorption of sweat in CF is only pathogenic under severe conditions such as heat waves, when excessive salt losses lead to heat prostration.

The ion transport defect in CF airway epithelia involves an increase in baseline Na reabsorption and a decrease in adrenergic-stimulated Cl secretion, however the Cl transport defect has received more attention. The gene that is mutated in CF was identified in 1989 (Rommens et al., 1989; Kerem et al., 1989; Riordan et al., 1989). As mentioned above, its protein product was named CFTR for cystic fibrosis transmembrane conductance regulator. When CFTR was transiently expressed in Hela cells using a Vaccinia/T7 system (Anderson et al., 1991), baculovirus-infected insect (Sf9) cells (Kartner et al., 1991), or stably expressed in Chinese hamster ovary cells (Tabcharani et al., 1991a), it conferred on

these cells a new cAMP-stimulated anion permeability that was not present before transfection. As mentioned above the channel that underlies this conductance was found to be an ohmic, low-conductance Cl channel (Kartner et al., 1991) identical to the one characterized previously in human fetal pancreatic duct (Gray et al., 1989) and T_{84} cells (Tabcharani et al., 1990). The fact that an identical channel is generated by CFTR expression in many different systems that normally do not have cAMP-stimulated Cl permeability suggested that the gene product itself is the low-conductance Cl channel. This hypothesis was strengthened by mutagenesis studies (Anderson et al., 1991) and proven by reconstituting pure protein into planar lipid bilayers (Bear et al., 1992; Tilly et al., 1992).

Cholera and secretory diarrhea

Cholera has been prevalent since ancient times and remains a major cause of death in developing countries. It is caused by a highly infectious, toxin-producing strain of bacteria (*Vibrio cholerae*) which causes severe diarrhea, dehydration, and circulatory collapse.

The *V. cholerae* adhere to and multiply on the surface of the small intestine where they produce cholera enterotoxin, a protein consisting of six subunits. One enterotoxin subunit binds the toxin to the surface membrane of the intestinal cells while another penetrates the lipid bilayer. Cholera toxin elevates adenylyl cyclase activity through its effect on the stimulatory G protein, G_S. This increases intracellular cAMP levels, inhibits NaCl absorption, and stimulates Cl secretion by hyperstimulating apical Cl channels. The combined effect is to dramatically increase net salt and fluid efflux into the lumen (Morris, 1986). Thus cholera and secretory diarrhea result from excess apical Cl conductance in epithelia, in contrast to cystic fibrosis, which results from insufficient apical Cl conductance. Interestingly, an increased resistance of CF heterozygotes to cholera in medieval Europe (500 to 1500 A.D.) has been proposed as an explanation for the high

incidence of CF in Caucasian populations (Romeo et al., 1989). It has been proposed that carriers of a defective CFTR gene have a selective advantage because they would have less cAMP-stimulated conductance and therefore less diarrhea (Romeo et al., 1989; Rodman and Zamudio, 1991).

4 Pharmacology of Cl channels

Channels can be characterized according to their biophysical properties (eg. voltage dependence, conductance, selectivity, and kinetics), their regulation by agonists and second messengers, or by their sensitivity to pharmacological agents. Understanding the pharmacology of single chloride channels can be useful when identifying their physiological role and may give insights into basic mechanisms of channel function. For example, the combination of mutagenesis and pharmacological studies can be used to identify regions of the protein that line the pore (Anderson et al., 1991; Tabcharani et al., in press). Several inhibitors of epithelial chloride conductance have been reported.

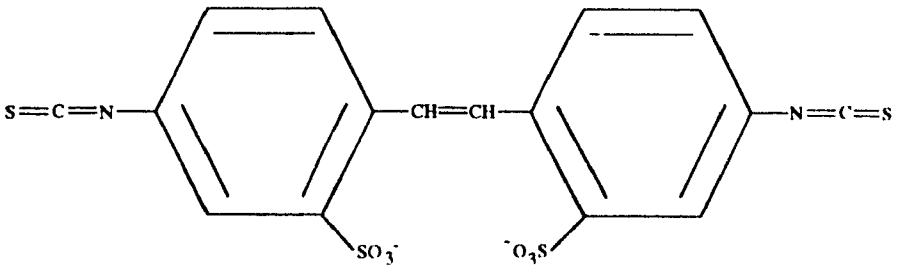
Stilbene isothiocyanates are a family of compounds that inhibit anion transporters. DIDS (4,4'-diisothiocyanostilbene-2,2' disulfonic acid, Fig. 1a) is an irreversible inhibitor of band 3-mediated anion exchange in red blood cells (Cabantchik et al., 1978), but it has also been shown to block Cl channels in the electric organ of *Torpedo californica* (White and Miller, 1979), A6 epithelial cells (Nelson et al., 1984), rabbit urinary bladder (Hanrahan et al., 1985), squid axon membrane (Inoue, 1985), neoplastic B lymphocytes (Bosma, 1986), and thick ascending limb of Henle's loop (Wangemann et al., 1986). Inhibition of Cl channels requires higher concentrations of DIDS than does inhibition of red cell anion exchangers ($\geq$10 μM vs <0.2 μM). Substitution of the isothiocyano group on DIDS with non-reactive substituents changes it into DNDS (4,4,-dinitrostilbene-2,2-disulfonic acid), which reversibly blocks the outward rectifier after its incorporation into planar bilayers (Bridges et al., 1989).

A second major family of Cl channel inhibitors is based on anthranilic acid. It includes anthracene-9-carboxylate (9-AC) and its derivatives diphenylamine-2-carboxylate (DPC), and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, Fig. 1b). 9-AC was found to inhibit Cl channels in skeletal muscle (Bryant and Morales-Aguilera, 1971; Palade and

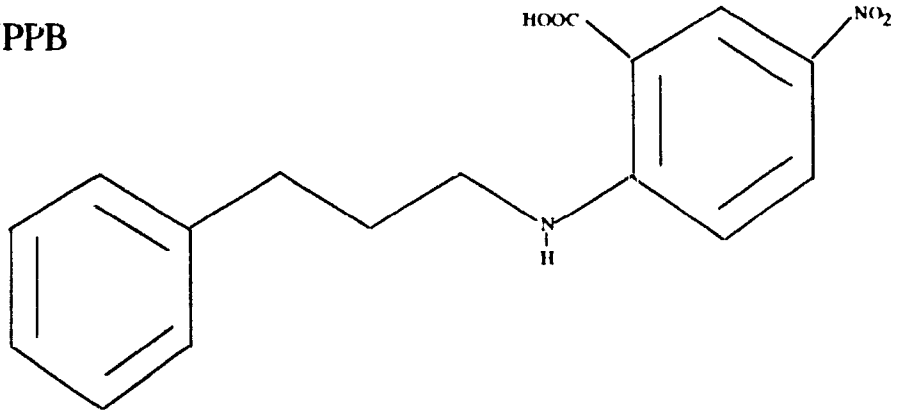
Barchi, 1977) and in the diluting segment of amphibian nephrons (Oberleithner et al., 1983). DPC inhibits Cl conductance in the red blood cell (Cousin and Motais, 1982) and the basolateral membrane of renal tubules (Distefano et al., 1985). Of the 200 related compounds that have been assayed on isolated rabbit nephrons, NPPB was found to be the most potent anthranilic derivative (IC_{50} = 80 nM; Wangemann, 1986). NPPB also blocks Cl channels in HT_{29} colon carcinoma cells (Dreinhöfer et al., 1988), rectal gland of the dogfish (Greger et al., 1987), airway epithelial cells (Kunzelmann et al., 1989), and bullfrog cornea (Reinach and Schoen, 1990), although with rather low and variable affinity in different preparations.

The third group of Cl channel inhibitors are the indanyloxy alkonoic acids, which were first developed to block brain tissue swelling after cranial injury (Cragoe et al., 1982). The most potent of these is IAA-94 (2-[cyclopentyl-6,7-dichloro-2,3 dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy]acetic acid or indanyl oxyacetic acid-94, Fig. 1c), which inhibits Cl conductance in bovine renal cortex and tracheal membranes (K_i of 1-2 μM) and has been used as a high-affinity ligand to isolate putative Cl channel proteins from tracheal and renal cortical membranes (Landry et al., 1987). Some of the compounds since have been shown to block renal Cl transport in perfused tubules (reviewed by Greger, 1990).

A: DIDS



B: NPPB



C: IAA-94

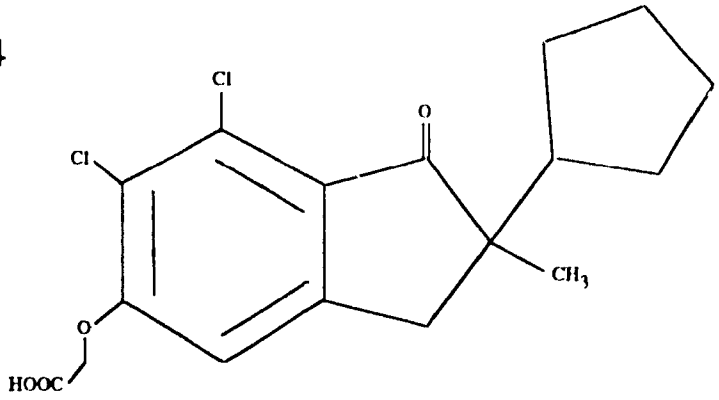


Figure 1: Chemical structures of Cl channel inhibitors used in this study

A: DIDS (4,4'diisothiocyanostilbene-2,2' disulfonic acid)
B: NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid)
C: IAA-94 (indanyl oxyacetic acid-94)

5 General mechanisms proposed for transepithelial secretion and cell volume regulation

Transepithelial secretion

Apical membrane chloride conductance plays an important role in secretion across most epithelial cell layers. Salt and water secretion by epithelia of the colon, airways, sweat gland, and pancreatic duct is driven by secondary active Cl transport. In transepithelial secretion, the NaKATPase in the basolateral membrane maintains intracellular Na concentration low, and Cl enters from the basolateral side via an electroneutral $Na^+/K^+/2Cl^-$ co-transporter. Apical Cl conductance is the rate limiting step during transcellular transport, and is controlled by the second messengers cAMP and calcium (Liedtke, 1989). Under resting conditions, apical and basolateral membranes both have relatively low permeability to Cl; however, apical Cl conductance is selectively increased during secretagogue stimulation and Cl moves down its electrochemical gradient to the lumen. Sodium ions follow Cl by diffusing paracellularly through the tight junctions and lateral intercellular spaces (see Fig. 2 and review by Dawson, 1991).

Cell volume regulation

The maintenance of cell volume is a physiological problem faced by virtually all cells because they contain charged macromolecules in their cytoplasm and therefore need some mechanism to prevent colloid osmotic swelling. The mechanisms used to reduce volume include the classical Na^+/K^+ pump, and K, Cl, and mino acid efflux pathways. Three factors are important when considering cell volume (Siebens and Kregenow, 1985; Hoffmann, 1987). First, water is usually near thermodynamic equilibrium across cell membranes therefore solute content is the main determinant of cell volume. Second, electroneutrality must be maintained on both sides of the membrane and may influence the

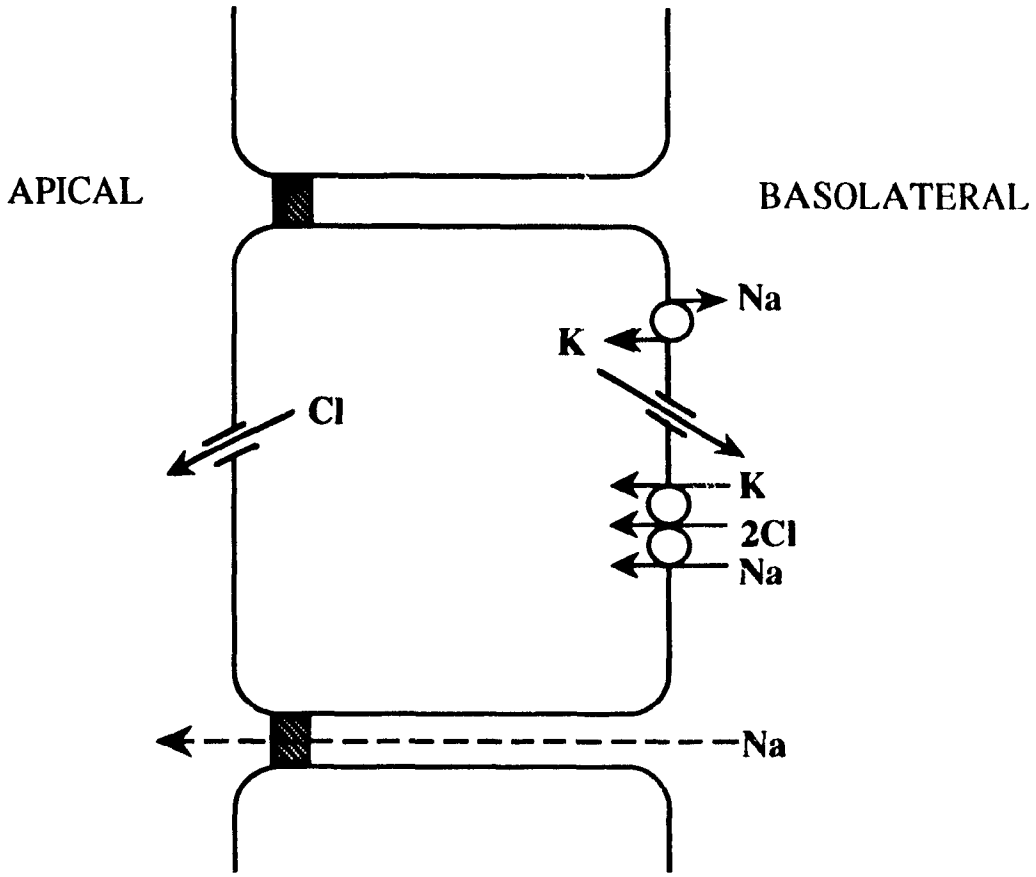


Figure 2: Model for active Cl secretion.

Transcellular transport requires apical Cl exit and outward basolateral K flow. The K that enters on Na/K/2Cl co-transporter and NaKATPase pump is recycled through K channels in the basolateral membrane. Cl leaves through the apical membrane down its net electrochemical gradient.

concentration of diffusible ions. Third, cell membranes cannot withstand large hydrostatic pressure gradients.

The initial change in cell volume (shrinkage or swelling) is usually due to the passive flow of water through the lipid bilayer and, in some cells, through specialized proteins that form water channels through the membrane. However the subsequent return of cells to their original volume in anisotonic medium requires activation of solute transporters, which vary among different cell types. The volume regulatory responses can be summarized as follows:

1. Regulatory Volume Increase (RVI)

Upon exposure to hyperosmotic solutions, there is an immediate loss of cell water and volume regulatory mechanisms are activated that mediate the net uptake of osmolytes and generate an influx of water, allowing the cell to return towards its initial volume (Fig. 3). One transport pathway involved in the regulatory volume increase (RVI) is electroneutral Na^+/H^+ exchange, which causes cells to gain one osmotically-active Na^+ for each proton lost from intracellular buffers. Alternatively, Na^+/H^+ exchange may be functionally coupled to Cl^-/HCO_3^- exchange, leading to NaCl uptake without a change in cell pH. Other transporters activated in particular cells during the RVI include $Na^+/K^+/2Cl^-$ co-transport and Na^+/Cl^- co-transport. These RVI mechanisms involve secondary active transport driven by the inward sodium gradient (Hoffmann, 1987). Much slower cell volume regulation (time scale of hours to days) occurs in renal medullary and brain cells, which accumulate organic solutes such as betaine or synthesize sorbitol in order to up-regulate their volume (Lohr et al., 1988; Nakanishi et al., 1988).

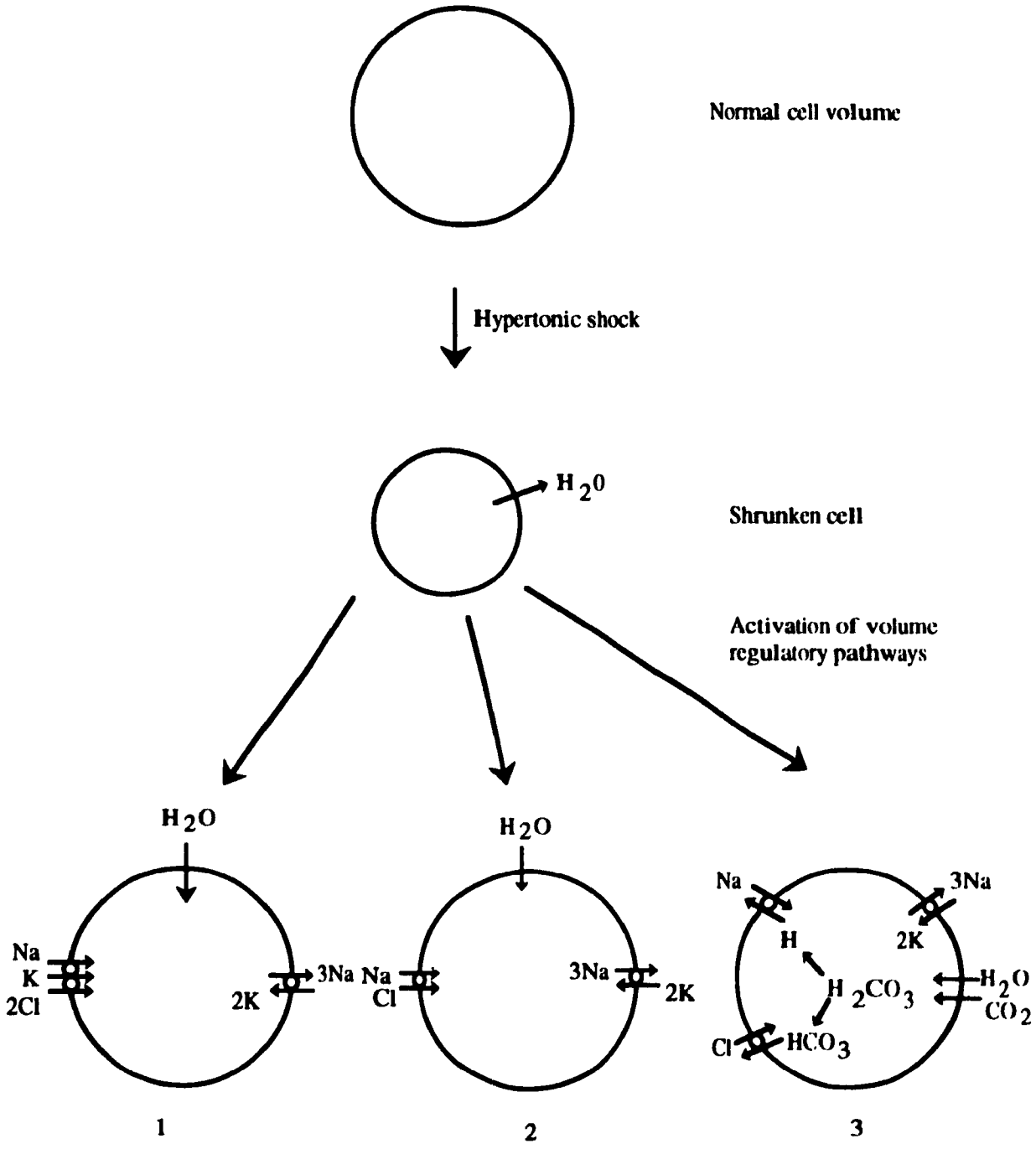


Figure 3: The regulatory volume increase (RVI).

The cell initially shrinks when extracellular osmolarity is increased. This figure shows three different volume regulatory mechanisms for restoring cells to their normal volume in the continued presence of hypertonic medium. 1) Na/K/2Cl co-transport. 2) NaCl co-transport. 3) Electroneutral Na/H and Cl/HCO_3 exchangers. This latter pathway results in NaCl uptake with no change in pH. In all three RVI pathways there is net uptake of NaCl.

2. Regulatory Volume Decrease (RVD)

When cells are swollen, they selectively increase membrane permeability to solutes. Common solute pathways for this response include K^+ and Cl^- conductances (presumably channels), electroneutral K^+/Cl^- co-transport, and ion exchangers (coupled K^+/H^+ and Cl^-/HCO_3^- exchangers allow KCl loss without altering pH, Fig. 4). Separate K^+ and Cl^- conductances are activated during RVD in human lymphocytes (Grinstein et al., 1982), frog urinary bladder (Davis and Finn, 1985), Ehrlich ascites tumor cells (Hoffmann et al., 1986), and MDCK cells (Rothstein and Mack, 1990). In cells that use K^+/Cl^- co-transport such as sheep LK cells (Dunham and Ellory, 1981), duck red blood cells (Kregenow, 1981), dog red blood cells (Parker, 1983), and *Necturus* gall bladder (Larson and Spring, 1984), the RVD involves loss of K^+ and Cl^- from the cell, driven by the K^+ concentration gradient across the cell membrane. Parallel K^+/H^+ and Cl^-/HCO_3^- exchanges occur when *Amphiuma* red cells undergo RVD (Cala et al., 1986).

Volume regulation is a particularly important housekeeping function for epithelial cells because they are exposed to external fluids having variable osmolarity. The detailed biochemical mechanisms that sense volume and signal the activation of transport pathways during RVI and RVD are still largely unknown although they must be very well tuned in rapidly transporting epithelial cells. The volume-regulatory channels have not been identified with certainty although, as discussed above, recent evidence suggests that the outward rectifying Cl channel and/or the ClC-2 chloride channels may be involved.

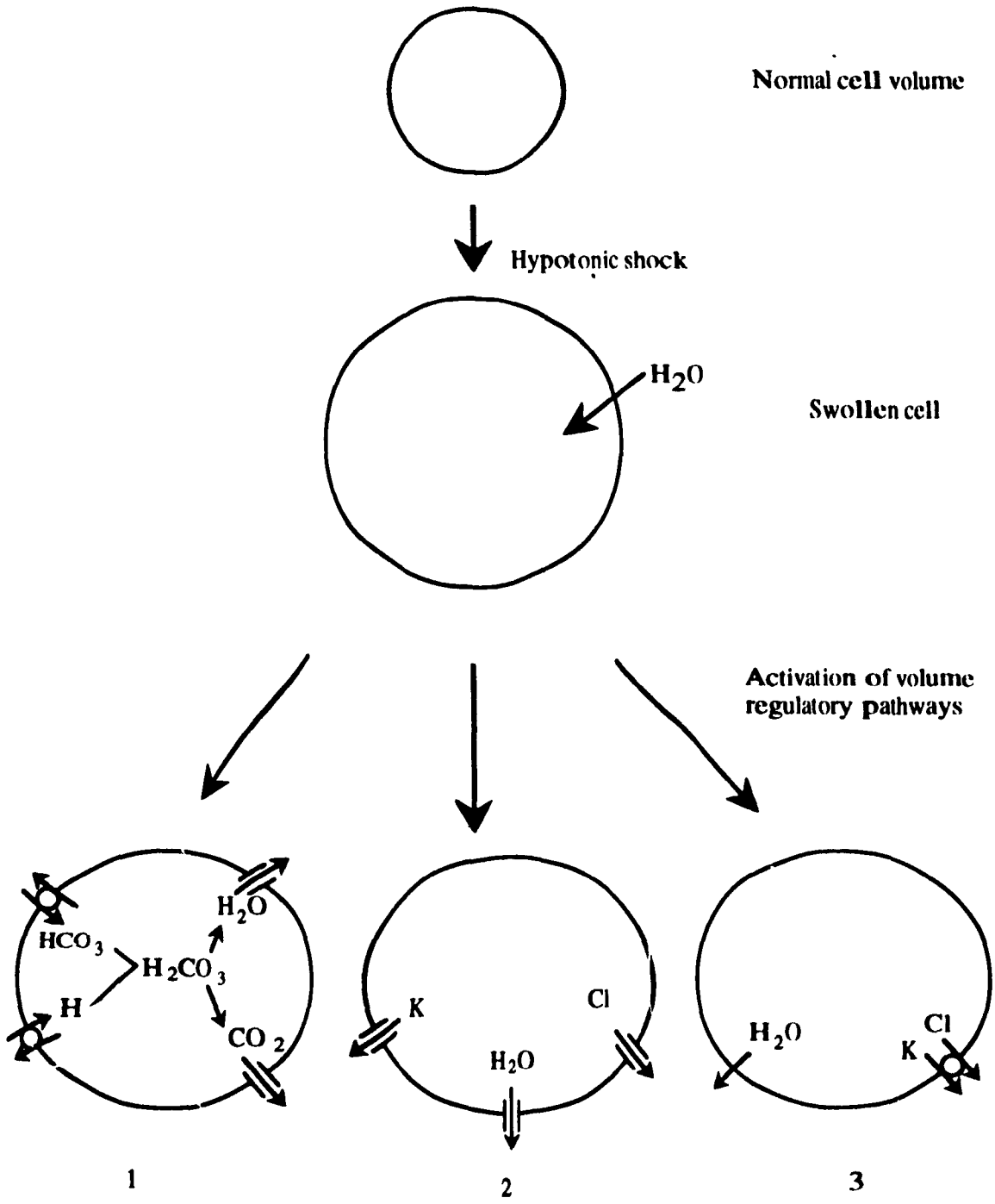


Figure 4: The regulatory volume decrease (RVD).

Model of ion fluxes in the regulatory volume decrease. 1) Electroneutral K/H exchange coupled to Cl /HCO_3, this model gives a loss of KCl with no change in pH. 2) Conductive K flux functionally coupled to conductive Cl flux. 3) Electroneutral KCl transport.

6 *In vitro* preparations for studying the epithelial Cl channels.

Cell preparation

Cl channels have been studied in freshly-isolated cells as well as in various cultured cell lines. However, the membrane of interest is often not readily accessible; the membrane may face the lumen of a microscopic tubule or it may be in close contact with the basement membrane. When epithelial cells are polarized in culture, their apical membrane faces "upward" and their basolateral membrane attaches to the porous support. Primary cultures are derived directly from tissue samples obtained from the organism; however it is difficult to obtain samples of some human tissues (e.g. pancreas, intestine) therefore transformed cell lines that grow indefinitely in culture are often used instead of primary cultures.

Primary cell culture

Primary cultures of human nasal polyp, dog trachea, human fetal pancreas, and shark rectal gland have been used to study apical Cl channels (see above). The tissue is dissociated into individual cells by mechanical and enzymatic disruption to obtain a relatively pure cell population, which is then plated on an appropriate substrate. Primary cell cultures sometimes maintain differentiated properties but have limited potential for replication unless they are immortalized or transformed. Transformation may occur spontaneously or can be induced by exposure of the cells to carcinogens (chemicals, viruses, or radiation).

Tumor cell lines

The main advantage of cell lines is that they are relatively easy to culture and store, and they can be cloned to produce a homogeneous cell population. On the other hand, transformed cells often develop abnormalities in chromosome number or structure, and

may spontaneously dedifferentiate after repeated passaging. Many epithelial cell lines have been characterized but only two were used in this study, T_{84} and PANC-1.

T_{84} cell line

The T_{84} cell line is the most widely used culture model for secretory epithelia. It was derived from a lung metastasis of a human colon carcinoma that had been serially transplanted in BALB/c nude mice. Original histological characteristics of the colon carcinoma were maintained throughout transplantation and, after 23 passages in athymic mice, the line was established (Murakami and Masui, 1980). T_{84} cells grow to confluence as monolayers and exhibit high tight junctional resistance. They have a columnar appearance and retain receptor-stimulated electrolyte transport mechanisms, therefore they provide an excellent model for secretion by the colonic crypts and similar tissues (Dharmsathaphorn et al., 1984).

PANC-1 cell line

PANC-1 is a human pancreatic ductal cell line which has been useful as a model for differentiation and mucin production. The line was initiated from a pancreatic carcinoma of ductal origin, has been maintained in culture for over two years, and has been subcultured up to 240 times without histological alterations. PANC-1 cells form polarized monolayers and have many differentiated features; including the apical microvilli, apically-localized cytoplasmic vesicles, epithelial intermediate filaments, and complete tight junctional complexes (Madden and Sarras, 1988). When grown on an appropriate substrate, PANC-1 cells resemble the principle cells of the pancreatic ductal epithelium. Biochemical analysis indicated that the PANC-1 cells have the same enzymatic profile as freshly-isolated rat and human pancreatic ducts.

Physiological techniques to study epithelial Cl channels

Patch clamp

Patch clamp techniques make it possible to study the properties of ion channels in detail, including its conductance, selectivity, kinetics, voltage dependence, and the effect of inhibitors. Patch clamping can resolve single ionic channels above background noise because the glass pipette tip forms a high resistance (>GΩ) seal with the cell membrane. Channels can be recorded in several configurations: whole cell, cell-attached, and after excision (inside-out or outside-out, see Fig. 5). The whole cell configuration makes it possible to observe and study the activity of all the channels in the cell, the cell-attached configuration enables one to study regulation of a channel under relatively physiological conditions, and excised patches are convenient for testing agonists and inhibitors, and other experiments where it is necessary to manipulate the voltage as well as solutions bathing the extracellular and intracellular sides of the membrane.

Short-circuit current

Transepithelial transport studies in Ussing-type chambers can provide a direct measure of net Cl secretion in some tissues, although it was originally developed to study active sodium transport by isolated frog skin (Ussing and Zerahn, 1951). In this method, electrical and chemical gradients across a monolayer or epithelium are eliminated by clamping the transepithelial potential at 0 mV and by placing identical solutions on both sides of the tissue (see Fig. 6). Under these conditions, any net flux must be due to active transport, and the short circuit current (I_{SC}) required to keep the transepithelial potential at 0 mV is the sum of all the electrogenic transport processes. It has been shown that I_{SC} provides an accurate measure of the rate of active Cl secretion in T_{84} cell monolayers (Dharmsathaphorn et al., 1984).

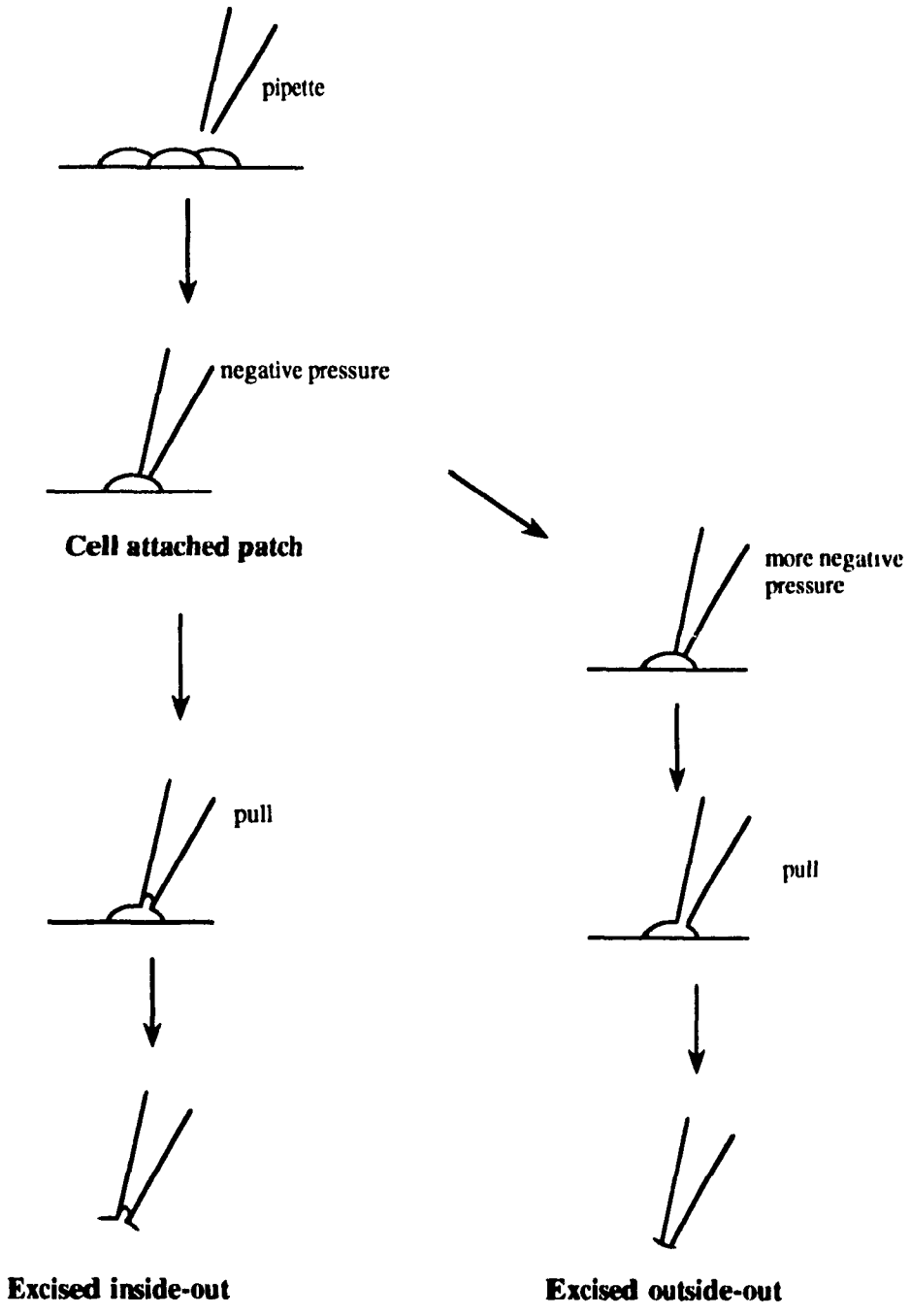
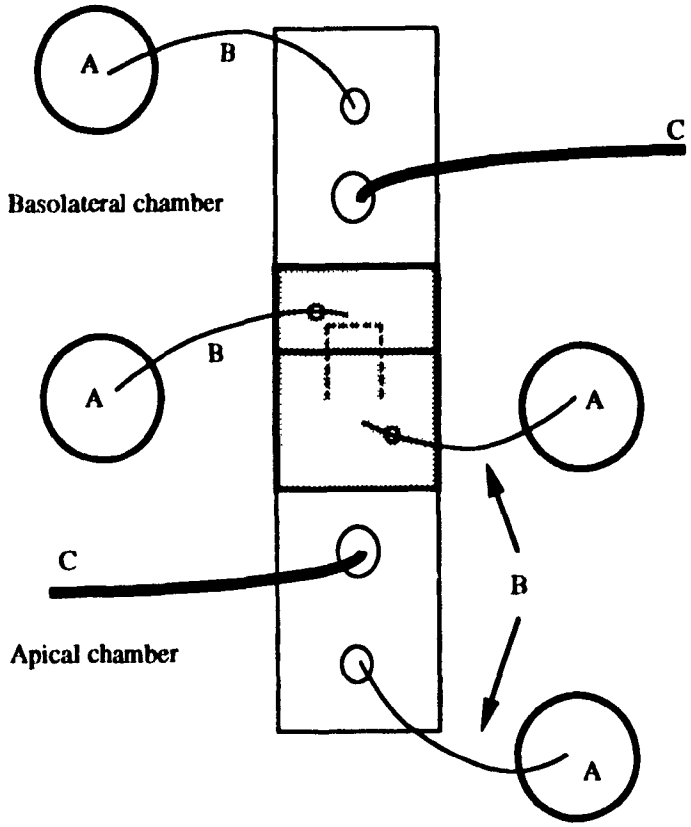


Figure 5: Patch clamp configurations.

This figure shows the sequence for obtaining different patch clamp configurations: cell attached, excised inside-out and outside-out.

Top view



Side view of chamber and culture insert with cell monolayer

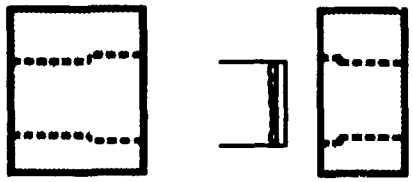


Figure 6: Schematic diagram of the Ussing chamber set up.

Top view (legend):
A: KCl filled wells containing voltage-sensing (calomel) electrode
B: KCl-agar bridges
C: Plastic tubing bringing 95% O_2/5% CO_2 to chamber

Cells are grown on collagen coated inserts and the apical and basolateral membranes are perfused with media.

Electronic cell sizing

Changes in cell volume can be assessed using electronic cell counting and sizing (Grinstein et al., 1982). In electronic cell sizing, a Coulter counter is used to determine the number of cells in a suspension and, assuming they are spherical, estimate their volume. The Coulter counter rapidly assays a large number of cells and generates a volume distribution curve. The median of the curve represents the average volume of individual cells. Actual cell volumes can be determined by calibrating the instrument, although aggregated cells, dead cells, and other debris may cause some error. Two experimental approaches have been used to study cell volume regulation. One approach is to study regulation under isotonic conditions by altering normal pump-leak relationships. The other approach, which was used in this thesis, is to alter cell volume by changing medium osmolality, and then monitor the volume recovery.

7 Purpose

The aim of this thesis was to identify the outwardly rectifying Cl channel in PANC-1 and T_{84} cells and to examine its possible role in chloride secretion and volume regulation using channel inhibitors. The approach was to identify the outward rectifier in patch clamp experiments and determine its sensitivity to blockers, then to assess the sensitivity of transepithelial transport and cell volume regulation to the same blockers under comparable conditions. Parts of this work have appeared (Low et al., 1990; Tabcharani et al., 1990).

II PHARMACOLOGY OF THE OUTWARDLY RECTIFYING Cl CHANNEL

Introduction

It is possible to classify channels according to their pharmacological properties and to use inhibitors to identify their functional roles. For example, toxins and Ca channel blockers such as the dihydropyridines, which are used clinically to treat heart arrhythmias and to relax vascular smooth muscle, have been useful in classifying different types of Ca channels (McClesky et al., 1986). Similarly, tetrodotoxin (TTX), a paralytic poison of some puffer fish, blocks action potential conduction in nerve and muscle and is a useful probe for the voltage-gated sodium channel. Finally, tetraethylammonium ion (TEA) has been shown to selectively block I_K in excitable cells and has been used to separate different microscopic K currents and to identify the pore region of voltage-gated K channels.

Unfortunately, highly specific blockers for particular types of epithelial Cl channels have not been identified, although the potencies of commonly used inhibitors may vary for different channel types. The outwardly rectifying Cl channel is sensitive to 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) and related disulfonic stilbene derivatives (Bridges et al., 1989), diphenylamine-2-carboxylate (DPC, Distefano et al., 1985) and its more potent derivative 5-nitro-2-(3 phenylprophyamino)-benzoate (NPPB, Wangemann et al., 1986), as well as a variety of other agents not usually considered as Cl channel blockers including verapamil and its derivatives, fatty acids, cytosolic extracts, and pH buffers (reviewed by Hanrahan et al., 1993).

This section looks at the effects of two inhibitors of chloride conductance, IAA-94 and NPPB, on single outwardly rectifying Cl channels. The goal of this section is to determine

the potency of each compound at the single channel level, as a prelude to further studies of the functional role of the outward rectifier in intact cells.

Materials and Methods

Cell culture

The T_{84} and PANC-1 cell lines were obtained from the American Type Culture Collection (ATCC) and used between passages 45 and 115. The T_{84} cell growth media was a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 media, supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. PANC-1 cell growth medium was DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Cells were grown on plastic for 4 days in a 37 °C, humidified, CO_2 incubator. To dissociate the cells, the culture medium was aspirated and cells were rinsed with 2 mL of a trypsin solution (0.5%) to remove remaining culture media and then incubated in 0.5 mL fresh trypsin solution for 10-15 minutes, depending on the degree of confluence. Cells were dispersed using a pipette and the cell suspension was counted and viability checked using a haemocytometer and the trypan blue exclusion method. Cells were plated on glass coverslips at a density of 10^6 cells/ml .

Patch clamp

Pieces of the glass coverslip were transferred into the patch clamp chamber, which was mounted on an inverted microscope (Olympus IMT-2), and viewed using phase-contrast optics. Solution changes were done by perfusing the chamber (0.5 mL volume) with at least 5 mL of the appropriate solution. Pipettes having resistances of 4-6 MΩ were constructed from Kimax-type glass capillaries using a two stage Narashige pipette puller

(Narashige, Tokyo, Japan). The pipette contained a chlorided silver wire, and the bath solution was grounded through an agar bridge having the same ionic composition as the pipette solution. Patches were obtained from the apical surface of cells. Experiments were performed at room temperature (20 - 22 °C).

Analysis

Single channel currents were amplified (Axopatch, Axon Instruments Inc., Foster City, CA) and stored on video tape (VC785C, Sharp Corp., Japan) using a pulse coded modulation-type recording adapter (DR384, Neurodata Instruments Co., New York). The data were low-pass filtered during playback using an eight pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA) and analyzed using a laboratory microcomputer system (Indec Systems, Sunnyvale, CA).

The number of channels in patches was estimated as the maximum number of simultaneous channels opening during long recordings under control conditions, when open probability was high. Single channel amplitude was estimated by visually fitting cursors to the open and closed levels as determined using amplitude histograms. Open probability (P_0) was calculated as the mean number of channels open averaged over 2 minutes, with a transition threshold set half way between the open and closed levels.

Solutions

The bath solution contained (in mM): 150 NaCl, 10 TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), and 2 $MgCl_2$, pH 7.4. The pipette solution was identical except that Mg was replaced with Ca. Solutions were kept at 4 °C and filtered before use. Inhibitors IAA-94 and NPPB were dissolved in dimethylsulphoxide (DMSO) to make 100 mM stock solutions, which were then aliquoted and stored at -20 °C. An appropriate volume of the inhibitor was used immediately before

experiments to prepare 10 mL of bath solution at the required concentration (maximal final DMSO concentration was 0.1%).

Results

Identifying the channel

Outward rectifiers were not seen in cell-attached patches, and were rarely recorded immediately after excision. However, they were often activated by holding patches at large depolarizing potentials (range +60 mV to +120 mV) for approximately 2 minutes. Channels became activated in 71% of the excised patches from PANC-1 cells after they had been voltage clamped at +120 mV for 2 minutes and then pulsed between positive and negative voltages (Fig. 1). On average, there were 2.2 ±0.2 channels per patch in PANC-1 cells in those seals that contained channels (n=26). The outward rectifier was somewhat less common in patches from T_{84} cells (present in 30% of patches after the same maneuvers), however its activation, conductance, and kinetics were indistinguishable from those from PANC-1 cells.

Currents recorded at depolarizing potentials were larger than those at hyperpolarizing potentials (Fig. 2), yielding an outwardly rectifying I/V relationship in symmetrical 154 mM Cl solutions (Fig. 3). The channel was mostly in the open state at all the potentials between -100 mV to +100 mV, and had a P_0 of 0.85 at +40 mV. The slope conductance at the reversal potential was 38 ± 3.1 pS when estimated between ±20 mV, 80.5 ± 6.4 pS between +40 mV and +80 mV, and 20.6 ± 3.7 pS between -40 mV and -80 mV.

Effect of NPPB

NPPB inhibited outward rectifiers from the cytoplasmic side of inside-out patches (Fig. 4). After recording channel activity in control solution at +40 mV the chamber was

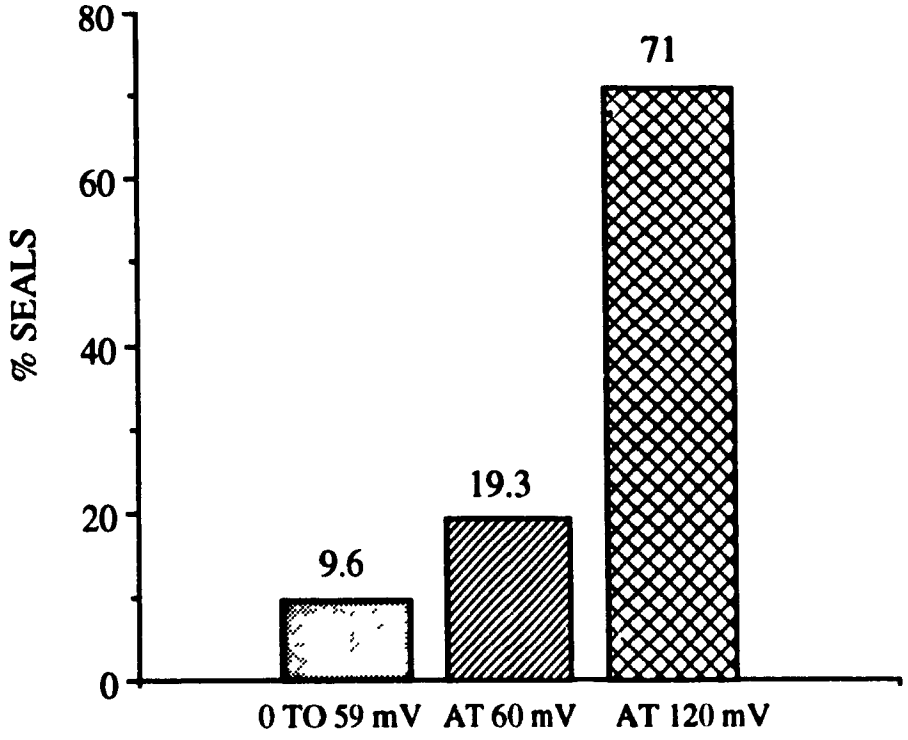


Figure 1: Fraction of inside-out patches from PANC-1 cells containing active, outwardly-rectifying Cl channels. After excision, spontaneous activation of the outward rectifier was observed immediately in 9.6% of those patches that were eventually found to contain the channel. If activation was not observed within 2 min, the patch was held at +60 mV for 2 minutes and then pulsed three times to ±60 mV. This caused activation of channels in 19.6% of patches. Finally, if there was still no activity, patches were then clamped at +120 mV and held for 2 minutes. Outward rectifiers were present in 26/31 seals.

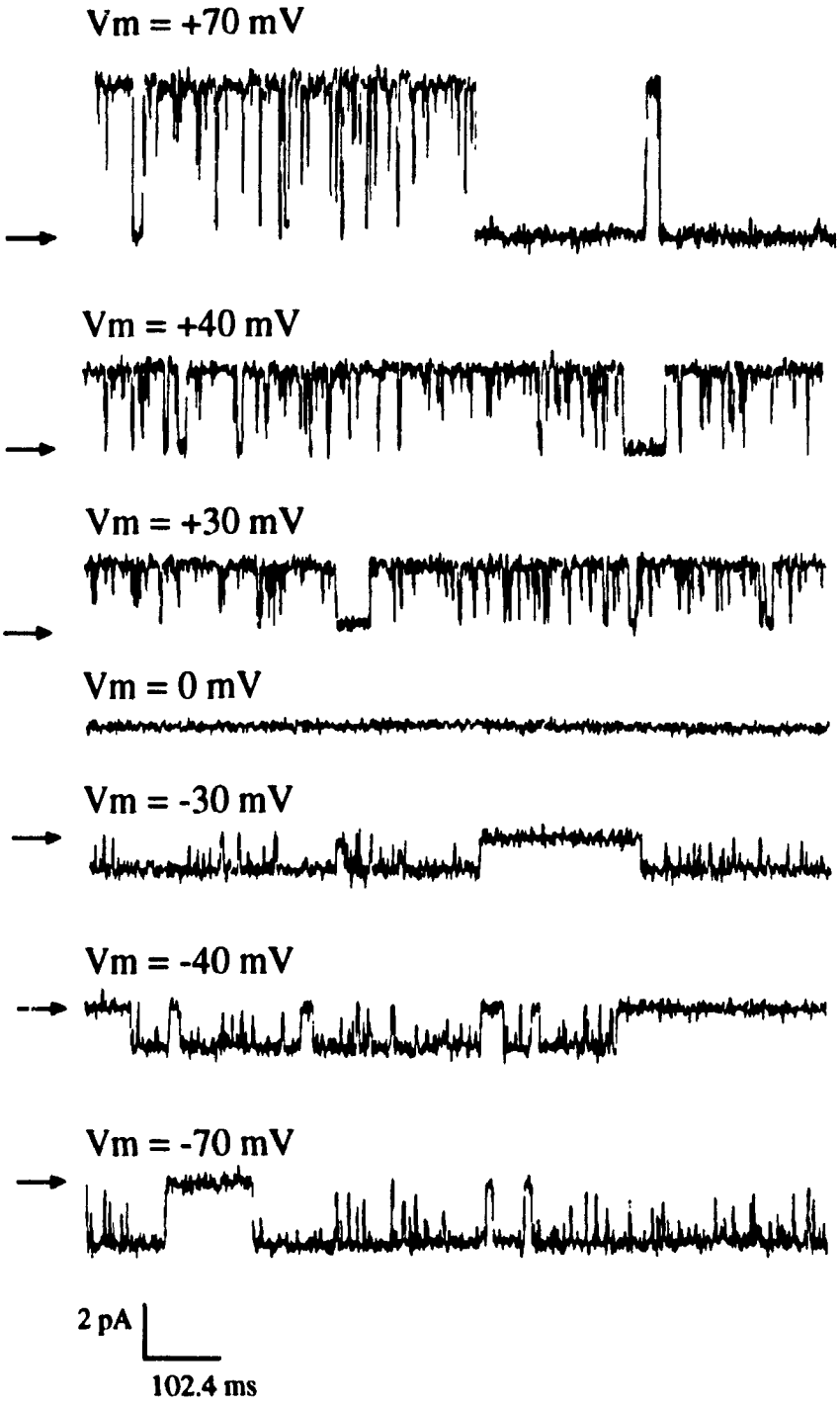


Figure 2: Traces of the outwardly rectifying Cl channel in patches excised from PANC-1 cells. The arrows mark the closed states. The channel is mostly in the open state at both depolarizing and hyperpolarizing potentials. At +40 mV, the channel has an open probability of 0.85.

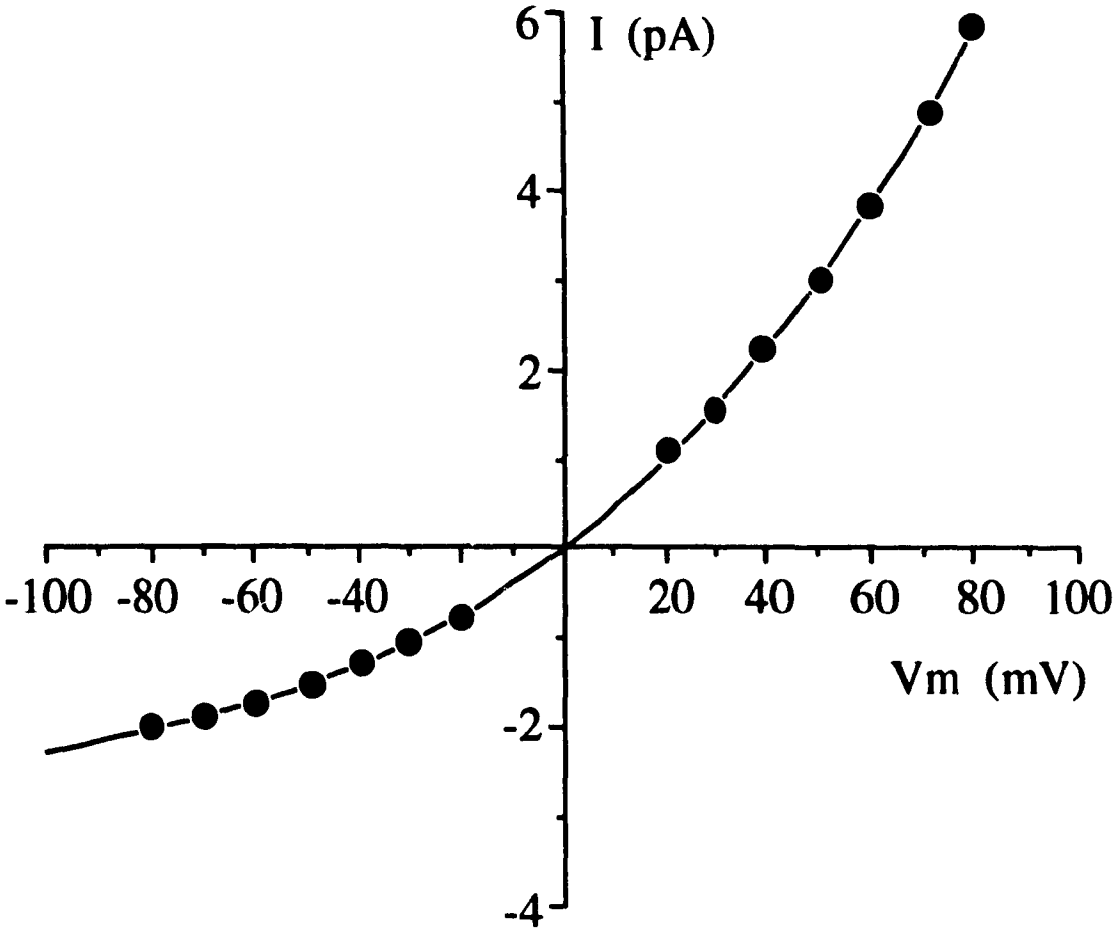


Figure 3: Current-voltage relation of the Cl channel measured using inside-out patches from PANC-1 cells. The channel rectifies at depolarizing potentials and has a conductance of 38 pS at the reversal potential in symmetrical NaCl. n=12 patches, standard error bars are within the symbols.

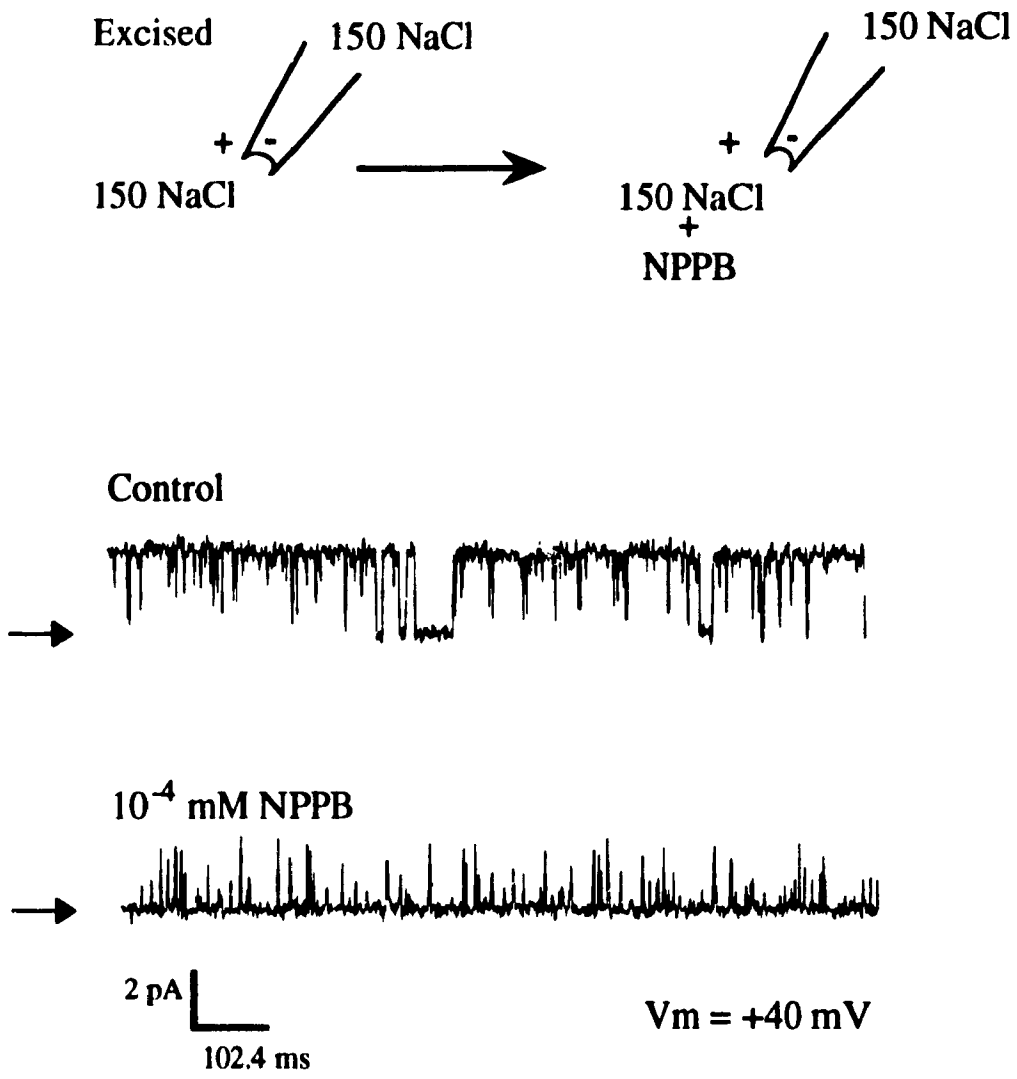


Figure 4: Diagram showing experimental conditions used to study effects of NPPB from the cytoplasmic side. The patch is excised in symmetrical 150 mM NaCl, then the patch is perfused with NaCl containing the required concentration of NPPB. Current traces obtained when the patch is held at +40 mV in control solution and 100 µM NPPB. Arrows indicate the closed level.

perfused with at least 10 mL of bath solution containing the desired final concentration of blocker. Increasing NPPB concentration on the cytoplasmic side of the membrane progressively decreased open probability with half-maximal inhibition (IC_{50}) at approximately 15 μM under these conditions (Fig. 5).

NPPB also inhibited from the extracellular side of the membrane, although this was difficult to quantitate using outside-out patches for technical reasons. Forming outside-out patches requires an additional step once a seal is obtained; first negative pressure must be applied to the pipette interior to rupture the membrane patch, then the pipette tip is retracted so that a small membrane patch reforms at the tip. Breaking into PANC-1 cells required very strong suction, which usually caused seals to become unstable. Therefore, the effects of extracellular NPPB were studied by adding it to the pipette solution and recording in the inside-out configuration. NPPB seemed to be more effective in blocking the channel from the extracellular side (with 1 μM external NPPB the open probability corrected for normal closures was 0.76 (P_0 was 0.59 ± 0.09, n=3), as compared to 0.88 (P_0 was 0.75 ±0.004, n=3) with internal NPPB). However, this difference may reflect voltage-dependence since the membrane potential (+40 mV) would favour inhibition by extracellular NPPB, which is negatively charged. Low concentrations of NPPB made the channel flicker while at higher concentrations (25, 50, 75, and 100 μM), channel openings were not observed (n=4).

Effect of IAA-94

IAA-94 had a potent inhibitory effect when applied to the cytoplasmic side of excised, inside-out patches. Addition of 100 μM IAA-94 to the bath caused brief closures (Fig. 6), but did not affect channel conductance significantly (Fig. 7). Inhibition of open probability by cytoplasmic IAA-94 was concentration-dependent and had an IC_{50} of 22 μM (Fig. 8). Extracellular IAA-94 was tested on inside-out patches by including IAA-94 in the pipette

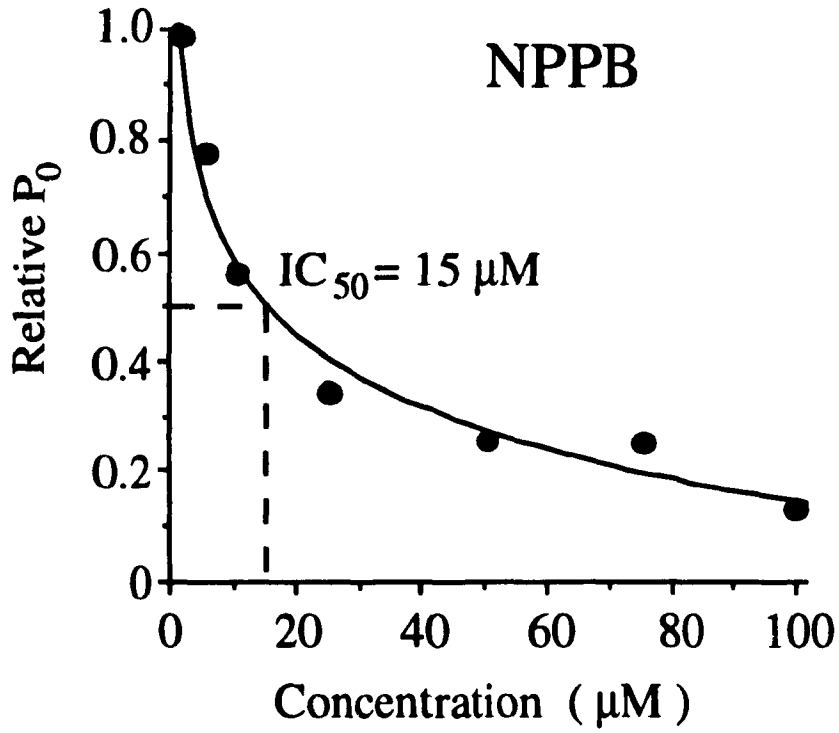


Figure 5: Concentration-dependent inhibition of open probability (P_0) by NPPB. Open probability at each concentration is expressed relative to P_0 measured in the absence of inhibitor, which was 0.83. The IC_{50} is the concentration of NPPB required to decrease P_0 by 50%. From the cytoplasmic side NPPB an apparent IC_{50} of ≈15 μM with the membrane potential clamped at +40 mV. n=3 at each concentration.

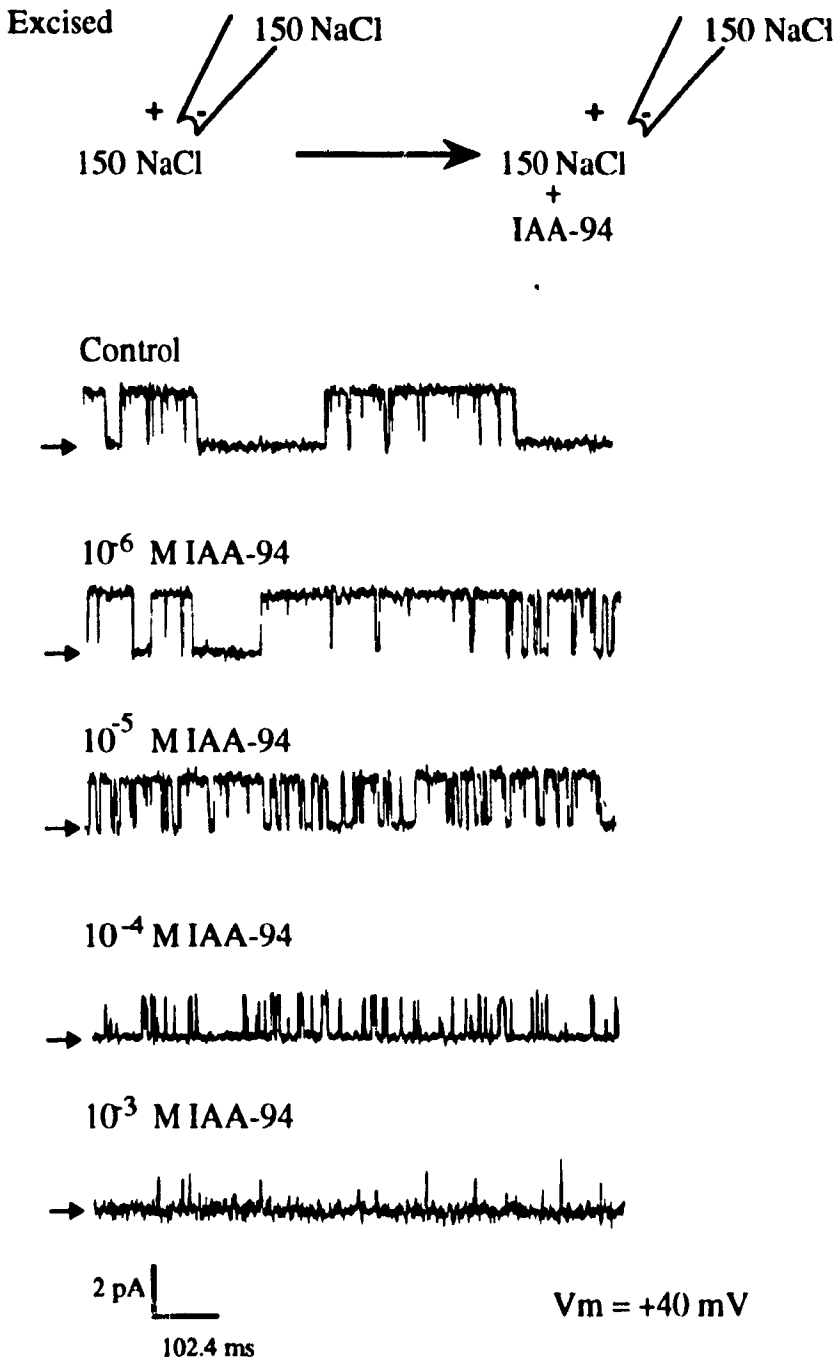


Figure 6: Diagram showing the recording configuration. Current traces obtained at a potential of +40 mV under control conditions (0 mM IAA-94) and with different concentrations of IAA-94 bathing the cytoplasmic side.

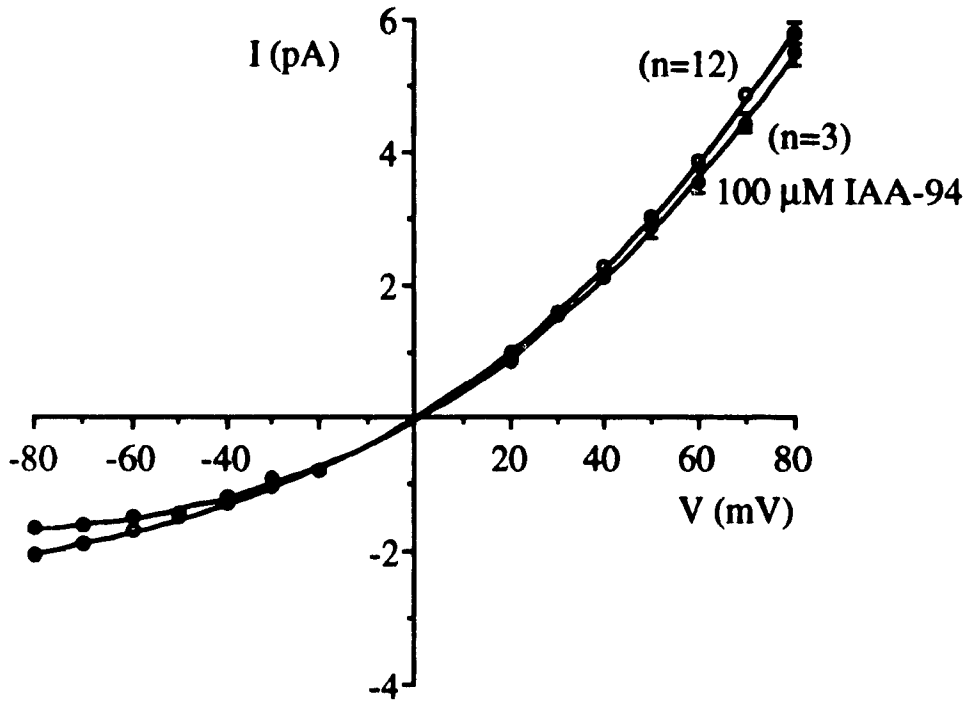


Figure 7: Comparison of I/V relationship with (o) 0 M IAA-94 (ie control conditions) and (•)100 µM IAA-94 on the cytoplasmic side. n=12 and 3, respectively, ± se. IAA- 94 does not affect conductance significantly.

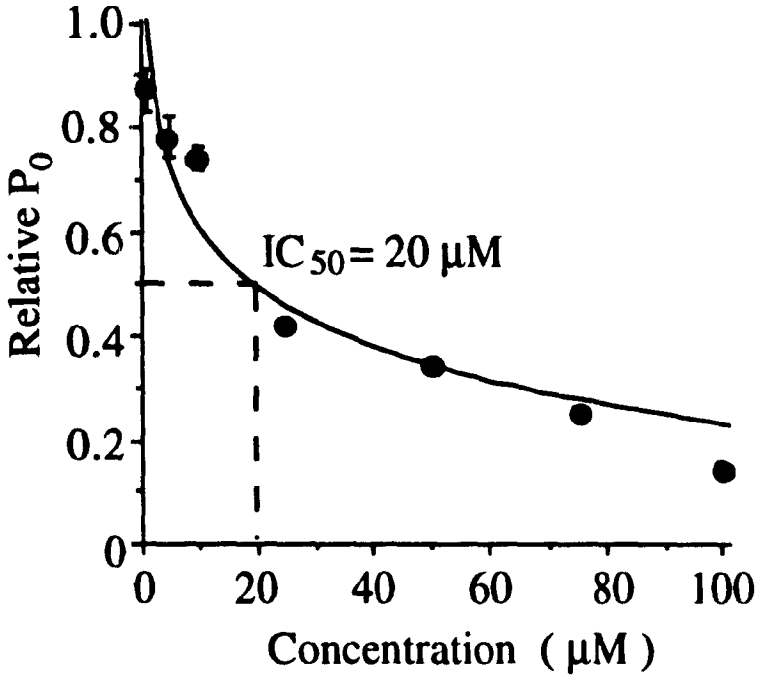


Figure 8: Concentration-dependence of IAA-94 inhibition of open probability from the cytoplasmic side after correction for normal (unblocked) closures. n=3, ± s.e. where larger than the symbol.

solution. As with NPPB, IAA-94 was also a more potent blocker from the outside (Fig. 9). The IC_{50} for external IAA-94 was 9 μM under these conditions at +40 mV (Fig. 10).

Discussion

An outwardly rectifying Cl channel has been identified in PANC-1 cells and its sensitivity to two known Cl channel inhibitors has been assessed. The channel was initially activated in excised patches by large positive potentials as reported by others (Schoumacher et al., 1987; Welsh et al., 1989; Tabcharani and Hanrahan, 1991a). IAA-94 and NPPB inhibited the outwardly rectifying channel from PANC-1 cells by causing the channel to become more flickery with little change in single channel conductance. Both compounds appeared to be more effective from the extracellular side. Although this difference could reflect the voltage-dependent block (all the present experiments were carried out at +40 mV), voltage would not explain discrepancies with the literature since Wangemann et al. (1986) reported that the IC_{50} for inhibition of conductance in the renal tubule by NPPB is only 80 nM despite a negative membrane potential, and Greger (1990) has proposed that NPPB blocks preferentially from the external surface.

The mechanisms of block by NPPB and IAA-94 are not fully understood, although low doses ($<10^{6}$ mol/L) of both inhibitors reduced the number of long-lasting open transitions without affecting single channel conductance (Figs. 4 and 6), as shown previously for NPPB by Dreinhöfer et al. (1988). They reported that NPPB binds to a site in the channel which changes the rate of transitions between normal conformations without inducing an additional blocked state. By contrast, Singh et al. (1991) concluded that NPPB acts as a fast blocker with an on/off rate of 1000 sec^{-1}, that it is equally potent from both sides of the colonic Cl channel in bilayer studies, and that it has an IC_{50} of 25 μM. As with NPPB, they found that a mixture containing IAA-94 and its stereoisomer IAA-95 were equipotent

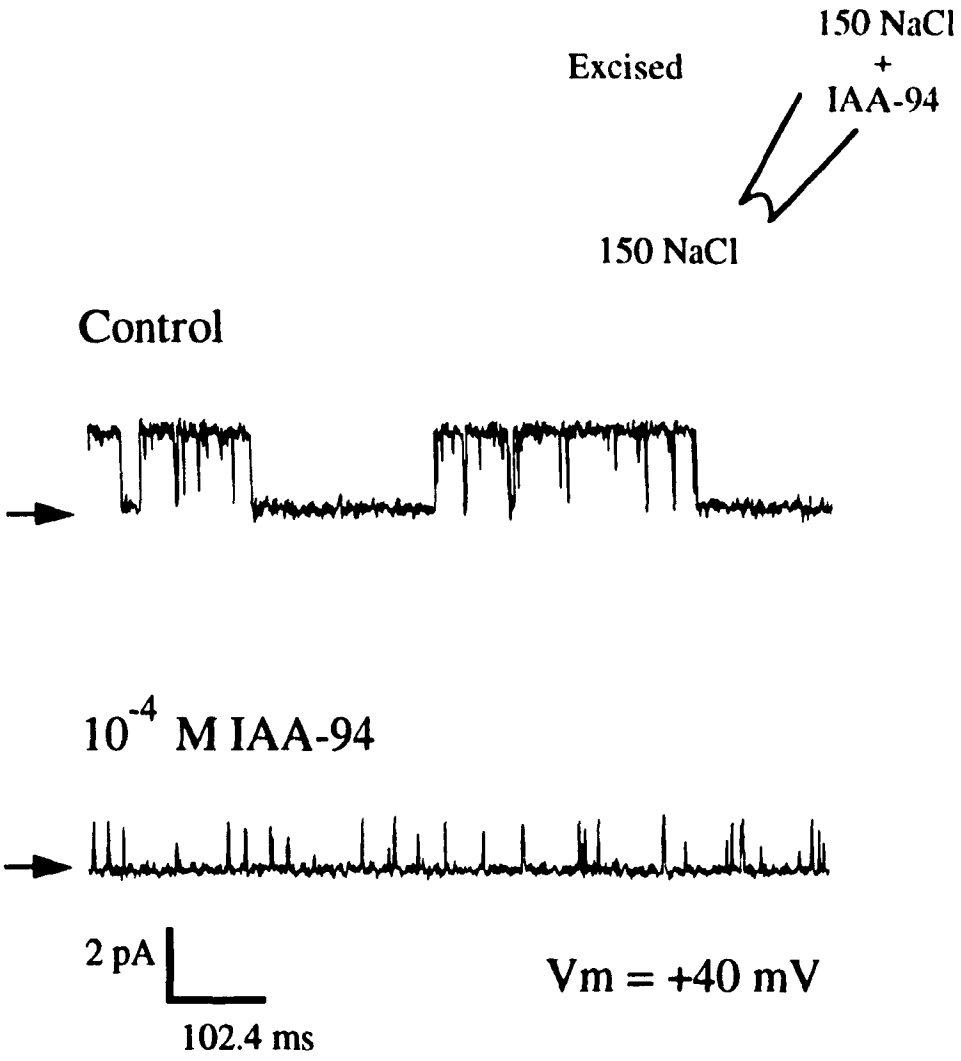


Figure 9: Current traces obtained at a membrane potential of +40 mV with IAA-94 bathing the extracellular side.

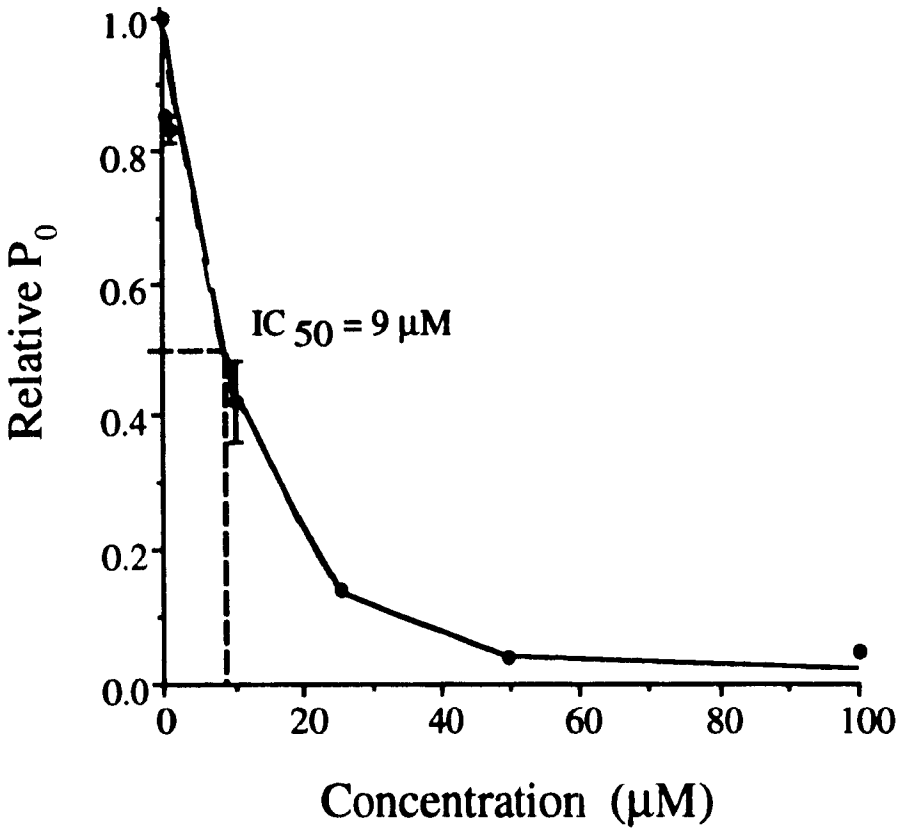


Figure 10: Concentration-dependence of IAA-94 inhibition of open probability from the extracellular side. n=3 ± s.e.

from either side of the bilayer and proposed a simple, linear closed-open-blocked kinetic scheme. According to Singh et al. (1991), IAA-94 does not need to permeate the cell membrane because it acts as an open channel blocker, apparently at a site that is accessible from either end of the channel.

Table 1 shows the range of IC_{50}s reported for inhibition of the outward rectifier in isolated membranes. There is some variation, however the concentrations needed are in the 2 to 30 μM range in most preparations. Elevating temperature to 37 °C , which activates the outwardly rectifying Cl channel (Hayslett et al., 1987; Welsh et al., 1989; Tabcharani and Hanrahan, 1991a), might affect the potency of inhibitors. Pope et al. (1991) showed that higher concentrations of IAA-94 or NPPB were needed to decrease ^{36}Cl uptake in membrane vesicles at higher temperatures (25 °C vs 4 °C) and longer incubation times were required (4 min. vs 10 min.). Regardless, all of the evidence suggests that the outward rectifier is sensitive to NPPB and IAA-94 at micromolar concentrations.

The pharmacologic results in this chapter and previous data showing that the outward rectifier is sensitive to external DIDS (Tabcharani et al., 1990) are used in the following two chapters to assess the role of the outward rectifier in Cl secretion and cell volume regulation by T_{84} cells.

Table 1: IC_{50} required for several cell types

a: NPPB

Extracellular		
0.1 μM	HT_{29}, resp. epith. cells	Tilmann et al., 1991
20 μM	HT_{29}	Champigny et al., 1992
Intracellular		
0.9 μM	HT_{29}, resp. epith. cells	Tilmann et al., 1991
1-10 μM	cultured airway cells	Li et al., 1990
15 μM	PANC-1	Low et al., 1990 (this work)
32 μM	cultured airway cells	Kunzelmann et al., 1989
Other preparations		
10 μM	inhibit ^{36}Cl uptake into membrane vesicles	Pope et al., 1991
25 μM	colonic channel in planar lipid bilayers (both sides)	Singh et al., 1991

b: IAA-94

Extracellular		
9 μM	PANC-1	Low et al., 1990 (this work)
Intracellular		
8 μM	HT_{29}, resp. epith. cells	Tilmann et al., 1991
20 μM	PANC-1	Low et al., 1990 (this work)
Other preparations		
2 μM	inhibit Cl transport in membrane vesicles	Landry et al., 1989
10 μM	inhibit ^{36}Cl uptake in membrane vesicles	Pope et al., 1991
17.7 μM	colonic channel in planar lipid bilayers (both sides)	Singh et al., 1991

III PHARMACOLOGY OF Cl SECRETION

Introduction

Apical chloride conductance is an essential feature of secretory epithelial cells; however, the nature of the channels mediating apical Cl conductance has been controversial (see Chapter 1). This chapter assesses the role of one candidate channel, the outward rectifier, by comparing its pharmacology with that of the short circuit current in T_{84} cells which is carried entirely by net chloride transport. Some of these results have been reported previously (Tabcharani et al., 1990).

Materials and Methods

Transport studies

T_{84} cells were grown on collagen-coated inserts in multiwell plates (0.45 μm pore size, 12 mm diameter, 1.13 cm^2 area) and cultured for 10-15 days in T_{84} medium as described in Chapter 2. Millicell-HA inserts were prepared by coating them with Type I collagen (Vitrogen 100, Collagen Corporation) and then fixing the collagen with 4% glutaraldehyde solution. Ussing-type chambers (Vangard International, Neptune, NJ) were fitted with custom-made teflon adaptors to hold the monolayers, which were short circuited using an epithelial voltage clamp (DVC-1000 Voltage/Current clamp, World Precision Instruments, Sarasota, FL), except when resistance was monitored by clamping the voltage to 10 mV for 2 seconds at 1 minute intervals.

Analysis

Monolayers having electrical resistances greater than 1000 Ωcm^2 and a stable baseline I_{SC} less than 5 μA for 10 minutes were used. Compounds were added as concentrated

stock solutions to the apical or basolateral side where indicated and mixed by bubbling vigorously with 95% O_2/5% CO_2.

Data was acquired using a C-Lab system (INDEC systems, Sunnyvale, CA) interfaced to a 386 computer, and analyzed using custom software to obtain the I_{SC} and voltage values immediately before each voltage pulse. Data files were transferred to a Macintosh IIsi computer for analysis using a spreadsheet program.

Solutions

The saline used in short-circuit current experiments contained (in mM): 115 NaCl, 25 $NaHCO_3$, 1.2 $CaCl_2 \cdot H_2O$, 1.2 $MgCl_2$, 0.4 KH_2PO_4, 2.4 K_2HPO_4 and 10 mM TES. The stock "cAMP mixture", which was used to stimulate Cl secretion, contained 50 mM dbcAMP (dibutyryl cyclic AMP), 1 mM IBMX (3-isobutyl-1-methylxanthine), and 1 mM forskolin. This mixture, which was stored at -20 °C, was diluted 100-fold during experiments to yield final concentrations of 0.5 mM dbcAMP, 10 μM IBMX, and 10 μM forskolin.

Stock solutions of the inhibitors IAA-94 and NPPB in dimethylsulphoxide (DMSO) were diluted 1000-fold when added to the Ussing chambers so that the final concentration of DMSO in the chambers never exceeded 0.4% (0.1% from the blocker stock solution, 0.3% due to the cAMP mixture). In control experiments, DMSO did not affect I_{SC} up to a final concentration of 1% (Fig. 1). DIDS was prepared as a 100 mM aqueous stock solution. Stock solutions were stored in aliquots at -20 °C. The pH of solutions was adjusted to 7.4 and Ussing chamber studies were performed at 37 °C.

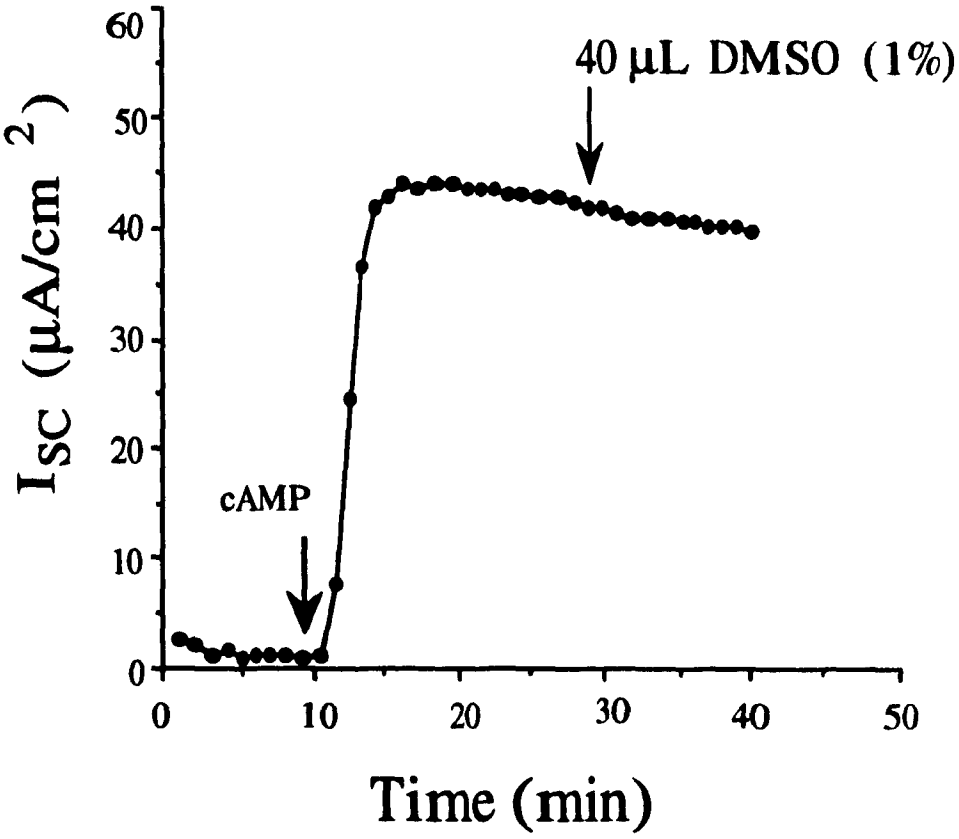


Figure 1: Lack of effect of vehicle(1% DMSO) on short-circuit current. The highest concentration of DMSO present during inhibitor studies was 0.4%.

Results

Activation of secretion by cAMP

Exposure of the apical side of the monolayer to the cAMP mixture increased the short-circuit current (I_{SC}) from 1.02 ± 0.33 $\mu A/cm^2$ to 38.68 ± 2.40 $\mu A/cm^2$ (n=16). Stimulation occurred within 2 minutes, then I_{SC} remained relatively stable for at least 30 minutes.

Effect of inhibitors on the short circuit current

Inhibition of apical chloride channels should lead to an abrupt fall in the short circuit current; however adding IAA-94, NPPB, and DIDS to the apical side of monolayers had little effect after 20 minutes exposure. IAA-94 (10 μM) had no effect at 10 μM and 100 μM IAA-94 caused only a slight inhibition of I_{SC}. Moreover the decline observed with 100 μM IAA-94 was gradual, suggesting it might be due to inhibition of some other process rather than to blockage of apical Cl channels (Fig. 2). There was a decrease of only 6.69 $\mu A/cm^2$ (16%) after exposure to 100 μM IAA-94 for 10 minutes. NPPB and DIDS had no detectable effect on Cl secretion when added to the apical side at the same high concentration (100 μM; Figs. 3 and 4, respectively).

Discussion

Using parallel studies of transepithelial transport and patch clamp, it is possible to compare the effects of Cl channel inhibitors on transepithelial secretion and on single channel activity. The single channel studies described in Chapter 2 indicated that 10 μM IAA-94 or NPPB strongly inhibit single outwardly rectifying Cl channels in excised patches. Previous studies have shown that DIDS is also a potent inhibitor of the outward rectifier (Tabcharani et al., 1990). The purpose of this chapter was to examine whether the

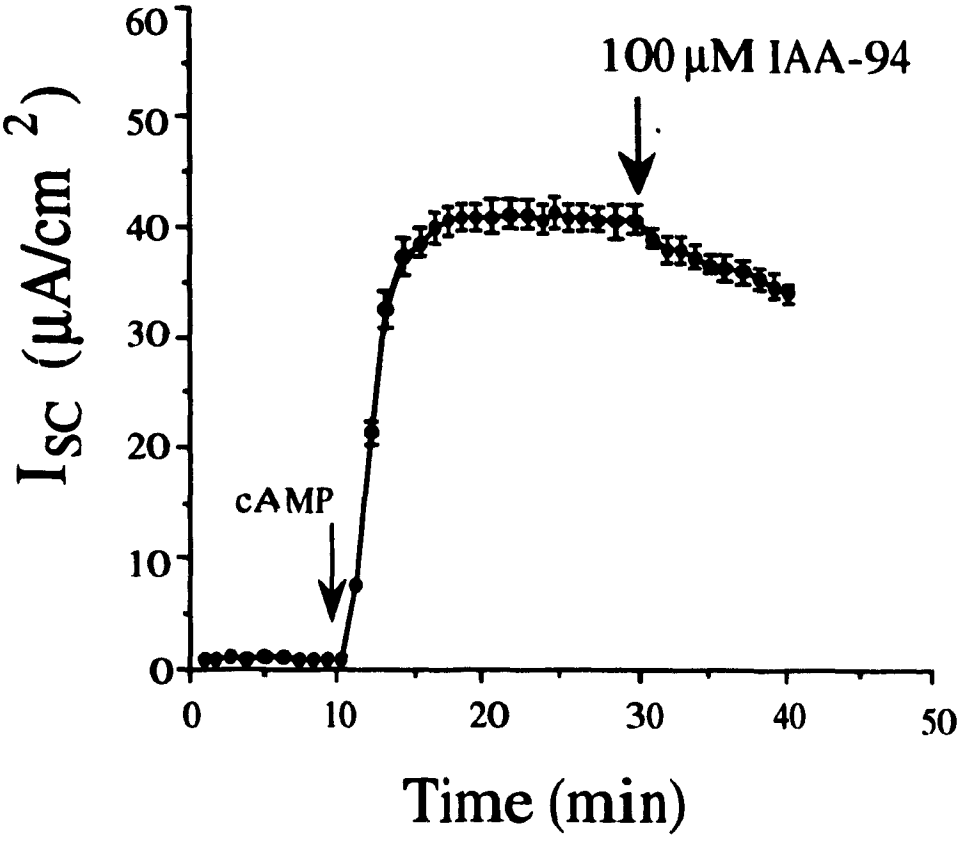


Figure 2: Effect of adding 100 μM IAA-94 to the apical side of T_{84} monolayers on short-circuit current. n=8 monolayers ± s.e.

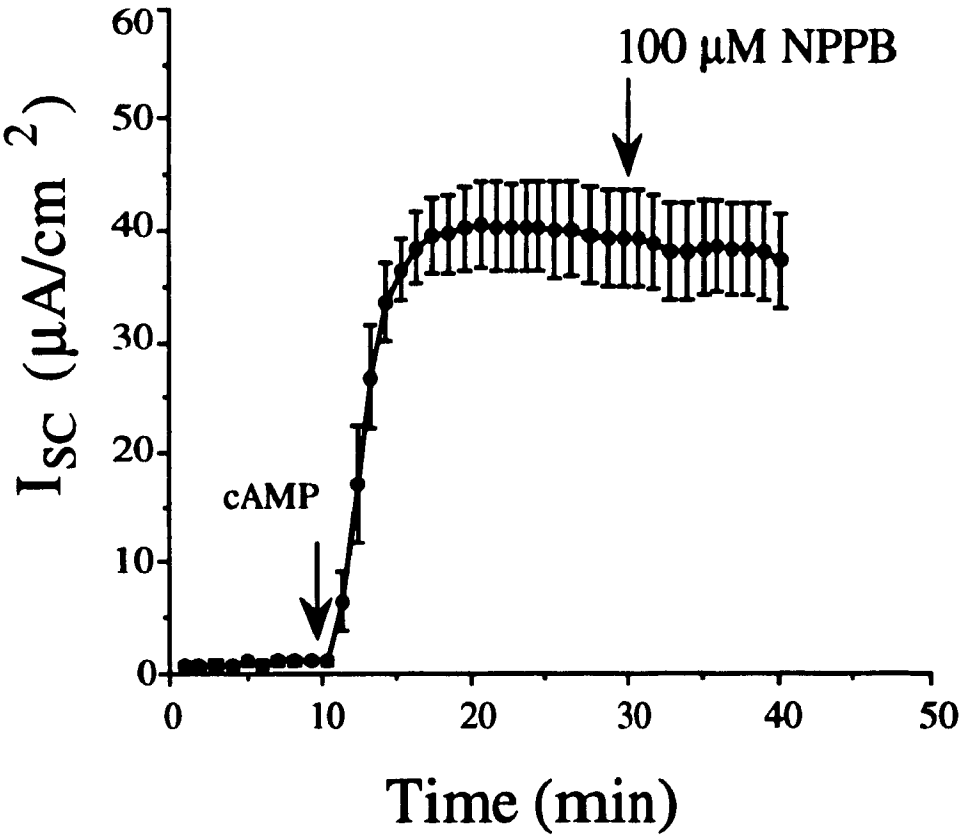


Figure 3: Effect of apical exposure to NPPB on Isc. NPPB (100 μM) did not change Isc after 10 minutes exposure (n=4 monolayers ± s.e, p>0.2).

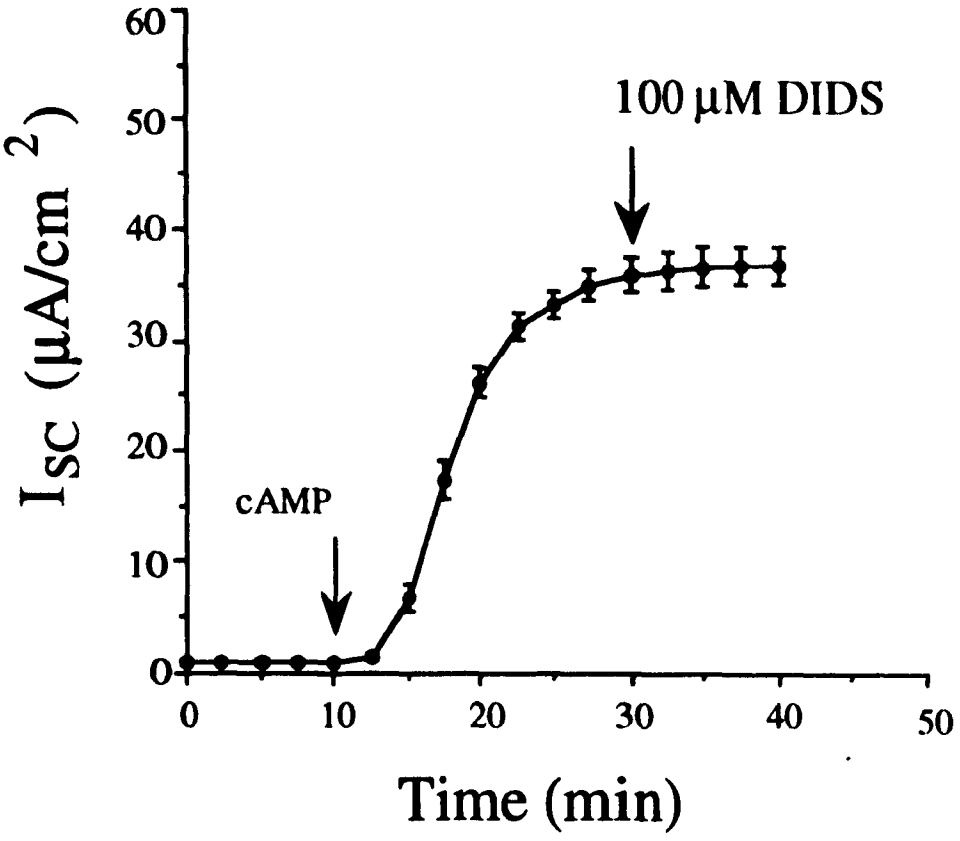


Figure 4: Effects of DIDS on short-circuit current. Addition of DIDS (100 μM) to the apical side did not cause any change in Isc after 10 min exposure. n=4 monolayers ± s.e.

same inhibitors would also inhibit I_{SC} in transepithelial studies, consistent with the hypothesis that the outward rectifier mediates Cl secretion in this tissue.

As shown in Figs. 2-4, IAA-94, NPPB, and DIDS had little effect on I_{SC} even at a concentration that was 10-fold higher than needed for inhibition of the outwardly rectifying Cl channel during the patch clamp studies described in Chapter 2. This suggests that some other Cl channel, such as the low-conductance Cl channel (CFTR) described above, mediates transepithelial secretion in T_{84} cells.

The effects of these chloride channel blockers on Cl secretion in previous studies have been variable. For example, high concentrations of NPPB were needed to inhibit I_{SC} in HT_{29}/B6 cells (300 μM; Kreusel et al., 1991) and ^{36}Cl efflux in T_{84} cells (IC_{50}=414 μM), whereas much lower concentrations were sufficient to block Cl conductance in perfused renal tubules (80 nM; Wangemann et al., 1986). Larrson et al. (1989) reported an IC_{50} of 100 - 200 μM for NPPB inhibition of VIP-stimulated I_{SC} in T_{84} monolayers, and NPPB had no effect on fluid secretion by the rat intestine at concentrations of 0.1 to 50 mM. Regardless, NPPB has been shown to have many actions, and if inhibition had been observed in intact cells it would have to be interpreted with caution. NPPB uncouples mitochondria and increases proton permeability of the plasma membrane in phagocytic cells (Lukacs et al., 1991), inhibits PGE_2 synthesis in mesangial cells (IC_{50}=25μM; Breuer and Skorecki, 1989), and reduces endogenous ATP levels in rabbit distal colon (100 μM; Keeling et al., 1991). On the other hand, Greger et al. (1991) has argued that NPPB effects are probably specific because one of its enantiomers (+) is a much more effective inhibitor than the other and NPPB inhibition was completely reversible in one study (Diener and Rummel, 1989). The "small" Cl channels in shark rectal gland (Gögelein et al., 1987) and porcine thyroid gland cells (Champigny et al., 1990) were not inhibited by NPPB.

The lack of effect of 100 μM DIDS on I_{SC} is consistent with previous studies of ^{36}Cl uptake into T_{84} monolayers (Mandel et al., 1986) where a similar stilbene isothiocyanate (SITS) was ineffective. On the other hand, Bridges et al. (1989) found that the derivative DNDS blocked the outward rectifier channel incorporated into planar lipid bilayers, and has recently extended those studies to DIDS (Singh et al., 1991).

Taken together with Chapter 3 of this thesis and data in the literature (e.g. Tabcharani et al., 1990), the results in this chapter strongly suggest that the outward rectifier does not mediate transepithelial Cl secretion in T_{84} cells.

IV ANION SELECTIVITY AND PHARMACOLOGY OF THE VOLUME REGULATORY Cl PATHWAY

Introduction

The ability to regulate cell volume is especially important for epithelia, which are exposed to external fluids having variable composition. Transient imbalances between the rates of solute entry and exit at opposite ends of the cell also cause volume stress during transepithelial transport. Many cells respond to swelling by activation of a regulatory volume decrease (RVD) in which osmotically active solutes are lost and water follows (see reviews, Siebens, 1985; Hoffmann, 1987). Although many solutes have been implicated in the RVD, early experiments on lymphocytes indicated that conductive K and Cl efflux pathways are activated by swelling (Grinstein et al., 1982), and this has since been confirmed for many other cell types (reviewed by Hoffmann, 1987). The purpose of this chapter is to investigate the role of the outward rectifier in cell volume regulation by determining the anion selectivity and inhibitor sensitivity of the volume regulatory anion conductance for comparison with the properties of the outward rectifier.

Materials and Methods

Preparation of the cells

T_{84} cells were cultured as described in Chapter 2 and dispersed into a suspension of single cells using a 0.5% trypsin solution. Trypsin activity was neutralized by the addition of 10 mL T_{84} media containing 5% FBS and the suspension was then centrifuged at 200 rpm for 5 min. The supernatant containing debris was discarded, and the cell pellet was resuspended in RPMI 1640 medium containing 10% bovine serum albumin (BSA) at pH

7.3. This suspension was equilibrated in a shaking water bath at 37 °C for 20-40 minutes before use in cell sizing studies.

Measurement of cell volume

Cell volume was estimated by electronic cell sizing using a Coulter counter (model Zm) and channelyzer (C-256). Fig. 1 shows the operation of the electronic particle counter. In this technique, cells are pulled through a small orifice (100 µm diam.) by a pump. As cells pass through, they interrupt a current which also flows through the opening. The size of each interruption is proportional to the volume of the particle. Aliquots of T_{84} cells were diluted to a final density of 30,000 cells/mL in NaCl solution, and mean cell volume (in picolitres; pL) was calculated as the median of a size distribution curve like the one shown in Fig. 2. The median was estimated as the midpoint between two cursors that had been set so as to bracket the peak. To study the changes in cell volume, relative cell volume was determined as the ratio of mean cell volume at time t to the mean cell volume at t=0 min. when the hypotonic shock began. Cellular volume was determined after 0.5, 2, 5, 10, and 20 minutes exposure to hypotonic solution. Volume determinations were calibrated using polystyrene latex beads of known sizes.

Solutions

The NaCl saline used during cell sizing experiments contained (in mM): 145 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 TES and 10 glucose. In high-K saline, sodium was completely replaced with potassium (148 mM K). To study the effects of anion replacement, a high-K saline was used and Cl was replaced with iodide, bromide, or nitrate. Stock solutions of IAA-94, NPPB, and gramicidin were prepared in DMSO at 100 mM, 100 mM, and 50 mM, respectively. DIDS was dissolved in water to make a stock solution of 100 mM. 5,8,11,14-eicosatetraynoic acid (ETYA) was dissolved in DMSO to make a stock concentration of 50 mM. All stock solutions were stored in the dark at -20 °C

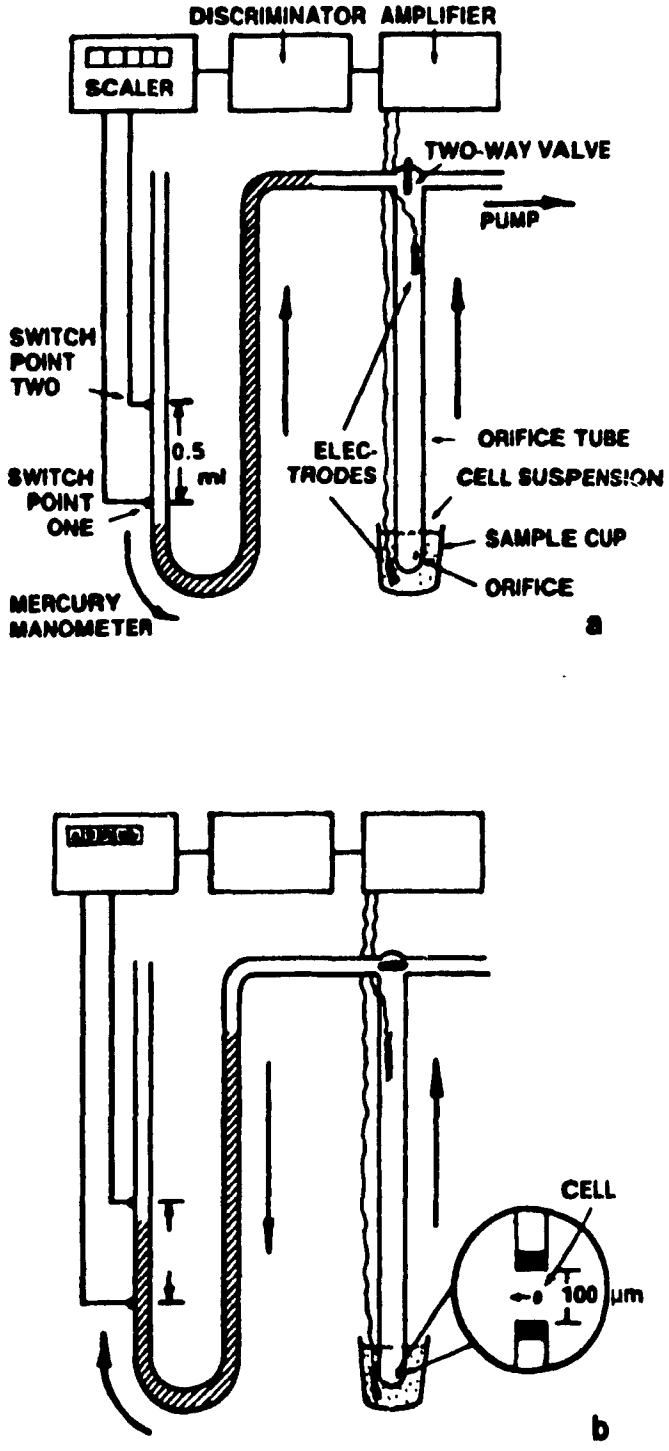
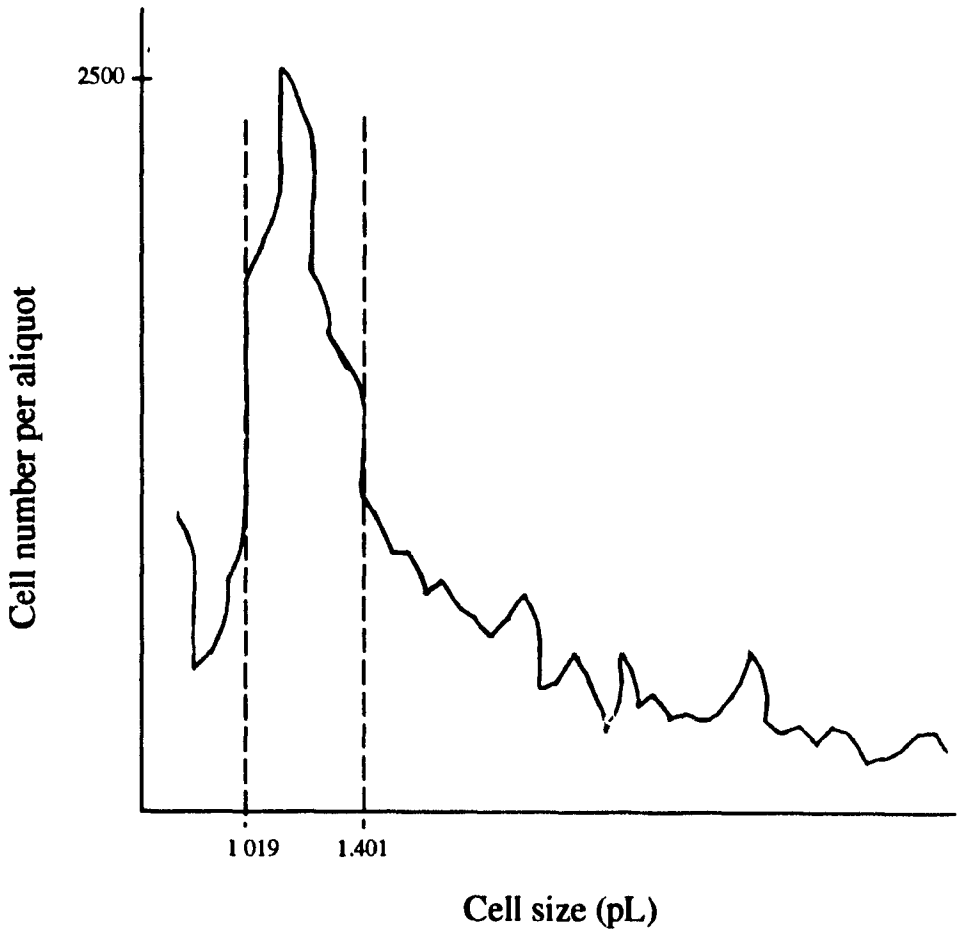


Figure 1: Schematic diagram illustrating the operation of the Coulter Counter.

a. The manometer is connected to the pump, bringing mercury up to the starting position.
b. Mercury returning to equilibrium draws sample up through the orifice and activates the count cycle.

Reproduced from Freshney, 1983.



Calculation of mean volume of T_{84} cells in aliquot

$V_1 = 1.019$ pL $\quad V_2 = 1.401$ pL

$\bar{V} = 1.21$ pL

Figure 2 : Volume distribution curve generated by the Coulter counter showing placement of cursors to determine mean volume of cells. The cursors (dashed lines) are positioned by the investigator on either side of the peak. A shift of the curve to the right indicates a volume increase.

except ETYA, which was stored at -70 °C. Inhibitors were thawed immediately before use. Experiments were carried out at 37 °C.

Results

T_{84} response to hypotonic shock

The average volume of T_{84} cells was 1.219 ±0.015 pL (n=30 experiments). The optimal cell density during cell sizing experiments was found empirically to be ~30,000 cells/mL. Lower cell densities gave a broad, diffuse histogram whereas densities >60,000 cells/mL caused the suspension to become cloudy and cells began to aggregate. Consistent readings were obtained if cells were allowed to equilibrate for 20 minutes in the shaking water bath at 37°C prior to sizing experiments. A robust RVD response to hypotonic challenge could be obtained for 2 hours following trypsinization; however, after this time, the swelling caused by a standard osmotic shock was reduced to less than 15% even though cells had normal resting volumes. T_{84} cells remained at a stable volume in the Coulter counter chamber for 10-15 minutes under isotonic conditions, and decreased by only 5% after 20 minutes (Fig. 3). When exposed to a 50% hypotonic shock the volume of T_{84} cells increased quickly by 26 ±0.03% and then shrank back to control levels after 10 min. of continued exposure to hypotonic saline (1.0 ±0.03). There was also a slow undershoot; that is, cells usually recovered to a slightly smaller volume (5-8% less than before osmotic shock), indicating a loss of intracellular solute during the RVD (Fig. 4).

K dependence of the regulatory volume decrease

Potassium efflux is proposed to be involved in the RVD of many cells. To study its involvement in T_{84} cells, Na in the bathing medium was replaced by K and cells were subjected to the same (50%) hypotonic shock as described above. Since intracellular K concentration is probably 120-150 mM, this maneuver should eliminate the electrochemical

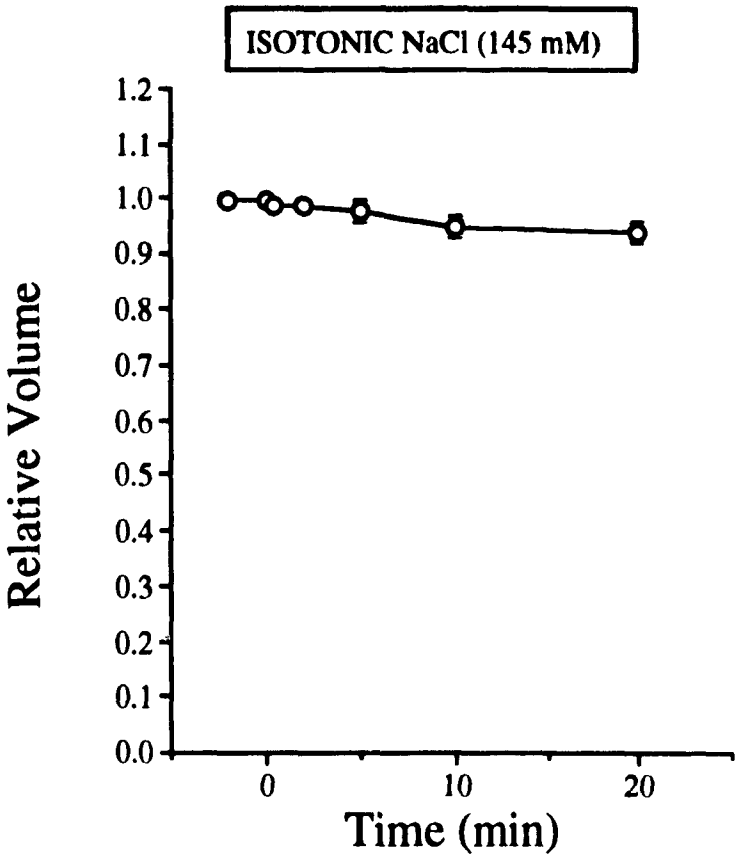


Figure 3 : Relative volume of T_{84} cells as a function of time under isotonic conditions (145 mM NaCl). After 20 minutes cell volume changed by only 5% under control conditions.

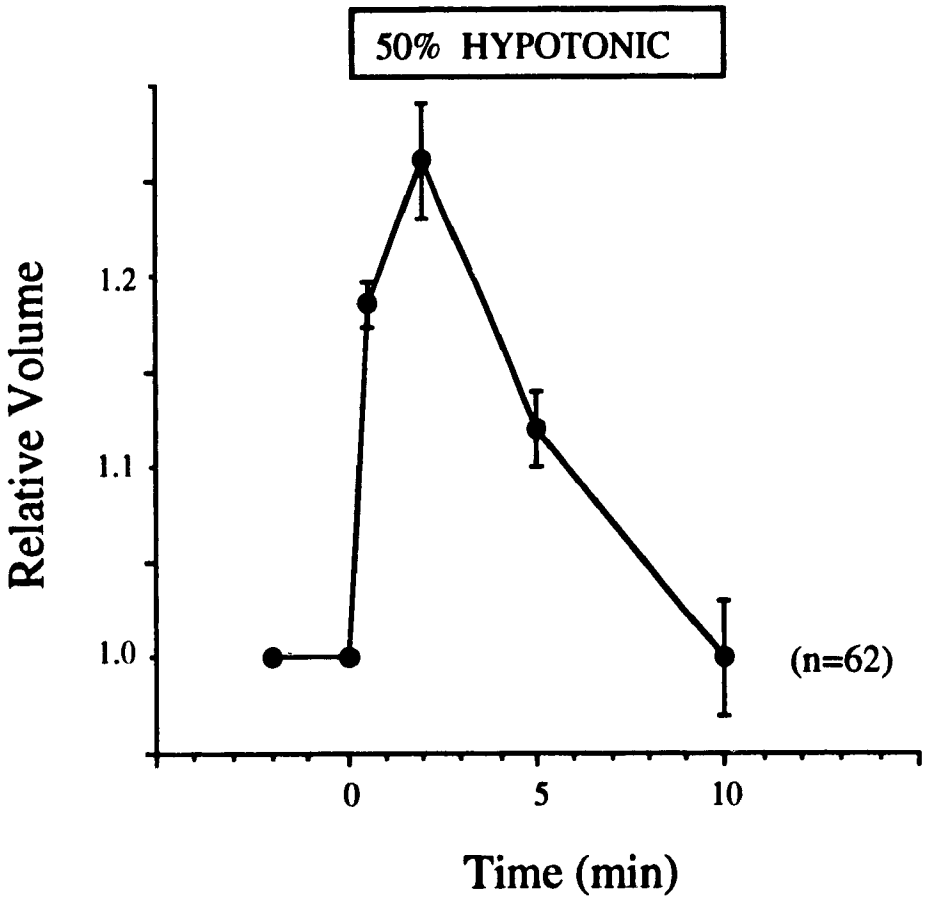


Figure 4: Response of suspended T_{84} cells to 50% hypotonic solution. Cells were initially bathed in isotonic NaCl media (145 mM), which was then diluted 50% with water at t=0 minutes. Relative volume increased to 1.26 ±0.03 of the control volume when measured 2 minutes after initiation of swelling.

gradient for K, thereby preventing net movement of K during the RVD. As shown in Figs. 5 and 6, cell volume regulation was indeed blocked under these conditions. In high K media, swelling began immediately during hypotonic challenge and continued to a volume of 1.37 ±0.03 pL after 2 min. and 1.49 ±0.04 pL after 10 min. These results suggest that the regulatory volume decrease requires K efflux and may involve activation of a K pathway in the plasma membrane.

Anion selectivity of the RVD

The RVD requires anion efflux, therefore it would seem that cells should be preloaded with other anions in order to assess their permeation through the pathway. However, this is problematic because many replacement anions are not carried by the $Na^+/K^+/2Cl^-$ co-transporter nor the Cl^-/HCO_3^- exchanger and there is no convenient way to ensure that all cellular chloride has been replaced by the test species. Therefore, the selectivity of the anion pathway activated in swollen cells was determined indirectly by studying the effects of extracellular anion substitutions on the rate of secondary swelling induced by hypotonic shock. These experiments were carried out in the presence of high-K saline and the cation-selective ionophore gramicidin. Under these conditions, the inward anion gradient provides a driving force for net salt influx and gives rise to secondary swelling (Fig. 7), however, anion entry is rate-limiting due to the cationophore. Thus the rate of secondary swelling in solutions containing different anions should indicate the relative permeability of the membrane to each anion.

Exposure to high K and gramicidin (2 μM) caused cells to swell more rapidly and to a 20% larger volume than exposure to high KCl saline alone (1.51 ±0.05 in high K alone vs 1.71 ±0.04 with gramicidin after 20 min., Fig. 8a). The hypotonic swelling response with gramicidin was not significantly different when cells were bathed in KBr rather than KCl saline (Fig. 8b). However, after 20 minutes, cell volume increased to 1.76 ±0.04 in

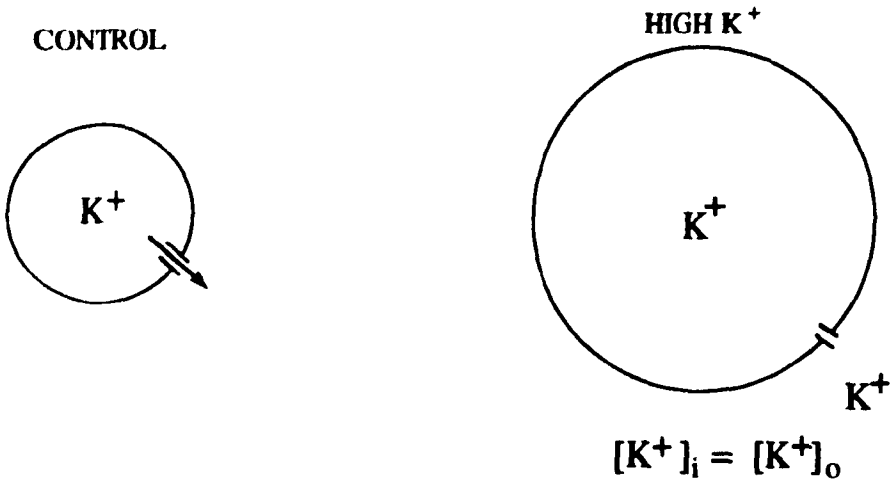


Figure 5: Cartoon showing the dependence of the regulatory volume decrease (RVD) on the transmembrane potassium gradient. The response is abolished when the net efflux of K is inhibited. K normally exits down its concentration gradient, however, in high-K solution there is no gradient and the movement of K is abolished.

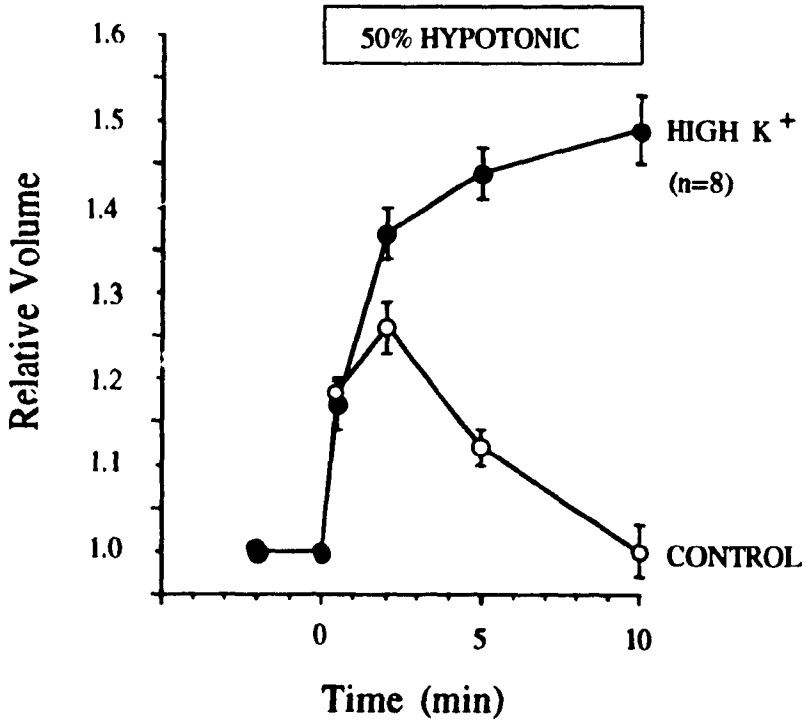


Figure 6: The dependence of the regulatory volume decrease on potassium. At t=0 minutes, T_{84} cells suspended in (○) NaCl or (●) KCl saline were given a hypotonic shock. The regulatory decrease to hypotonic shock was eliminated in high K media and cells were 49% larger than in control (NaCl) medium after 10 min exposure to hypotonic shock. n=8 ±s.e.

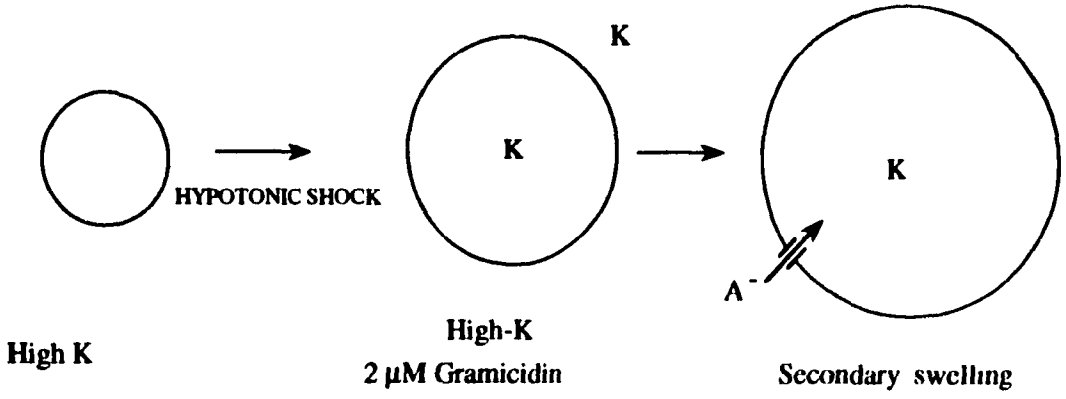


Figure 7: Cartoon illustrating the method used to assess the anion selectivity of the Cl pathway in swollen cells. Cells were bathed in high-K saline and hypotonically shocked by diluting the bath 50%. The ionophore gramicidin was also present to increase cation permeability, so that the anion conductance would be the pathway that limits subsequent (secondary) swelling. The rate of influx of different anions provides a measure of the relative permeability to each anion.

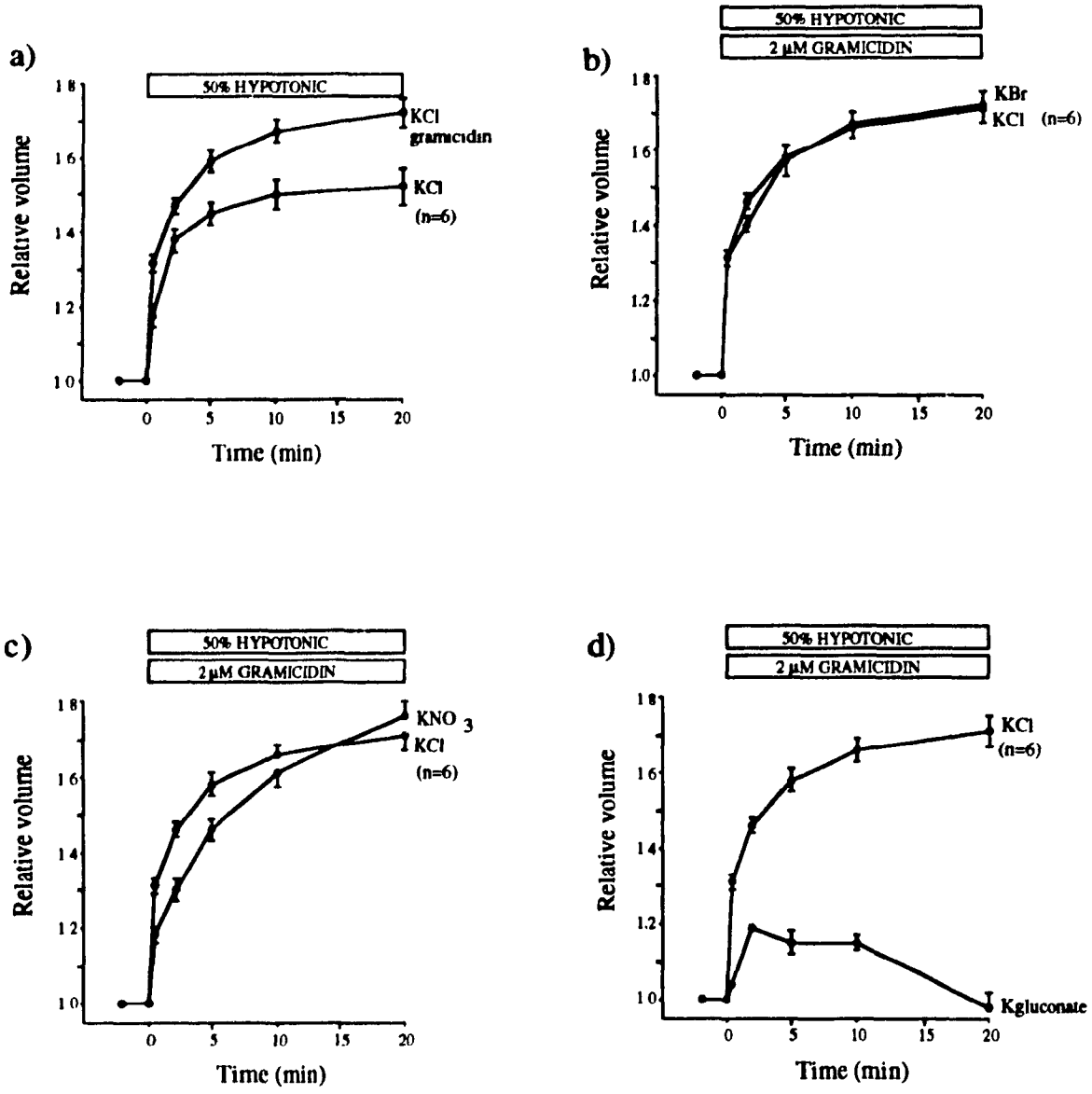


Figure 8: Anion selectivity

Cells were given a hypotonic shock in high K medium at *t*=0 minutes. Open circles (o) show that the cells swell and remain large in KCl medium, they cannot regulate their volume under these conditions.

a) Addition of gramicidin in the same KCl medium (at *t*=0 minutes) increases the rate and the amount of swelling. Cl swelling is increased in the presence of gramicidin (20%).
b) The degree of swelling in the presence of external Br was similar to that with Cl.
c) The degree of swelling in the nitrate solution was higher indicating high permeability to nitrate.
d) The cells did not swell in gluconate saline, which indicates the volume-activated anion conductance is not significantly permeable to gluconate.

KNO_3 saline and did not plateau, suggesting that NO_3^- was slightly more permeant than Cl^- (Fig. 8c). On the other hand, gluconate was less permeant; i.e. cells did not show any sustained increase in volume, only a transient swelling which probably reflects rapid movement of water into the cell following hypotonic shock. Thus the anion selectivity sequence for the swelling induced pathway under these conditions is NO_3>Br=Cl>>gluconate. It was not possible to determine iodide permeability because its effects were inconsistent, seven out of 16 preparations had a small secondary swelling response while the other nine did not.

Effect of Cl channel inhibitors on the RVD

The sensitivity of the RVD to several Cl channel inhibitors was assessed using the conventional swelling protocol, that is, by adding the inhibitor to the standard NaCl saline at the same moment the bath was diluted. All the inhibitors caused a dose dependent reduction in the RVD and increased the average maximal "swollen" size from 26% to 31% above that of control, unswollen cells.

IAA-94 had no effect on the RVD when tested at 10 μM (1.02 ±0.02, n=8, Fig. 9a), although this concentration was sufficient to block outward rectifiers during patch clamp studies (Chapter 2). Intermediate levels of inhibition were observed with 50 μM and 100 μM IAA-94 (Figs. 9b,c); in each case the cells swelled to a larger maximum size in the presence of IAA-94 and then partially recovered. The maximum volume at 2 min. with 100 μM IAA-94 was 1.29 ±0.02, as compared with 1.26 ±0.03 in the presence of 10 μM IAA-94. However at very high concentrations (1 mM) IAA-94 did inhibit the RVD, causing the cells to remain 30% (1.30) larger indefinitely when exposed to hypotonic medium. The effect of NPPB on cell volume responses to hypotonic shock was also tested at several concentrations and was also found to inhibit the RVD and increase the peak cell volume at

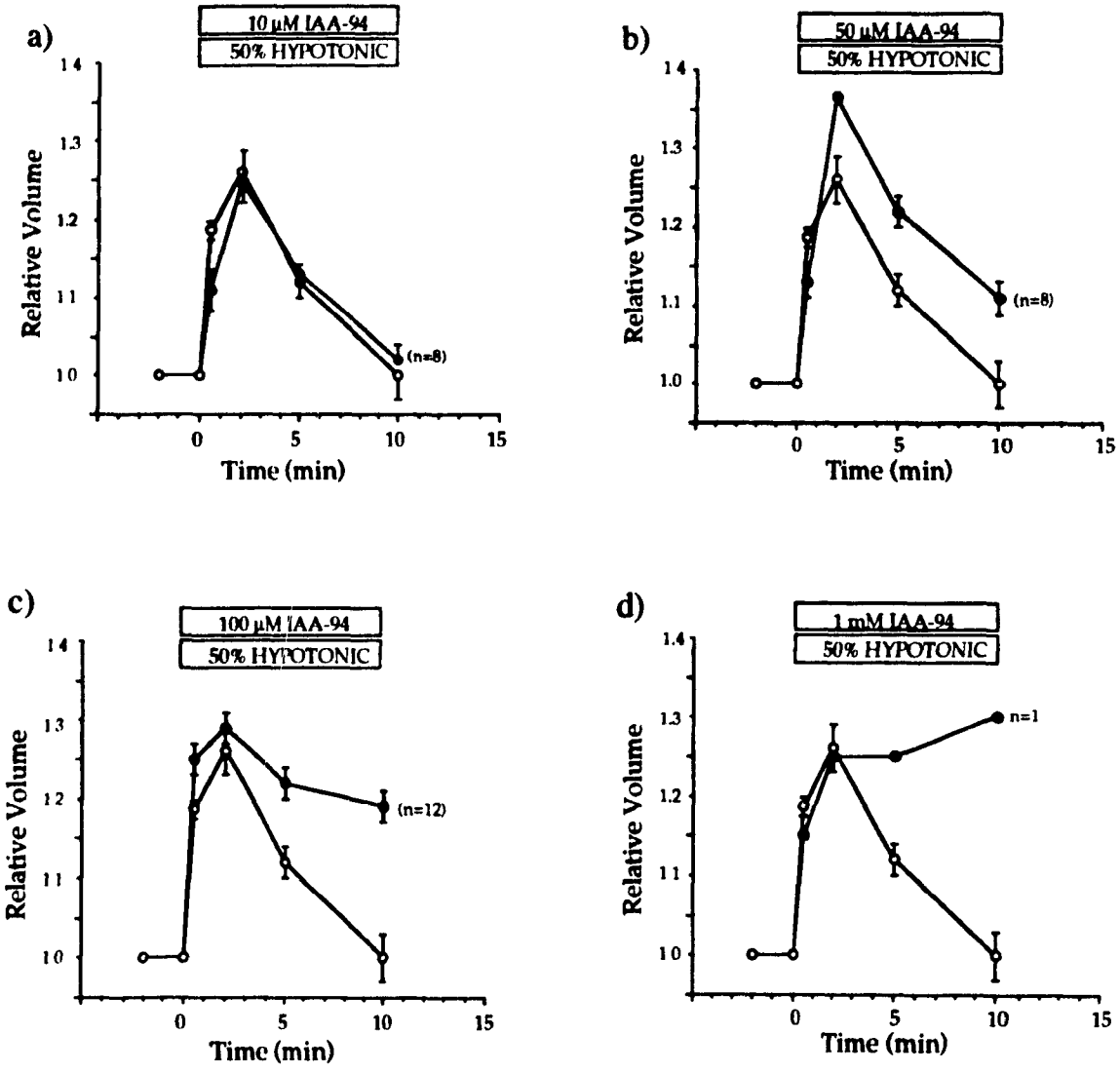


Figure 9: Effect of the Cl channel inhibitor IAA-94 on the regulatory volume decrease (RVD) in T84 cells.

Single cell suspensions were hypotonically swollen at t=0 minutes. The volume response in the absence of inhibitor (o) is compared with those in the presence of inhibitor (•).

a) 10µM IAA-94,
b) 50 µM IAA-94,
c) 100 µM IAA-94,
d) 1 mM IAA-94.

Mean ±s.e. and the number of preparations is indicated in each panel. Inhibitor was added at t=0 minutes.

high NPPB concentrations. The average peak volume was 1.21 ±0.02 in the presence of 50 μM NPPB and 1.3 ±0.04 with 100 μM NPPB (Fig. 10).

Finally, DIDS inhibited the RVD, although it was a much less potent blocker than either IAA-94 or NPPB. Peak volume was not larger in the presence of 50 μM DIDS, and cell volume recovered to within 10% of the resting size even in the presence of 100 μM DIDS (Fig. 11), a concentration that abolishes activity of the outward rectifier from these cells (Tabcharani et al., 1990).

Arachidonic acid metabolites have been implicated in the RVD (eg. Lambert et al., 1987) therefore the effects of ETYA, an inhibitor of arachidonic acid incorporation into cell lipids (Taylor et al., 1985), was also tested for its ability to block cell volume regulation. ETYA was a potent inhibitor of the RVD. It inhibited the RVD completely at 40 μM and caused cells to remain fully swollen (35% larger than control conditions) after 10 min. exposure to hypotonic solution (Fig. 12).

Discussion

The results in this chapter provide the first direct evidence that T_{84} cells regulate their volume following hypotonic shock, and that this regulatory volume decrease requires passive K and Cl effluxes. The single channel basis of the K pathway activated during the RVD was not investigated in this thesis although an inwardly-rectifying, calcium-insensitive K channel activated by hypotonic shock has been identified in T_{84} cells (Tabcharani and Hanrahan, 1991b). The relative selectivity of the Cl pathway among anions determined by following secondary swelling was found to have the sequence NO_3>Br=Cl>>gluconate. Several anion transport blockers inhibited the RVD, but only at very high concentrations. An inhibitor of arachidonic acid metabolism was also found to reduce the RVD.

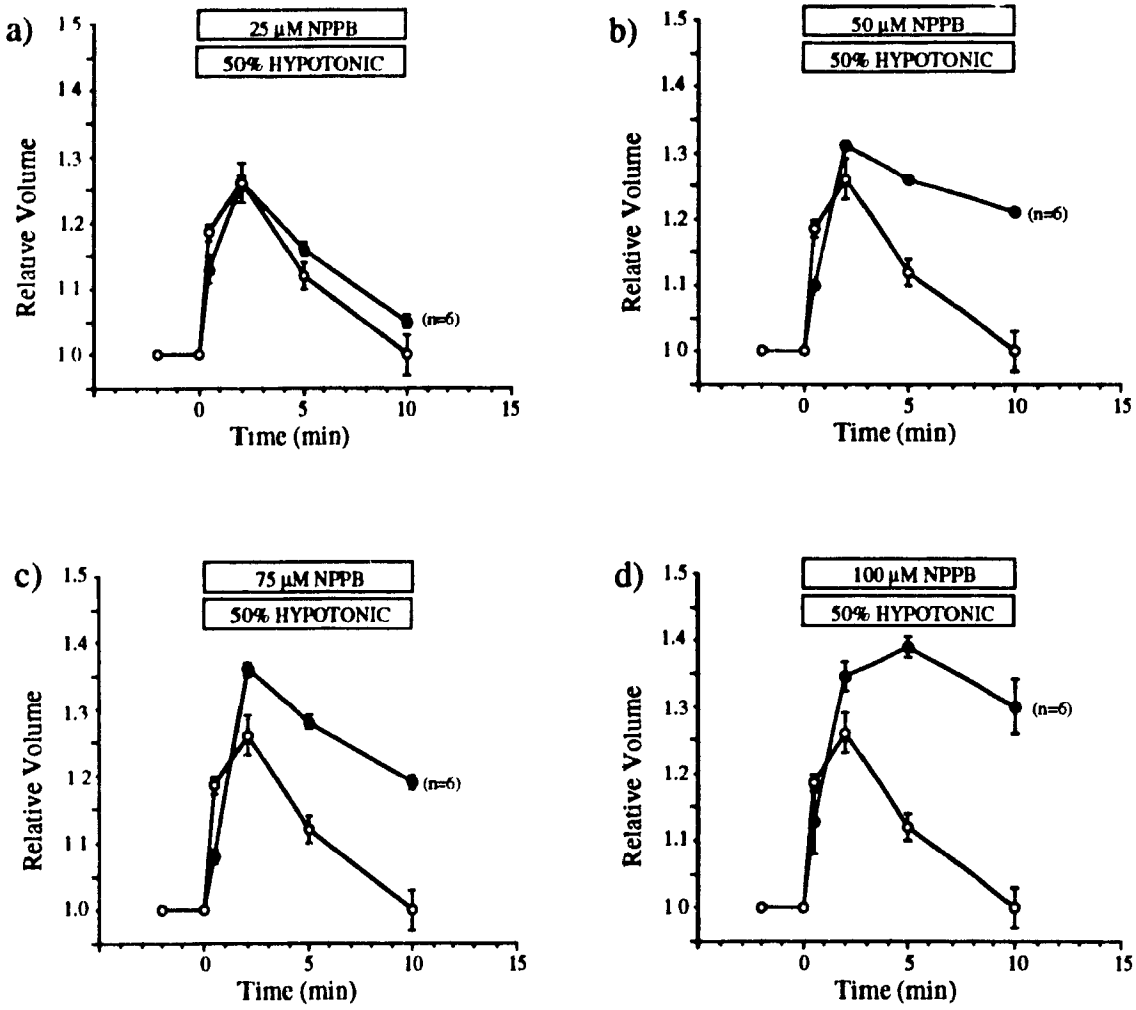


Figure 10: Effect of the Cl channel inhibitor NPPB on the RVD in T84 cells.

Same conditions as in Fig. 9 except NPPB was added. The concentrations used were

a) 25 µM NPPB,
b) 50 µM NPPB,
c) 75 µM NPPB,
d) 100 µM NPPB

Means ±s.e., n=6 preparations.

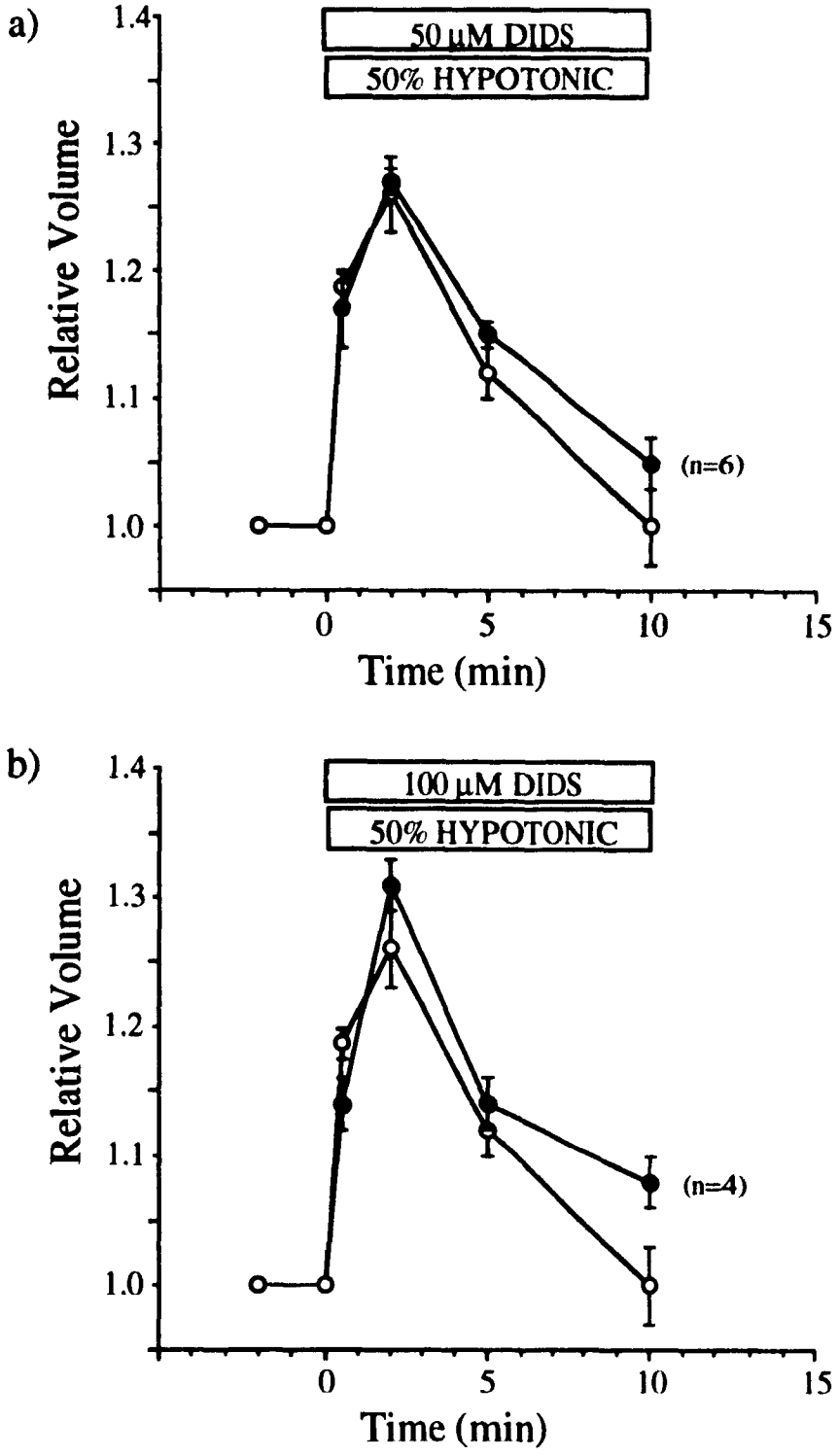


Figure 11: Effect of the Cl channel inhibitor DIDS on the RVD in T_{84} cells.

Same conditions as in Figs. 9 and 10, except DIDS was used.

a) 50 µM DIDS,
b) 100 µM DIDS.

Mean ±s.e., number of preparations are shown in each panel.

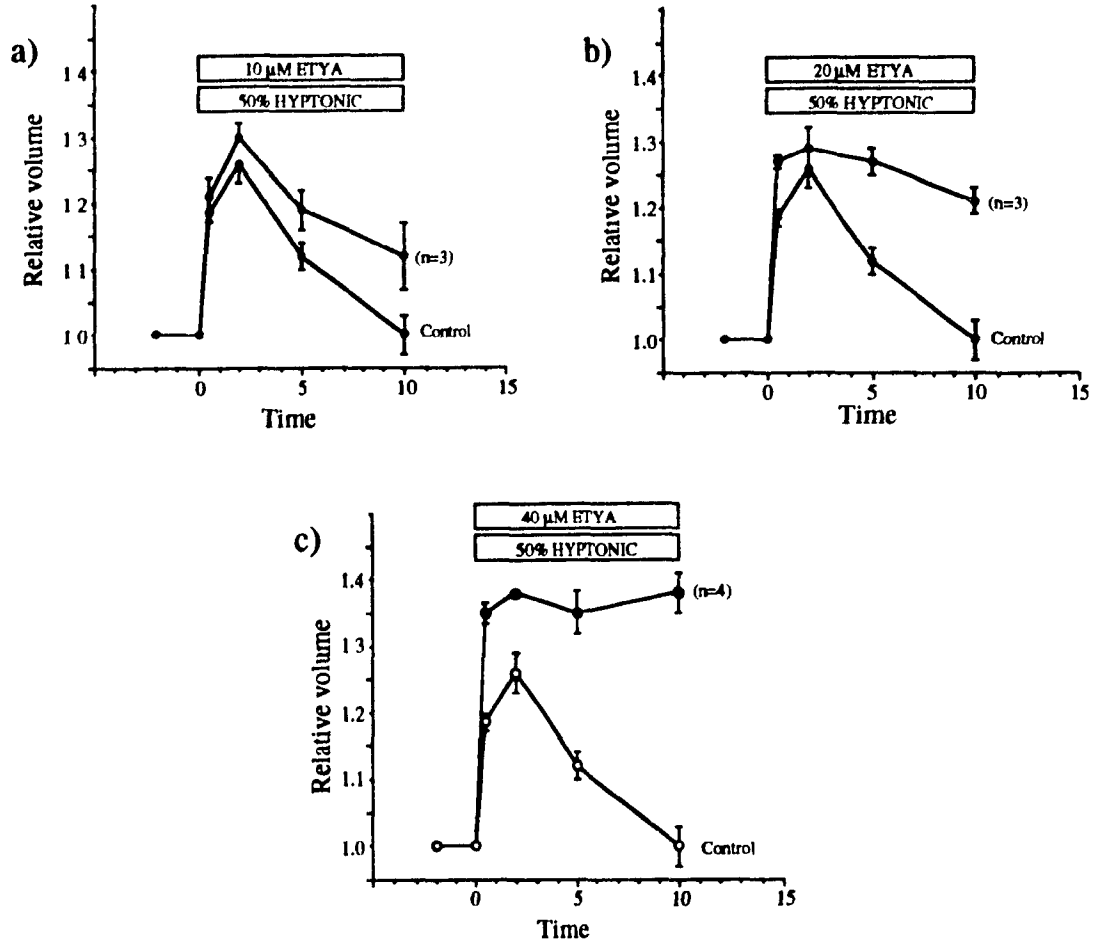


Figure 12: Effect of ETYA on the RVD.

Same conditions as in Figs. 9, 10, and 11 except that ETYA was used,

a) 40 µM ETYA,
b) 20 µM ETYA,
c) 10 µM ETYA.

Mean ±s.e. and number of preparations are shown in each panel.

The solutes lost during the RVD depend on cell type but most commonly they are K, Cl, and various amino acids (reviewed by Chamberlin and Strange, 1989). The signal that triggers the RVD is uncertain, although arachidonic acid metabolites (Lambert et al., 1987), hormones (Haussinger and Lang, 1990), and intracellular calcium (Eveloff and Warnock, 1987; Foskett and Spring, 1985; Wong and Chase, 1986; Rothstein and Mack, 1990) have been shown to influence the RVD in different cells. Separate K and Cl pathways have been implicated in the RVD of lymphocytes (Grinstein et al., 1982), Madin-Darby canine kidney (MDCK) cells (Ritter et al., 1991, Rothstein and Mack, 1990), *Necturus* gallbladder epithelium (Furlong and Spring, 1990), and guinea pig villus cells (MacLeod and Hamilton, 1991), although the Cl conductances activated have not been characterized electrophysiologically. Several groups have identified a Cl conductance in swollen cells that has some macroscopic properties consistent with the outward rectifier described in Chapter 2. Worrell et al. (1989) identified an outwardly rectifying whole cell Cl conductance in T_{84} cells that depends on bath osmolality. Solc and Wine (1991) identified a swelling-induced whole cell current in T_{84} cells that resembled the outward rectifier with respect to its ion selectivity and outward rectification, but the single channels had a higher conductance compared to outward rectifiers usually observed in excised patches.

Comparisons between macroscopic anion conductance and single channels in this chapter suggest that the outward rectifier does not mediate the Cl pathway which is activated during the RVD in intact T_{84} cells. The selectivity of the swelling-activated anion pathway (NO_3>Br=Cl>> gluconate) differed slightly from that determined for the outward rectifier in many systems in that Br was not more permeant than Cl (Reinhardt et al., 1987; Hanrahan and Tabcharani, 1989; Li et al., 1990; Halm and Frizzell, 1992). Also, the RVD and outward rectifier had different sensitivities to the inhibitors IAA-94, NPPB, and especially DIDS. IAA-94 or NPPB (100 μM) caused cells to swell more initially and

partially inhibited the subsequent RVD, however inhibition was modest at 50 μM or 75 μM considering that the outward rectifier would be almost completely inhibited at these concentrations. Moreover, DIDS had little effect on the RVD even at 100 μM, a concentration that abolishes activity of the outward rectifier (Tabcharani et al., 1990). These results suggest the outward rectifier is not involved in the RVD, although it should be noted that 100 μM DIDS has been reported to block the RVD in MDCK cells (Rothstein and Mack, 1990), OK cells (Knoblauch et al., 1989), and the swelling-activated transepithelial Cl secretion in T_{84} cells (N. Simmons, pers. commun.).

There are alternative explanations for the lower potency of blockers against the volume regulatory anion conductance and slightly different selectivity as compared to single outward rectifiers. If the swelling-activated Cl conductance is not rate-limiting during the RVD due to the presence of a parallel (DIDS-insensitive) leak pathway, the leak pathway might allow some volume regulation even when the volume-activated Cl channels are inhibited by DIDS. There is no way to be certain that the anion influx pathway responsible for the secondary swelling is the same one that mediates the RVD. Variations in the magnitude of the leak component among tissues might explain different apparent sensitivities of the RVD to DIDS and other inhibitors. A second possibility arises because the outward rectifier is normally studied in excised patches after activation by non-physiological stimuli (reviewed by Hanrahan et al., 1993). These maneuvers might artifactually enhance the channel's sensitivity to DIDS in patch clamp experiments. For these reasons, the possibility that the outward rectifier contributes to cell volume regulation cannot be rigorously excluded despite discrepancies in blocker potency.

Interestingly, the properties of the RVD match those of the ClC-2 chloride channel more closely than those of the outward rectifier. Like the RVD in T_{84} cells (this thesis) the ClC-2 channel is only weakly sensitive to 9-AC and DPC (~50% at 1 mM) and is almost

insensitive to 1 mM DIDS (Thiemann et al., 1992). ClC-2 also has similar permeability to Cl and Br, consistent with the swelling-activated anion conductance in T_{84} cells observed here; although it should be noted that a selectivity ratio assessed by secondary swelling is probably more equivalent to a conductance ratio than to an equilibrium permeability ratio, which would depend on different properties of the channel. Expression of ClC-2 in T_{84} cells has been reported (Gründer et al., 1992).

Although T_{84} cells swelled rapidly during exposure to hypotonic (50%) medium, they did not double in size as expected for an ideal osmometer. The most likely explanation for this finding is that cells did not reach maximum volume until 2 min. after hypotonic shock, by which time the RVD was already in progress. This would account for the submaximal swelling response and seems reasonable in view of reports that RVD in other cells can be elicited by only 5-15% swelling (Hoffmann, 1987). The observation that high concentrations of NPPB and IAA-94, which inhibited the RVD, also increased the peak of the initial swelling response would be consistent with this hypothesis, since inhibiting the RVD during the early phase would permit a larger initial swelling response. Another possible explanation for the apparent deviation from ideal behaviour is the fact that cells are not perfectly spherical and some of the volume increase might be accommodated by microscopic unfolding at the cell surface without changing the apparent macroscopic cell diameter significantly.

The intracellular signals involved in controlling cell volume have not yet been identified although leukotrienes, diacylglycerol, PKC, and cAMP have been implicated in Ehrlich ascites tumor and other cells in the opening of volume regulatory channels (Lambert et al., 1987). The preliminary studies in this chapter using ETYA are consistent with the involvement of arachidonic acid metabolites in the T_{84} cell RVD; however, further studies are needed to confirm this and to identify the signaling molecule. If ClC-2 mediates Cl

conductance during the RVD, the signaling pathway must ultimately interact with the short, amino-terminal end of the protein which has been identified as the "volume sensor" region of the channel.

In summary, these studies of the selectivity and pharmacology of the swelling-activated Cl pathway do not exclude a role for the outward rectifier in regulating T_{84} cell volume; however, they show that the properties of the swelling activated anion pathway are most consistent with those of the ClC-2 chloride channel. Further patch clamp studies of T_{84} cells, and of cells transfected with ClC-2 or antisense oligonucleotides to ClC-2 are needed to confirm this hypothesis.

V CONCLUSION

The intent of this study was to define the physiological role of the outwardly rectifying Cl channel in epithelia using a pharmacological approach. Previous single channel studies have shown that this channel is abundant in the apical membrane of PANC-1 and T_{84} cells. The single channel characteristics are identical in both cell types and closely resemble outward rectifiers in epithelia from the airways, sweat gland, and fetal pancreas. The patch clamp results in Chapter 2 of this thesis show that the channel is highly sensitive to the inhibitors IAA-94 and NPPB. The insensitivity of the transepithelial short-circuit current to these blockers in Chapter 3 rule out the involvement of this channel in Cl secretion by T_{84} monolayers; inhibitors that decrease the open probability of single channels to zero had no effect on I_{SC} at similar concentrations. This conclusion, which was published previously (Tabcharani et al., 1990), has been confirmed by many laboratories and strengthened by the finding that CFTR is itself a secretory chloride channel having very different properties from those of the outward rectifier. An alternative physiological role for the outward rectifier, cell volume regulation, was then tested in Chapter 4. Electronic cell sizing experiments showed that IAA-94 and NPPB both inhibit cell volume regulation by T_{84} cells in a concentration-dependent manner, but at significantly higher concentrations than required for inhibition of the outward rectifier. This discrepancy was especially obvious for DIDS, which was at least ten-fold more potent against single outwardly-rectifying anion channels than it was against the RVD.

The present results provide strong evidence against a role for the outward rectifier in Cl secretion across T_{84} cells, and also argue against a major role in cell volume regulation. On the other hand, the cell sizing studies are consistent with a volume regulatory role for another type of Cl channel (ClC-2) which has recently been cloned and is expressed in T_{84} cells. In the absence of a specific blocker, identification of the gene encoding the outward

rectifier will probably be needed before a definite conclusion can be reached regarding its physiological role.

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