J. Membrane Biol. 147, 173–183 (1995)

The Journal of
Membrane
Biology

Volume-activated Chloride Currents in Pancreatic Duct Cells

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Received: 10 December 1994/Revised: 5 May 1995

Abstract. We have used the patch clamp technique to study volume-activated Cl^- currents in the bicarbonate-secreting pancreatic duct cell. These currents could be elicited by a hypertonic pipette solution (osmotic gradient 20 mOsm/l), developed over about 8 min to a peak value of 91 ± 5.8 pA/pF at 60 mV (n = 123), and were inhibited by a hypertonic bath solution. The proportion of cells which developed currents increased from 15% in freshly isolated ducts to 93% if the ducts were cultured for 2 days. The currents were ATP-dependent, had an outwardly rectifying current/voltage (*I-V*) plot, and displayed time-dependent inactivation at depolarizing potentials. The anion selectivity sequence was: $ClO_4 = I = SCN > Br = NO_3 > Cl > F > HCO_3 >$ gluconate, and the currents were inhibited to a variable extent by DIDS, NPPB, dideoxyforskolin, tamoxifen, verapamil and quinine. Increasing the intracellular Ca^{2+} buffering capacity, or lowering the extracellular Ca^{2+} concentration, reduced the proportion of duct cells which developed currents. However, removal of extracellular Ca^{2+} once the currents had developed was without effect. Inhibiting protein kinase C (PKC) with either the pseudosubstrate PKC (19–36), calphostin C or staurosporine completely blocked development of the currents. We speculate that cell swelling causes Ca^{2+} influx which activates PKC which in turn either phosphorylates the Cl^- channel or a regulatory protein leading to channel activation.

Key words: Pancreatic duct cells — Patch-clamp — Whole-cell recording — Volume-sensitive Cl^- current

Introduction

Volume-activated Cl^- channels play a key role in the mechanism by which cells limit the volume change that occurs in response to conditions that cause cell swelling (for reviews *see* [20, 29]). Activation of these Cl^- channels (in concert with K^+ channels) leads to efflux of Cl^- and K^+ from the cell, loss of cell water by osmosis, and a return of cell volume towards the normal, isotonic, value. This process is known as regulatory volume decrease (RVD). Cell swelling and a subsequent RVD can be initiated either by exposing cells to a hypotonic solution or by stimulating the transport of osmotically active solutes into the cell (e.g., as occurs following activation of Na-glucose and Na-amino acid transporters). Such conditions would be frequently encountered by epithelial cells lining the gut and proximal renal tubule and in these cells RVD mechanisms are well developed and have been extensively studied [29].

Pancreatic duct cells secrete the bicarbonate ions that are found in pancreatic juice (for a review *see* [3]). We have recently shown that these cells contain both cAMP-activated (cystic fibrosis transmembrane conductance regulator, CFTR) [15] and Ca^{2+}-activated Cl^- conductances [17, 33]. The channels that underlie these conductances are located on the apical plasma membrane of the duct cell, and play a key role in cAMP- and Ca^{2+}-mediated bicarbonate secretion [3]. Under physiological conditions pancreatic duct cells should not be exposed to circumstances which cause cell swelling; pancreatic juice is isotonic with plasma and, as far as we know, these cells do not absorb either amino acids or sugars [3]. Thus there is no *a priori* reason why pancreatic duct cells should need an RVD mechanism. Therefore, we were surprised to find that under certain conditions these cells exhibit a large volume-sensitive Cl^- conductance. In this report we detail the basic characteristics of this conductance, and investigate how its activity and its expression are regulated.

Some of our observations have been presented in preliminary form [43, 44].

Materials and Methods

Pancreatic Duct Cells

The isolation, culture, and separation of rat pancreatic ducts into single epithelial cells is described in detail elsewhere [2, 15, 17]. In brief, the

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pancreas of Wistar rats, fed on a standard laboratory diet, was dissociated with collagenase and small interlobular pancreatic ducts were microdissected from the tissue fragments. The isolated ducts were usually maintained in culture for up to 2 days as previously described [2], and then dissociated into single cells for electrophysiological studies [15, 17]. In experiments to determine the effect of culture time, cells from freshly isolated ducts were also used.

Electrophysiology

The cell preparations were transferred to a tissue bath (volume 1.5 ml) mounted on a Nikon Diaphot inverted microscope and viewed using phase contrast optics. Current recordings were made at 21–23°C from single duct cells using the whole-cell configuration of the patch clamp technique as previously described [15, 17]. Pipettes were pulled from borosilicate glass (Clarke Electromedical, UK) and had resistances, after fire polishing, of between 2 and 5 MΩ. Seal resistances were typically between 10 and 30 GΩ.

Whole-cell currents were recorded with an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, FRG) usually by employing two basic voltage-clamp protocols. (i) During continuous recording, the membrane potential was held at 0 mV and then alternately clamped to ± 60 mV for 1 sec. Between each pulse there was a 1-sec interval at the holding potential. (ii) To obtain current-voltage (*I-V*) relationships the membrane potential was held at 0 mV and then clamped over the range ± 100 mV in 20 mV increments. Each voltage step lasted 500 msec and there was an 800 msec interval at the holding potential between steps. Data were filtered at 1 kHz and sampled at 2 kHz with a Cambridge Electronic Design 1401 interface (CED, Cambridge, UK), and stored on either a digital tape recorder or the computer hard disk.

Instantaneous *I-V* plots were constructed using the average current measured over a 2 msec period starting 4 msec into the voltage pulse. The currents were not leak corrected. Series resistance (R_s) was typically two to three times the pipette resistance, and R_s compensation (40–70%) was routinely used. Membrane potentials (V_m) have been corrected for current flow (I) across the uncompensated fraction of R_s using the relationship: $V_m = V_p - IR_s$, where V_p is the pipette potential. Junction potentials were measured and the appropriate corrections applied to V_m as previously described [15, 17]. Reversal potentials (E_{rev}) and conductance data were obtained from *I-V* plots after fitting a third order polynomial using least squares regression analysis. E_{rev} was obtained by interpolation, and whole-cell conductances have been calculated between E_{rev} and ± 60 mV. Anion permeability ratios were derived from E_{rev} values using the Hodgkin-Katz modification of the Goldman equation [15, 16].

The input capacitance of isolated single cells was routinely measured using the analogue circuitry of the EPC-7 amplifier and compensated prior to the start of recording. The average input capacitance of cells was 3.1 ± 0.1 pF (n = 123). Capacitance values were used to calculate current density which is expressed as picoamperes per picofarad (pA/pF).

Solutions and Chemicals

To block the duct cell potassium conductance, we routinely used a caesium-rich pipette solution which contained (mM): 155.0 CsCl, 2.0 $MgCl_2$, 0.2 EGTA, 1.0 ATP and 10.0 N-2-hydroxyethylpiperazine-N′-ethanesulphonic acid (HEPES), pH 7.2. The calculated free calcium concentration in this solution was 7.1 nM, and its osmolarity, measured using a freezing point depression osmometer (Roebling) was 310 mOsm/l. Pipette solutions were filtered through a 0.2 μm membrane filter before use. The standard bath solution contained (mM): 138.0 NaCl, 4.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 5.0 glucose, 10.0 HEPES, pH 7.4, osmolarity 290 mOsm/l. A Ca^{2+}-free solution was prepared by reducing the standard bath $CaCl_2$ concentration to 602.5 μM and adding 1 mM EGTA (0.1 μM calculated free Ca^{2+}). Bath solution changes were accomplished by gravity feed from reservoirs at a flow rate of 5–6 ml/min.

A 50 mM stock solution of 1,9-dideoxyforskolin (Calbiochem) and a 10 or 20 mM stock solution of the carboxylate analogue 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (a gift from Prof. R. Greger, Frieburg, FRG) were made up fresh daily in dimethyl sulfoxide (DMSO), and diluted to the required concentration in the standard bath solution. Initial control experiments showed that volume-sensitive Cl^- current was unaffected by a bath solution containing 0.5% DMSO (n = 3). Dideoxyforskolin solutions were sonicated briefly before use. The stilbene derivative 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), verapamil and quinine (all from Sigma) were dissolved directly into the bath solution at the required concentration. Gadolinium was added to the bath solution as $GdCl_3$ (Aldrich Chemical) at a final bath concentration of either 50 or 100 μM. Three inhibitors of protein kinase C (PKC) were used in this study. The synthetic peptide pseudosubstrate, PKC(19–36) (Calbiochem) [21], was prepared as a 1 mM stock solution in 0.5 M acetic acid and then diluted in the pipette solution to a final concentration of 10 μM. Acetic acid alone had no effect on the whole cell currents. The membrane permeable inhibitors of PKC, staurosporine [39] and calphostin C [4] (both from Sigma), were made up as 5 mM stock solutions in DMSO and diluted in bath solution to the required final concentrations. Calphostin C was activated by incubation with the duct cells under fluorescent light for about 1 hr [4]. The peptide inhibitor of protein kinase A, PKI (5–24) (Calbiochem) [8] was dissolved directly in the pipette solution. All other chemicals were purchased from commercial sources and were of the highest purity available.

Statistics

Significance of difference between means was determined using the Mann Whitney *U* test. Significance of difference between the number of cells responding to a particular maneuver was assessed using the Fischer's exact test. The level of significance was set at $P \leq 0.05$. All values are expressed as mean ± SE (number of observations).

Results

Activation of the Volume-Sensitive Currents

We found that once a whole-cell recording was established under nonswelling conditions (135 mM CsCl in the pipette, 270 mOsm/l; with the standard bath solution), the GΩ seal was invariably lost if the cells were exposed to a hypotonic shock (20% dilution of the bath medium). Therefore, in all the experiments described here, swelling of the duct cells, and thus activation of the volume-sensitive currents, was induced by using a pipette solution which contained 155 mM CsCl, making it approximately 20 mOsm/l hypertonic to the standard bath medium. We were able to confirm that cell swelling occurred under these conditions by direct visual observation using ×400 phase contrast optics.

Figure 1*A* shows the time course of current activation with the hypertonic pipette solution. Immediately

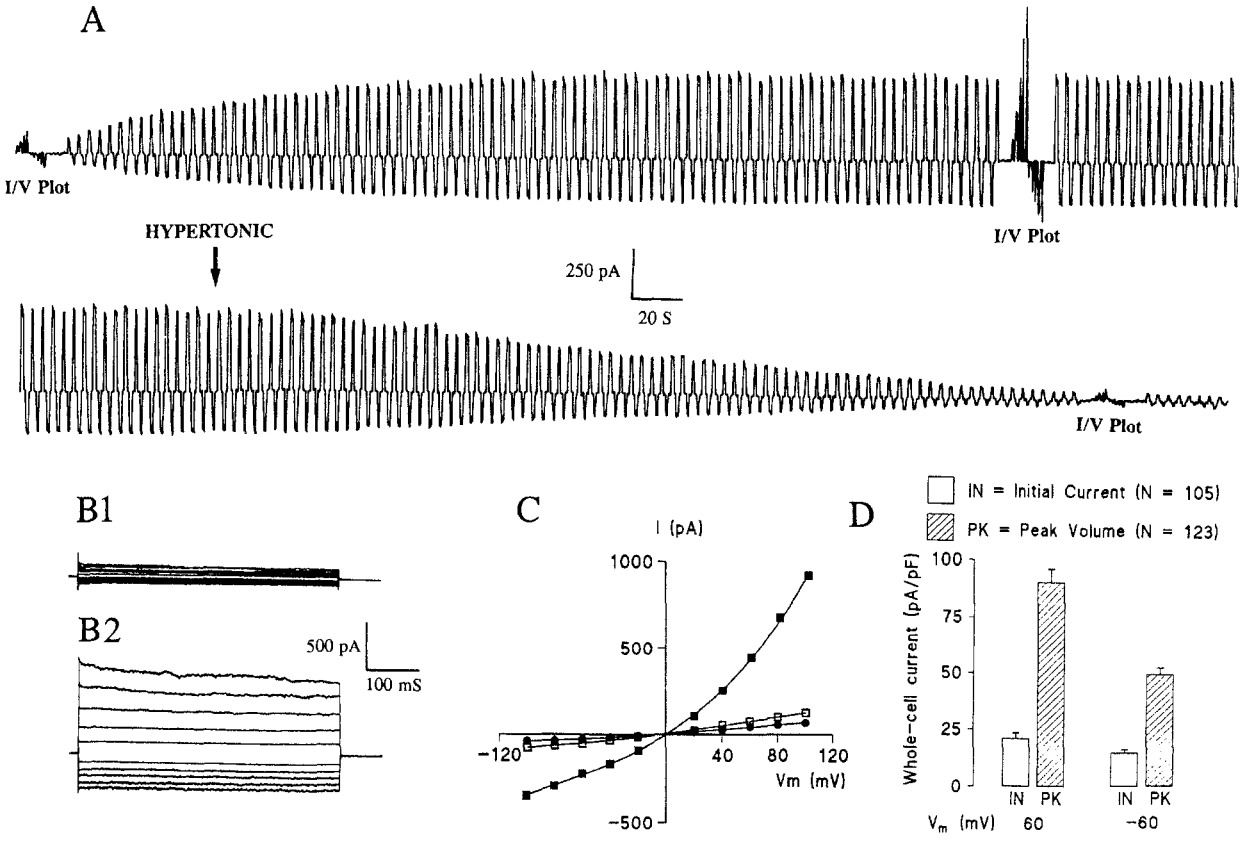

Fig. 1. The effects of internal hypertonicity (pipette, 310 mOsm/l; bath, 290 mOsm/l) on whole-cell current. (*A*) continuous recording starting 30 sec after the establishment of the whole-cell configuration. Current developed steadily, in this case reaching a peak amplitude after approximately 240 sec. Raising bath osmolarity to a level hypertonic to the pipette solution (384 mOsm/l) reversed the response. Recording was obtained using the ± 60 mV protocol and was interrupted by the ± 100 mV protocol (*see* Materials and Methods) as indicated to establish *I-V* relationship. (*B*) currents elicited by the ± 100 mV protocol immediately after establishment of whole-cell configuration (*B1*) and after the full development of volume-sensitive currents (*B2*). Recordings were obtained from the same cell as *A* (as indicated). (*C*) instantaneous current-voltage relationship (currents measured 5 msec after the onset of step voltage) corresponding to the *I-V* plots indicated in *A* and the currents shown in *B*. Open squares, initial currents; filled squares, peak volume-sensitive currents; filled circles, inhibition of volume-sensitive currents by hypertonic bath medium. (*D*) summary of values obtained for initial current (measured within 90 sec of establishing whole-cell configuration) and peak current. Currents were measured at E_{rev} ± 60 mV and have been normalized to cell input capacitance.

on establishing a whole cell recording small currents were observed (Fig. 1*A* and *B*1). These initial currents then increased with time to reach a peak value after about 5 min (Fig. 1*A*). The peak currents showed time-dependent inactivation at depolarizing step potentials of ⩾60 mV (Fig. 1 *B*2). Inactivation could be optimized by applying a holding potential of −80 mV and stepping the voltage to 120 mV. Under these conditions, the current decayed to about 60% of its original value after 500 msec, while full inactivation took longer than 10–15 sec (*data not shown*). Volume-sensitive currents also displayed an outwardly rectified instantaneous *I-V* plot (Fig. 1*C*), and an E_{rev} close to the calculated equilibrium potential for Cl^- of 1.7 mV (Fig. 1*C*). Similar results were obtained using a pipette solution containing 155 mM NMDG-Cl (20 mOsm/l hypertonic to the bath) indicating that current development is not simply caused by the presence of Cs^+ ions inside the cell. Peak currents under these conditions were 115 ± 39 pA/pF and −61 ± 20 pA/pF at +60 mV and −60 mV respectively (n = 6). Increasing the pipette CsCl concentration from 155 to 170 mM (osmolarity 328 mOsm/l, i.e., now 38 instead of 20 mOsm/l hypertonic to the standard bath solution) had no appreciable effect on either the time course of development or the peak amplitude of the currents. Finally, Fig. 1*A* also shows that adding 90 mM sucrose to the standard bath solution (osmolarity 384 mOsm/l, i.e., 74 mOsm/l hypertonic to the pipette solution) slowly inhibited the currents, confirming that they are regulated by the osmotic gradient across the cell membrane. Similar results were obtained in six other experiments. Taken together, these data suggest that rat pancreatic duct cells possess a volume-sensitive chloride conductance that is fully activated by a transmembrane osmotic gradient of 20 mOsm/l.

Responses of the type illustrated in Fig. 1*A* occurred

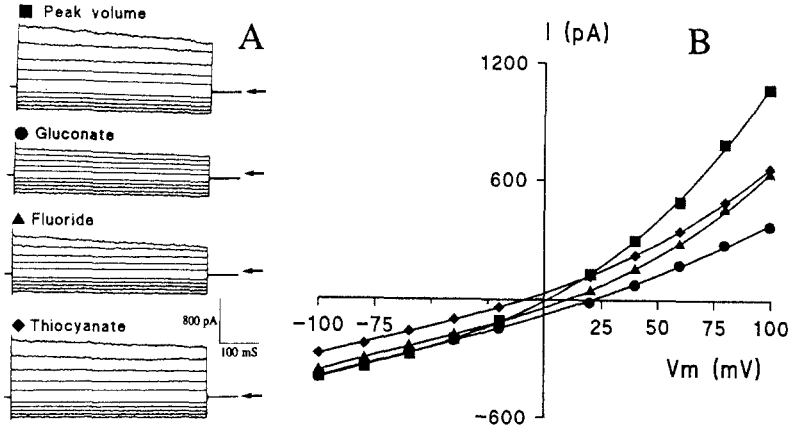

Fig. 2. Anion selectivity of volume-activated currents. (*A*) representative data from a single cell showing currents evoked using the ± 100 mV protocol described in Materials and Methods. Currents were recorded 60 sec after replacement of 100 mM bath chloride with the indicated anion. Arrows indicate zero current level. (*B*) instantaneous *I-V* plots from currents shown in *A*.

Table. Anion selectivity of volume-activated whole-cell currents

Anion	Reversal potential shift	P_x/P_{Cl}
Perchlorate (5)	−9.9 ± 1.0	1.86 ± 0.09
Iodide (5)	−9.5 ± 1.5	1.82 ± 0.14
Thiocyanate (6)	−8.0 ± 0.9	1.69 ± 0.08
Bromide (6)	−4.3 ± 1.2	1.41 ± 0.10
Nitrate (8)	−3.6 ± 0.6	1.35 ± 0.04
Chloride	0.0	1.0
Fluoride (8)	6.8 ± 0.8	0.74 ± 0.38
Bicarbonate (4)	9.4 ± 0.9	0.62 ± 0.04
Gluconate (64)	16.9 ± 0.5	0.35 ± 0.02
Low NaCl (20)	18.2 ± 0.7	0.30 ± 0.03

Values are means ± SE with the number of observations in parentheses. Relative permeability ratios (P_x/P_{Cl}) were calculated from the shifts in the reversal potential as described in Methods.

in 80% (123/154) of cells tested using the 155 mM-CsCl pipette solution, demonstrating that volume-sensitive currents are present in the majority of rat pancreatic duct cells. In this series of experiments, the initial currents measured 21.0 ± 2.5 pA/pF at 60 mV, and −14.7 ± 1.6 pA/pF at −60 mV (n = 105) (Fig. 1*D*). In 43 of these cells (41%) the initial current rectified slightly in the outward direction. In the remaining cells, initial currents were very small (< 10 pA/pF at ± 60 mV) and had linear *I-V* plots. Usually, the current began to increase within 3 min of establishing a whole-cell recording, reaching peak amplitude after a mean time of 8.7 ± 0.4 min (n = 118). The average peak currents were 91.0 ± 5.8 pA/pF and −49.9 ± 3.0 pA/pF at 60 and −60 mV respectively (n = 123) (Fig. 1*D*). The mean E_{rev} for all the volume-sensitive currents recorded was −1.80 ± 0.35 mV (n = 123).

Ionic Selectivity of Volume-Sensitive Currents

The anion selectivity sequence was examined by replacing 100 mM of the bath NaCl with the sodium salts of various anions. The anion permeabilities relative to chloride were then calculated from the shifts in E_{rev} using the Goldman-Hodgkin-Katz equation. We have assumed that anion replacement does not alter any cation conductance in the duct cells. Figure 2*A* shows currents from a representative experiment on a single duct cell in which extracellular chloride was partially replaced by gluconate, fluoride and thiocyanate. From the corresponding *I-V* plots (Fig. 2*B*), it can be seen that gluconate and fluoride caused a positive shift in E_{rev} indicating that these anions are less permeable than chloride. In contrast, thiocyanate caused a negative shift in E_{rev} indicating a higher permeability than chloride.

The Table summarizes all the permeability data that yield an anion selectivity sequence of: $ClO_4 = I = SCN > Br = NO_3 > Cl > F > HCO_3 >$ gluconate. Although gluconate was the least permeable anion tested, the $P_{gluconate}/P_{chloride}$ permeability ratio of 0.35 ± 0.02 (n = 64) indicates that the volume-sensitive conductance selects for chloride over gluconate only by a factor of 2.9. Furthermore, removal of 100 mM extracellular NaCl, while maintaining bath osmolarity by the addition of sucrose, caused E_{rev} to shift by 18.2 ± 0.7 mV (n = 20), a value appreciably lower than that predicted by the Nernst equation for a perfectly chloride-selective conductance (28.3 mV). Thus although the major component of the volume-sensitive current in pancreatic duct cells is carried by Cl^- ions, the conductance is also permeant to large anions such as gluconate, and also exhibits some cation permeability (Table).

Pharmacological Inhibition of Volume-Sensitive Current

Figure 3 compares the effects of DIDS, NPPB, dideoxyforskolin (all at 50 μM) and tamoxifen (5 μM) on peak volume-sensitive currents. DIDS and NPPB are commonly employed as chloride channel blockers, whereas dideoxyforskolin and tamoxifen have only recently been reported to inhibit volume-sensitive currents in other cells [41, 42]. The inhibitory effect of 50 μM-DIDS was apparent immediately and the block was voltage depen-

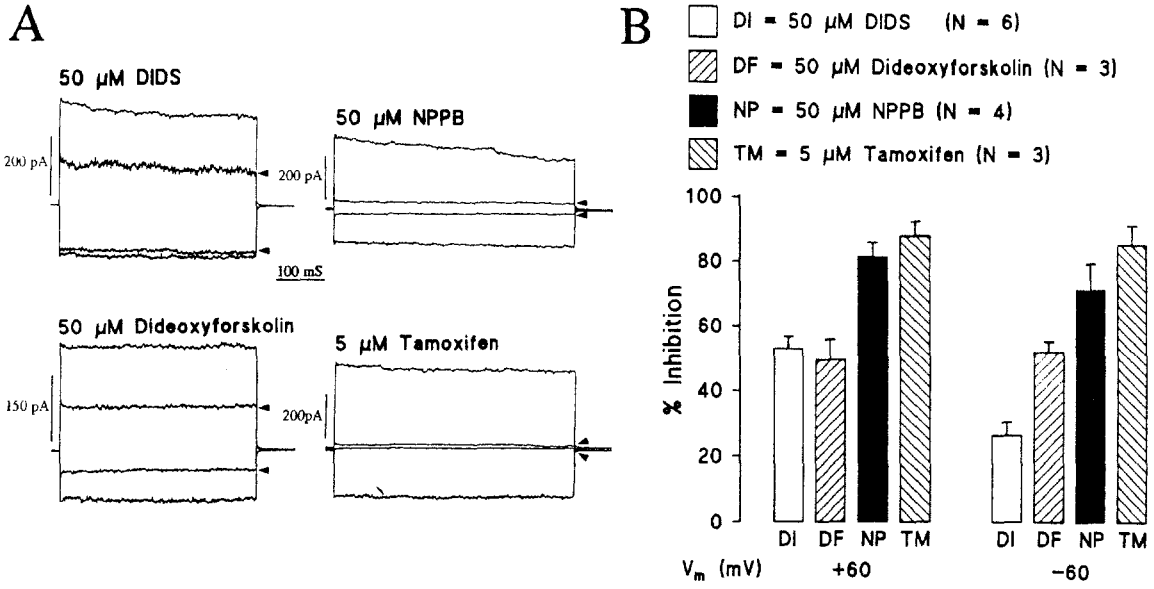

Fig. 3. Inhibition of volume-activated currents. (*A*) representative whole-cell current traces in response to +60 or –60 mV voltage pulses (recorded during the ± 100 mV protocol described in Materials and Methods). Traces represent control currents (no symbols) and currents recorded after the application of a given drug (arrowheads). Measurements were taken after 2–3 min of exposure to the drug, except for DIDS where recordings were normally taken within 30 sec. (*B*) summary of inhibition data measured at E_{rev} ± 60 mV. Data are represented as the percentage inhibition of control currents.

dent; the degree of current inhibition being 53 ± 4% at 60 mV compared to 26 ± 4% at –60 mV (n = 6) (Fig. 3*A* and *B*). In contrast to DIDS, block by the other drugs was voltage independent (Fig. 3*A* and *B*), and inhibition occurred with a slower time course, reaching a maximum value approximately 120 sec after application. The average percentage current inhibition observed with these compounds at 60 and –60 mV respectively was 82 ± 4% and 72 ± 8% for NPPB (n = 4); 50 ± 6% and 52 ± 3% for dideoxyforskolin (n = 3); and 88 ± 5% and 85 ± 6% for tamoxifen (n = 3) (Fig. 3*B*). Figure 4 shows dose-response data for the various inhibitors. The IC_{50} (i.e., the concentration which gives 50% current inhibition) for DIDS was 52 μM at 60 mV and 357 μM at –60 mV. The IC_{50} values for dideoxyforskolin and NPPB were approximately 51 μM and 27 μM respectively.

Verapamil also inhibited the volume-sensitive chloride current, but was only effective at high concentrations. For example, 500 μM verapamil caused an inhibition of 51 ± 7% and 38 ± 6% at 60 and –60 mV respectively (n = 6). Quinine proved to be a poor inhibitor of the conductance, with a concentration of 500 μM causing a mean decrease in current amplitude of 18 ± 6% at +60 mV and 13 ± 4% at –60 mV (n = 3).

Regulation of Volume-Sensitive Currents

Culture Time

Most of the experiments described in this study were performed on epithelial cells isolated from intact ducts that had been maintained in culture for either 1 or 2 days. However, to check whether the duration of culture had any influence on the volume-sensitive currents we isolated cells from ducts that had been cultured for different times. The proportion of isolated cells in which volume-sensitive currents could be elicited with the 155 mM CsCl pipette solution were: 15% (3/20) in freshly isolated ducts, 75% (9/12) in ducts cultured for 1 day, and 93% (13/14) in ducts cultured for 2 days (ducts from 3 different animals at each time point). The proportion of responding cells was significantly greater in ducts that had been cultured for 1 or 2 days as compared to fresh ducts ($P < 0.001$; Fisher's exact test). The difference in the proportion of responding cells between cells cultured for 1 day and 2 days was not significant. There were no significant differences in peak current amplitude with culture time.

ATP

The development of volume-activated currents was entirely dependent upon the presence of ATP (1 mM) in the pipette solution. Omission of ATP prevented the development of currents in response to both the 155 mM CsCl (0/4) and the 170 mM CsCl pipette solutions (0/4).

Extracellular and Intracellular Ca^{2+}

Figure 5 illustrates the effect of extracellular Ca^{2+} on the development of volume-sensitive currents. All of these data were obtained from the same batch of cells. In the control group (2 mM extracellular Ca^{2+}), 80% (8/10) of

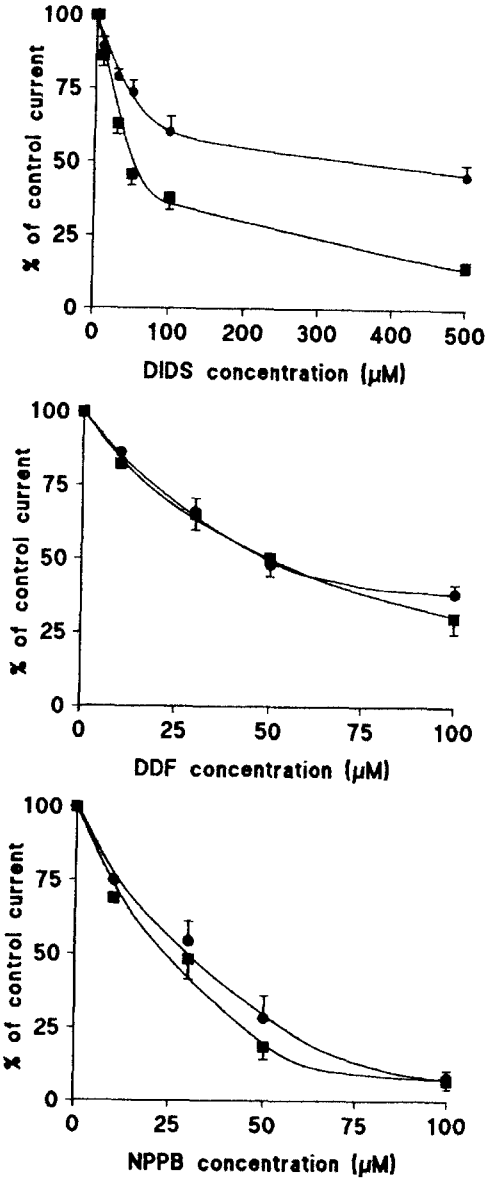

Fig. 4. Effects of increasing doses of DIDS, dideoxyforskolin (DDF) and NPPB on volume-activated current. Current density was measured at +60 mV (squares) and −60 mV (circles) voltage steps. Values are mean ± SE for $n \geq 3$.

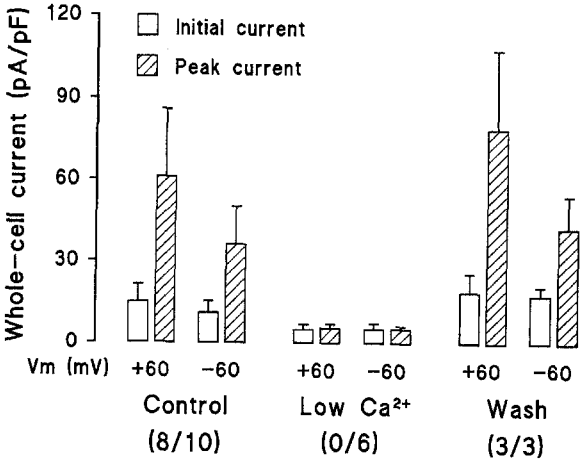

Fig. 5. Effect of removal of bath Ca^{2+} on the activation of volume-sensitive Cl^- currents under cell swelling conditions. Currents were measured at E_{rev} ± 60 mV and have been normalized to cell capacitance.

the cells developed currents. However, after removal of bath Ca^{2+} (calculated free Ca^{2+} concentration 0.1 μM) none of the cells responded (0/6). Currents of similar magnitude to the control currents could once again be elicited in 100% of cells (3/3) following reapplication of the standard bath medium containing 2 mM Ca^{2+} (Fig. 5).

We also investigated the importance of extracellular Ca^{2+} in maintaining the volume-sensitive currents by removing bath Ca^{2+} after the currents had been activated. The effect of this maneuver was rather variable. There was either no effect on the currents (3/9 cells), or a slight decrease in current amplitude (maximum reduction of 40% and 33% at 60 and −60 mV respectively) which occurred over a period of up to 5 min (6/9 cells). However, it is unlikely that this slow reduction in current amplitude is directly attributable to the lack of extracellular Ca^{2+} because it was not reversed by readdition of Ca^{2+} to the bath (4/4 cells). These experiments with bath Ca^{2+} show that extracellular Ca^{2+} is required to activate the volume-sensitive Cl^- channels. However, once they are activated, bath Ca^{2+} is not required to maintain the channels in the open state.

We attempted to confirm that activation of the volume-sensitive conductance resulted from an increase in intracellular Ca^{2+} by increasing the pipette EGTA concentration. With a transmembrane osmotic gradient of 20 mOsm/l, and a pipette solution containing 0.2 mM EGTA, 80% (123/154) of isolated duct cells developed a volume-sensitive conductance (*see above*). Increasing the intracellular Ca^{2+} buffering capacity by elevating the pipette EGTA concentration to 20 mM (keeping the osmolarity constant by adjusting the CsCl concentration to 130 mM) reduced the proportion of duct cells which developed a chloride conductance to 47% (7/15) ($P < 0.01$, Fisher's Exact test). The peak current amplitude in these responding cells was 89.2 ± 21.4 pA/pF and −42.3 ± 9.0 pA/pF at 60 and −60 mV respectively. These current densities are comparable to those obtained with 0.2 mM EGTA in the pipette solution (*see above*), suggesting that EGTA itself does not block the chloride channels. Taken together, we think our data provide evidence for Ca^{2+} influx, and an increase in intracellular Ca^{2+} concentration being required to activate the volume-sensitive chloride conductance.

A possible route of Ca^{2+} influx during cell swelling is through stretch-activated cation channels. Since these channels can be blocked by Gd^{3+} in epithelial cells [11],

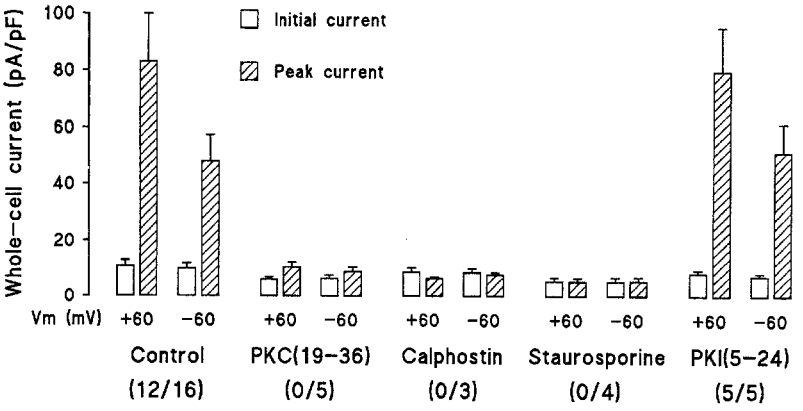

Fig. 6. Summary of the effects of 10 μM PKC(19–36), 500 nM calphostin C, 200 nM staurosporine and 10 μM PKI(5–24) on volume-sensitive Cl^- current activation. Peak current was measured after 419 ± 128 sec in control cells, after 245 ± 38 sec in cells treated with PKI(5–24), and after 600 sec for all other treatments. Currents were measured at E_{rev} ± 60 mV and have been normalized to cell capacitance.

we tested the effect of this cation on the development of volume-activated currents in pancreatic duct cells. In this series of experiments, volume-sensitive Cl^- currents developed in 73% (8/11) of duct cells under control conditions, compared with 67% (4/6) in cells that were pre-incubated with 50 or 100 μM Gd^{3+}. Moreover, neither 50 μM (n = 1) nor 100 μM Gd^{3+} (n = 2) had any effect when applied to cells that had already developed volume-sensitive currents. These data suggest that Ca^{2+} influx into pancreatic duct cells during cell swelling does not occur via stretch-activated cation channels. However, current densities were affected by Gd^{3+}. Initial currents were higher in Gd^{3+}-treated cells being 29.5 ± 7.1 pA/pF and −23.2 ± 7.9 pA/pF at 60 and −60 mV respectively (n = 3), compared with 9.6 ± 2.7 pA/pF and −9.5 ± 2.4 pA/pF (n = 8) for control cells at the same holding potentials. Interestingly, peak volume currents were smaller in Gd^{3+} treated cells being 48 ± 10 pA/pF and −28 ± 7 pA/pF (n = 4) compared with 94 ± 15.4 pA/pF and −54 ± 9.8 pA/pF (n = 8) for control cells. We have as yet no explanation for these phenomena, but they cannot be attributed to differences in cell capacitance.

Protein Kinases

In this series of experiments we examined whether the signaling pathway which activates the volume-sensitive Cl^- currents might involve protein kinases as well as Ca^{2+}. Figure 6 summarizes data from a series of experiments in which we tested the effects of various agents known to inhibit protein kinase A (PKA) and protein kinase C (PKC). Using the standard pipette solution, volume-sensitive Cl^- currents developed normally in 12/16 (75%) of cells tested (Fig. 6). Adding 10 μM of the specific PKA inhibitor peptide, PKI(5–24), to the pipette solution had no effect on volume-sensitive current generation, indicating that PKA is not involved in activating the channels (Fig. 6). However, when the pipette contained 10 μM PKC(19–36), a specific inhibitor of PKC, development of the currents was completely inhibited (Fig. 6). Moreover, bath application of either 500 nM calphostin C or 200 nM staurosporine, which are membrane permeable inhibitors of PKC, also inhibited current development (Fig. 6). Taken together, these results suggest that PKC plays an important role in the activation of volume-sensitive Cl^- currents in rat pancreatic duct cells.

Discussion

We have previously described cAMP-activated (CFTR) [15, 17] and Ca^{2+}-activated Cl^- conductances [17, 33] in pancreatic duct cells. The channels that underlie these conductances are located on the apical plasma membrane of the duct cell, and play a key role in cAMP- and Ca^{2+}-mediated bicarbonate secretion [3]. Our major finding in this study is that pancreatic duct cells possess a third Cl^- conductance, which can be activated by cell swelling. We found that we could activate this current using a pipette solution which was 20 mOsm/l hypertonic to the bath medium. Under these conditions, we observed an initial current on establishing the whole-cell recording which, in 80% of cells tested, increased about 4.5-fold over a period of about 9 min. The peak volume-sensitive outward current measured about 90 pA/pF at 60 mV which is comparable to the CFTR current density in these rat cells [15]. Once developed, the volume-sensitive current could be inhibited by a hypertonic bath solution. We did not make quantitative measurements of duct cell volume changes in response to the transmembrane osmotic gradients we imposed. However, we were able to confirm that cell swelling occurred with the hypertonic pipette solution by direct visual observation of the cells under phase contrast optics.

Biophysical Characteristics of the Volume-Sensitive Cl^- Current

The major biophysical properties of the volume-sensitive conductance are an outwardly rectifying *I-V* relationship, and time-dependent inactivation at strong depolarizing potentials. These features are typical of swelling in-

duced Cl^- currents previously described in other epithelial cells (e.g., airway [7, 27, 34, 35, 38], sweat gland [38], epididymis [6], gut [24, 38, 46], ciliary ocular epithelium [47] and distinguish this conductance from the duct cell conductances regulated by cAMP (CFTR) and Ca^{2+} [15, 17, 33]. This suggests that the volume-sensitive, cAMP-activated (CFTR) and Ca^{2+}-activated conductances in pancreatic duct cells are mediated by distinct ion channels. We did not investigate the single-channel basis of the volume-sensitive currents in pancreatic duct cells. However, outwardly rectifying Cl^- channels with a conductance of 50–60 pS have been identified on sweat gland, airway and T84 cells that had been swollen by a hypotonic challenge [38]. These channels have the same biophysical characteristics as the whole-cell currents we have recorded from pancreatic duct cells [38].

Anion replacement experiments confirmed that the volume-sensitive current in rat pancreatic duct cells was Cl^- selective. The anion permeability sequence was $ClO_4 = I = SCN > Br = NO_3 > Cl > F > HCO_3 >$ gluconate. This is essentially similar to the anion permeability sequence of volume-activated currents in other cell types, and in particular the sequence $I > Br > Cl$ is characteristic of these channels [6, 7, 9, 24, 34, 46]. In contrast, the anion selectivity sequence of the CFTR channel in rat pancreatic duct cells is $Br > Cl > I$, providing further evidence that the volume-sensitive current represents a distinct conductance pathway [15]. A significant gluconate permeability has been described for volume-sensitive chloride channels in other cells, including epithelial cell lines [9, 34] and NIH3T3 fibroblasts transfected with MDR1 [41]. However, the magnitude of the gluconate permeability in these cells is smaller than we found for pancreatic duct cells where $P_{gluconate}/P_{chloride}$ was 0.35. This high gluconate permeability indicates that the channel pore has a diameter greater than 0.54 nm [34].

There is evidence in the literature, based on single-channel recording and the effects of hypotonic shock on short circuit current across epithelial layers, that volume-sensitive Cl^- channels may be located on either the basolateral [10] or the apical plasma membrane of epithelial cells [13, 27, 30, 38]. The whole-cell recording technique we have employed in this study cannot provide information about the spatial localization of ion channels. However, we suspect that the volume-sensitive Cl^- channels are located on the basolateral, rather than the apical plasma membrane of the duct cell since bathing isolated pancreatic ducts in a hypotonic medium does not stimulate fluid secretion (M. Moffatt, R. Green & B.E. Argent, *unpublished observations*).

Inhibitors of the Volume-Sensitive Cl^- Current

The volume-sensitive conductance in pancreatic duct cells was inhibited by DIDS, NPPB, dideoxyforskolin, tamoxifen, verapamil and quinine. DIDS, NPPB, dideoxyforskolin and tamoxifen were all relatively effective and inhibited the currents by between about 50 and 85%. The effect of DIDS occurred immediately and, as previously reported for the action of stilbene derivatives on some other epithelia, was voltage-dependent [6, 24, 38]. In contrast, the inhibitory effects of NPPB, dideoxyforskolin and tamoxifen took several minutes to develop, and were voltage independent. Compared to the other compounds we tested, verapamil and quinine proved to be poor inhibitors of the volume-sensitive conductance, with a dose of 500 μM reducing the current by 50% and less than 20% respectively. Verapamil and quinine are much more effective inhibitors of volume sensitive currents in 3T3 fibroblasts transfected with P-glycoprotein (*see below*), 100 μM-verapamil producing a 65–80% inhibition and 20 μM-quinine a 20–25% inhibition [41].

We tested the effects of verapamil and 1,9-dideoxyforskolin because these compounds have been shown to reverse drug resistance mediated by P-glycoprotein [14], and to inhibit outwardly rectifying, time- and voltage-dependent, volume-sensitive Cl^- currents in P-glycoprotein transfected cells [31, 41]. Indeed, it has recently been proposed that P-glycoprotein acts as a regulator of the volume-sensitive Cl^- channel [18], although several studies have questioned the link between expression of P-glycoprotein and volume-sensitive Cl^- conductances [1, 23, 30, 35, 45]. Nevertheless, our finding that verapamil, 1,9-dideoxyforskolin and tamoxifen all inhibited the volume-sensitive Cl^- currents in pancreatic duct cells is consistent with an involvement of P-glycoprotein. Moreover, the fact that we could detect volume-sensitive Cl^- currents in only a minority (15%) of cells obtained from fresh ducts is consistent with the finding that expression of P-glycoprotein cannot be detected in rat pancreatic ducts using in situ hybridization techniques [40]. The increased proportion of duct cells exhibiting volume-sensitive currents following in vitro culture could be explained by P-glycoprotein expression being upregulated under these conditions.

Regulation of the Volume-Sensitive Cl^- Current

We found that pipette ATP was required for activation of volume-sensitive Cl^- currents in rat pancreatic duct cells. The most likely explanations for this finding are that ATP either binds to the channel or acts indirectly (i.e., as a phosphate donor) in the channel activation mechanism. ATP is also required for the activation of volume-sensitive Cl^- channels in Hela cells [9] and NIH3T3 fibroblasts [12], but not to maintain the channels in the open state [12]. ATP appears not to be required for the activation of volume-sensitive Cl^- currents in a number of other cell types, including human airway epithelia [7,

27], human sweat gland [38], rat epididymis [6], ciliary epithelia [47], T84 cells [19], and the lung cancer cell line H69AR [23]. These discrepant findings could be explained if the rate at which endogenous ATP is lost after establishing a whole-cell recording varied from cell to cell. However, ATP is not required for activation of volume-sensitive anion currents in outside-out patches derived from H69AR cells [23]. Effective removal of ATP from the membrane should occur when using this patch configuration [23]. Therefore, the possibility exists that there are two classes of volume-sensitive Cl^- channels that can be distinguished on the basis of their ATP dependence.

Cell swelling has been reported to increase intracellular calcium concentration $[Ca^{2+}]_i$ in a number of different cells types (for a review *see* [29]). However, the usual finding is that the volume-sensitive Cl^- channels that are thought to be involved in RVD are Ca^{2+}-independent [6, 24, 27, 38, 47], although exceptions to this rule do exist [20]. We found that increasing the Ca^{2+} buffering capacity of pancreatic duct cells, by increasing the pipette EGTA concentration from 0.2 to 20 mM, almost halved the number of cells that developed volume-sensitive currents. These results are consistent with an increase in intracellular Ca^{2+} concentration being required to activate the volume-sensitive conductance. Variability in the rate at which EGTA diffused from the pipette to the intracellular compartment might explain why 47% of the cells still responded when the pipette solution contained 20 mM EGTA. Thus, in the responding cells the cytoplasmic Ca^{2+} buffering capacity during the first few seconds of whole-cell recording might have been too low to prevent triggering of the currents. Additional evidence for an involvement of Ca^{2+} comes from the observation that bathing the cells in a Ca^{2+}-free medium completely abolished current activation. The simplest interpretation of this finding is that Ca^{2+} influx from the extracellular space plays a role in activating the Cl^- channels. Removal of extracellular Ca^{2+} is known to abolish the rise in $[Ca^{2+}]_i$ that occurs following a hypotonic challenge in many cell types [29], and also to abolish RVD [26, 36]. Based on the effect of pharmacological blockers, Ca^{2+}-selective channels, voltage-activated Ca^{2+} channels, and stretch-activated nonselective cation channels have all been proposed for the swelling-induced Ca^{2+} entry pathway [29]. We have not made a detailed investigation of the Ca^{2+} entry pathway in pancreatic duct cells. However, Gd^{3+} ions, which block stretch-activated nonselective cation channels in epithelial cells [11] and inhibit RVD in frog proximal tubule cells [36] did not inhibit development of the volume-sensitive Cl^- currents in pancreatic duct cells, although the magnitude of the currents was reduced by about 50% in the presence of Gd^{3+}.

Once the volume-sensitive current in pancreatic duct cells had been activated neither removal of extracellular Ca^{2+}, nor addition of Gd^{3+}, had any effect on current amplitude. This suggests that Ca^{2+} is only involved in events occurring early on in the mechanism of current activation and is reminiscent of the 'calcium window' effect in proximal tubule cells where Ca^{2+} influx must occur within 1 min of cell swelling for RVD to ensue [28]. Taken together, our data suggest that Ca^{2+} influx, and an increase in intracellular Ca^{2+} concentration, are involved in activating volume-sensitive Cl^- channels in pancreatic duct cells. However, the Ca^{2+} requirement is a transient feature and once the channels are activated, Ca^{2+} is not necessary to keep them in the open state.

The transient nature of the Ca^{2+} requirement and the dependence on ATP suggested to us that a phosphorylation step might be involved in the activation of volume-sensitive Cl^- currents. In support of this idea, we found that including either PKC(19–36), a pseudosubstrate which inhibits PKC [21], in the pipette solution, or calphostin C [4] or staurosporine [39] in the bath solution, completely inhibited development of the currents. Previously, PKC has been shown to activate outwardly rectifying Cl^- channels in membrane patches excised from airway epithelial cells [22, 25], although it is not clear whether these channels are the same as those that have been identified on swollen epithelial cells [38]. PKC has also been implicated in the regulation of a Cl^- conductance that controls cell volume regulation in response to Na^+-nutrient cotransport, but not hypotonic challenge, in enterocytes [26]. Moreover, in frog proximal tubule cells, RVD is accelerated by the phorbol ester PMA (which stimulates PKC activity) [36], and PKC(19–36) inhibits a volume-sensitive Cl^- conductance [37]. However, in contrast to all of these earlier observations which suggest that PKC can upregulate volume-sensitive chloride channels, Hardy *et al.* [18] have recently reported that PKC inhibits the same channels via phosphorylation of p-glycoprotein. Nevertheless, taken together these data suggest that PKC is likely to play an important role in regulating volume-sensitive Cl^- channels in epithelial cells.

PKC is known to be a Ca^{2+}-sensitive enzyme [32], so perhaps the simplest hypothesis to explain the role of Ca^{2+}, ATP and PKC in activating the volume-regulated conductance in pancreatic duct cells is as follows: cell swelling leads to Ca^{2+} influx and the resultant increase in $[Ca^{2+}]_i$ activates PKC which in turn phosphorylates either the Cl^- channel itself or a regulatory protein. In this model Ca^{2+} is only required for the initial activation of the conductance which is consistent with the results we have obtained. We cannot entirely exclude the possibility that the volume-sensitive Cl^- channels are directly activated by membrane stretch. However, on exposure to hypotonic solutions epithelial cells swell quickly with the maximum volume change usually occurring in less than 1 min (for example *see* [28, 36]). This contrasts with the slow activation of the Cl^- current (over a period of about 9 min) that we observed following the establishment of a whole-cell recording.

Physiological Role of the Volume-sensitive Cl^- Current in Pancreatic Duct Cells

Under normal conditions, pancreatic duct cells should not be exposed to conditions which cause them to swell; pancreatic juice is isosmotic with plasma, and, as far as we know, the cells do not have an absorptive function [3]. Moreover, in secretory epithelia the activation of electrolyte secretion usually leads to cell shrinkage due to a net loss of electrolytes [5]. Thus it is difficult to envisage a physiological role for the volume-sensitive Cl^- conductance in pancreatic duct cells. In agreement with this conclusion, the swelling-induced conductance was only observed in a minority (15%) of epithelial cells derived from freshly isolated ducts. However, if ducts were cultured for 1 or 2 days the number of cells that expressed the conductance was increased to 75% and 93% respectively. These data suggest that expression of either the volume-sensitive Cl^- channels, or some component of the regulatory mechanism (or both), markedly increases with time in culture. The nature of the stimulus which triggers upregulation of the volume-sensitive Cl^- conductance is a matter of speculation. However, during culture, pancreatic ducts seal and inflate due to fluid secretion into the closed luminal space [2]. It is possible that duct inflation stretches the epithelial cells and, by increasing the tension in their plasma membranes, mimics a hypotonic stimulus.

We thank David Stephenson for skilled technical assistance, and Dr. Malcolm Hunter for useful discussions. This work was funded by the National Institutes of Health (grant No. DK 43956), and the Cystic Fibrosis Trust.

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