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Secretin-Regulated Chloride Channel on the Apical Plasma Membrane of Pancreatic Duct Cells

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Summary. Using the patch clamp technique we have identified a small conductance ion channel that typically occurs in clusters on the apical plasma membrane of pancreatic duct cells. The cell-attached current/voltage (*I/V*) relationship was linear and gave a single channel conductance of about 4 pS. Since the reversal potential was close to the resting membrane potential of the cell, and unaffected by changing from Na^+-rich to K^+-rich pipette solutions, the channel selects for anions over cations in cell-attached patches. The open state probability was not voltage-dependent. Adding 25 mM-bicarbonate to the bath solution caused a slight outward rectification of the *I/V* relationship, but otherwise, the characteristics of the channel were unaffected. In excised, inside-out, patches the *I/V* relationship was linear and gave a single channel conductance of about 4 pS. A threefold chloride concentration gradient across the patch (sulphate replacement) shifted the single channel current reversal potential by −26 mV, indicating that the channel is chloride selective. Stimulation of duct cells with secretin (10 nM), dibutyryl cyclic AMP (1 mM) and forskolin (1 μM) increased channel open state probability and also increased the number of channels, and/or caused disaggregation of channel clusters, in the apical plasma membrane. Coupling of this channel to a chloride/bicarbonate exchanger would provide a mechanism for electrogenic bicarbonate secretion by pancreatic duct cells.

Key Words chloride channels · secretin · pancreas · ducts · bicarbonate · secretion

Introduction

Bicarbonate ions are secreted by the stomach, small intestine, liver and pancreas (for reviews *see* Flemstrom & Garner, 1982; Case & Argent, 1986; Erlinger, 1987). By neutralizing gastric HCl, they provide the correct pH environment for the digestion of food (Fordtran & Locklear, 1966) and also form, in combination with mucus, an alkaline unstirred layer, which protects the upper gastrointestinal tract from ulceration (*see* Allen et al., 1986). While it is known that bicarbonate can be secreted from gastrointestinal epithelia by electroneutral and electrogenic mechanisms (*see* Flemstrom & Garner, 1982) neither the transport processes involved, nor their regulation, have been fully defined.

Pancreatic bicarbonate is secreted by the epithelial cells that line the small interlobular and intralobular ducts of the gland (*see* Case & Argent, 1986). This epithelium forms an excellent model system in which to study the cellular mechanisms of gastrointestinal bicarbonate transport since: (i) it is homogeneous consisting of one epithelial cell type (Arkle et al., 1986); (ii) there is easy access to both the apical and basolateral membranes of the duct cell; and (iii) bicarbonate transport is acutely controlled by the peptide hormone secretin, which uses cyclic AMP as an intracellular messenger (*see* Case & Argent, 1986).

Here we show that rat pancreatic duct cells possess clusters of small conductance (4 pS) chloride channels on their apical plasma membrane. Stimulation with secretin, dibutyryl cyclic AMP and forskolin increased channel open-state probability and also increased the number of channels, and/or caused disaggregation of channel clusters, in the apical plasma membrane. Coupling of this channel to a chloride/bicarbonate exchanger at the same location would provide a mechanism for electrogenic bicarbonate secretion from the duct cell.

Some of our observations have previously been reported in preliminary form (Argent, Gray & Greenwell, 1987*b*).

Materials and Methods

Isolation of Pancreatic Ducts and Ductal Epithelial Cells

Small interlobular and intralobular pancreatic ducts were isolated from the glands of copper-deficient rats as previously de-

scribed (Arkle et al., 1986). Copper-deficiency causes a noninflammatory atrophy of enzyme-secreting acinar cells (80% by volume of the pancreas) but leaves the duct cells (4% by volume of the gland) structurally and functionally intact (*see* Arkle et al., 1986). As a starting point for duct isolation, this preparation has two advantages over the copper-replete rat pancreas: (i) the glandular content of potentially harmful digestive enzymes is markedly decreased; and (ii) the ratio of ductal to acinar tissue in the pancreas is markedly increased. Up to fifty ducts can be microdissected from each gland (Arkle et al., 1986), and they can be maintained in culture for up to three days (Argent, et al., 1986). We have previously shown that these isolated ducts possess morphological, biochemical and secretory characteristics typical of ducts within the intact copper-replete rat pancreas (Argent et al., 1986; Arkle et al., 1986).

Isolated epithelial cells were prepared by incubating cultured ducts for 30 min at 37°C in Dulbecco's modified Eagles medium (Flow Laboratories, Rickmansworth, U.K.) containing 4.5 U/ml papain (P-3125, Sigma, Poole, U.K.). The ducts were then incubated for 5 min at room temperature in a Ca^{2+} and Mg^{2+}-free, HEPES-buffered (pH 7.4), Na^+-rich Ringer solution containing 2 mM EGTA, after which single cells or cell clusters were teased from the epithelium using sharpened stainless steel needles. Before use in electrophysiological experiments, the isolated cell preparations were incubated for 1 hr at room temperature in Dulbecco's modified Eagles medium.

Single Channel Current Recording

Single-channel current recordings from cell-attached and inside-out membrane patches were made at 21–23°C using the patch clamp technique (Hamill et al., 1981). Most experiments were performed on epithelial cells within cultured ducts. The basolateral membrane was exposed by microdissecting away the surrounding connective tissue, while access to the apical plasma membrane was gained by tearing a flap in the wall of the duct. Ducts were immobilized in the tissue bath (volume 1.5 ml) using a glass suction pipette and a fine glass rod. Solution changes were accomplished by gravity feed from reservoirs at a flow rate of 5 ml/min.

Once studies on intact ducts had established that the chloride channel was located only on the apical plasma membrane, some experiments were also performed on isolated duct cells. Although apical/basal structural polarity is probably lost in this preparation, their use greatly facilitated our ability to maintain cell-attached recordings for the 15 to 20 min required to observe reversible effects of hormone stimulation on channel activity.

Recording pipettes were constructed from 1.2 mm o.d. borosilicate glass (GC120F15, Clarke Electromedical, Pangbourne, U.K.) using a vertical electrode puller (Bioscience, Sheerness, U.K.) and had resistances of between 8 and 15. MΩ, and external tip diameters of 1–2 μm. An agar bridge (4% wt/vol agar in 150 mM NaCl) connected the Ag : AgCl reference electrode to the bath solution. Single-channel currents were amplified using an EPC-7 patch-clamp system (List Electronic, Darmstadt, FRG), the head-stage of which was mounted on a Narishige MO-103 hydraulic manipulator (Narishige Scientific Instrument Laboratory, Tokyo, Japan), and the signals stored on a digital tape recorder (Lamb, 1985). For viewing and analysis, these data were low pass filtered at 100–300 Hz (Fern EF5-02 in damped mode; Barr and Stroud, Glasgow, Scotland) and displayed on a digital storage oscilloscope (Model 1425; Gould Advance, Hainault, U.K.). Permanent records were made using a graphics plotter (Model 7470A; Hewlett Packard, San Diego, U.S.A.). Potential difference across the patch is referenced to the outside, extracellular face, of the membrane. Outward current, the flow of positive charge from the inside to the outside of the membrane, is indicated as an upward deflection on the records.

Frequently, the recording pipette and bath contained different solutions at the start of an experiment, and/or the bath solution was changed during the course of an experiment. Under these conditions, liquid junction potentials developed between the pipette and the bath solution, and also between the agar bridge and the bath solution. These junction potentials were measured as described by Hughes et al. (1987), and the appropriate corrections applied to our data.

Data Analysis

Permeability data was derived using the Goldman constant field equation (Goldman, 1943)

$$I = \frac{(ZF)^2 PV}{RT} \cdot \frac{(C_o - C_i e^{\frac{ZFV}{RT}})}{(e^{\frac{ZFV}{RT}} - 1)} \tag{1}$$

where I is current (C · sec^{-1} · cm^{-2}), P is permeability (cm · sec^{-1}), V is membrane potential (volts), C_o and C_i are the concentrations of the conducting ion on the outside and inside of the membrane respectively (mole · cm^{-2}), and Z, F, R, and T have their usual meanings.

When $C_o = C_i$ the Goldman equation simplifies to:

$$I = \frac{-(ZF)^2 PC \cdot V}{RT} . \tag{2}$$

The chloride permeability of the channel was calculated from Eq. (2) using the single channel current at a given membrane potential obtained from linear current/voltage (I/V) plots on inside-out patches. Because channel current has units of C · sec^{-1} both sides of the simplified Goldman equation must be multiplied by cm^2, and thus the units of channel permeability are cm^3 · sec^{-1}.

Kinetic analysis of channel activity was complicated by the fact that the channel usually occurred in clusters. Only a few patches were obtained with only a single channel present, and we were unable to record from these for long enough to obtain a sufficient number of events to allow a statistical analysis of open state probability, mean open and mean closed times using conventional amplitude threshold techniques (Sigworth, 1983).

We therefore employed the method described by Vivaudou, Singer and Walsh (1986) for the analysis of channel kinetics in multichannel patches. Assuming a value (N) for the number of channels in a patch, the open state probability is given by the fractional area under the single channel current events. Channel cycle time is then determined from the total number of transitions, the assumed number of channels, and the time duration of the recording. Finally, mean open time and mean closed time are calculated from the cycle time and the open-state probability. This method of analysis will only give correct absolute values for open and closed times if the channel obeys a simple kinetic scheme with one open and one closed state. If more complex schemes are involved, the absolute values for open and closed

times will be incorrect, however, changes in these two parameters will still be detected.

When analyzing experiments, we have assumed that the total number of channels in a patch remained constant, and was equal to the largest number of simultaneous single channel current steps. We confirmed that this value was reasonable by comparing the measured state-time probability to a set of binomial probability distributions with the same value for the product (number of channels · open state probability), the closest fit yielding an estimate of the number of channels in the patch. These values for channel number obtained by binomial analysis suggested that the number of channels in a patch did not change following exposure of duct cells to stimulants during continuous cell-attached recordings. All analyses were performed on a microcomputer (BBC Master, Acorn Computers, Cambridge, U.K.) using 8-bit digitized data sampled at either 2.2 or 5 kHz.

Solutions and Chemicals

The extracellular-type Na^+-rich solution contained (mM): 138 NaCl, 4.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 5 glucose, and 10 HEPES pH 7.4. In some experiments, the chloride in this solution was partially replaced with either sulphate or bicarbonate. These solutions had the following compositions (mM). Sulphate replacement: 41.5 NaCl, 4.5 KCl, 47.25 Na_2SO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 5 glucose, and 10 HEPES pH 7.4. Bicarbonate replacement: 121 NaCl, 4.5 KCl, 25 $NaHCO_3$, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 5 glucose. This bicarbonate-containing solution was gassed with 5% CO_2/95% O_2 to maintain pH at 7.4. The intracellular-type K^+-rich solution contained (mM): 145 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 5 glucose, and 10 HEPES pH 7.4. Glucose was omitted from these solutions when they were used in the recording pipette.

Hormones and drugs were made up as either 100- or 1000-fold concentrated stock solutions, and 1.5 or 15 μl microinjected directly into the tissue bath as required. Secretin (Karolinska Institute, Stockholm, Sweden) was dissolved in 150 mM-NaCl containing 1% (wt/vol) bovine serum albumin, dibutyryl cyclic AMP (Sigma, Poole, U.K.) in the Na^+-rich extracellular type solution, and forskolin (Sigma, Poole, U.K.) in absolute ethanol. None of these solvents had effects on channel activity when injected alone into the tissue bath.

All other chemicals were purchased from commercial sources and were of the highest purity available.

Statistics

Channel conductance data were obtained from linear *I/V* plots by least squares regression analysis. Significance of difference was determined using Student's *t* test, the level of significance being set at $P < 0.05$. All values are expressed as mean ± SEM (number of observations).

Results

Single-Channel Currents from Cell-Attached Membrane Patches

Typically, cell-attached patches on the apical surface of epithelial cells in interlobular ducts were either quiescent or contained clusters of up to nine active channels. In the experiment shown in Fig. 1*A*, three active channels were present and their open-state probability was not voltage dependent. However, there was an increase in the number of fast closing events during inward current flow at hyperpolarizing membrane potentials. These fast events are not clearly resolved in Fig. 1*A* due to the degree of filtering required to clearly observe the very small single-channel currents.

Filling the recording pipette with either a Na^+-rich or a K^+-rich solution gave linear *I/V* plots with the channel currents reversing at pipette potentials of 5.6 +/− 0.4 (n = 14 patches from 8 cells) and 1.7 +/− 3.7 mV (n = 11 patches from 8 cells), respectively (Figs. 1*B* and 1*C*). These reversal potentials were not statistically different. However, the single channel conductance with a Na^+-rich solution in the pipette (3.9 +/− 0.7 pS) was slightly smaller than that observed with a K^+-rich solution in the pipette (4.5 +/− 0.7 pS) (P = 0.01).

When 25 mM-bicarbonate was in the bath solution (Figs. 1*D* and 1*E*), the reversal potential was not markedly affected (Na^+-rich pipette solution, −1.5 mV, n = 2 patches from 2 cells; K^+-rich pipette solution, 0.2 +/− 0.6 mV, n = 3 patches from 2 cells). However, the presence of this ion did cause a reduction in the size of the inward currents measured at positive pipette potentials (hyperpolarizing membrane potentials), compared to the outward currents measured at negative pipette potentials (depolarizing membrane potentials) (Figs. 1*D* and 1*E*). Thus the channel became an outward rectifier, with the conductance obtained by linear regression analysis of outward and inward currents being 4.2 +/− 0.3 pS and 2.4 +/− 0.1 pS (P = 0.001), respectively, for Na^+-rich pipette solutions, and 5.9 +/− 0.5 pS and 2.1 +/− 0.2 pS (P = 0.001), respectively, for K^+-rich pipette solutions. It is unlikely that these effects of bicarbonate result from a direct action of the ion on the channel, since we did not observe a similar rectification with inside-out patches (*see below*). However, it is possible that the presence of external bicarbonate caused a change in either intracellular pH or intracellular chloride concentration and that this affected current flow through the channel.

Because the *I/V* relationship was not markedly affected by changing the cation in the pipette solution, and the single channel currents always reversed at voltages close to the membrane potential of the cell (pipette potential, V_p = 0 mV), it is likely that the channel selects for anions over cations in cell-attached patches.

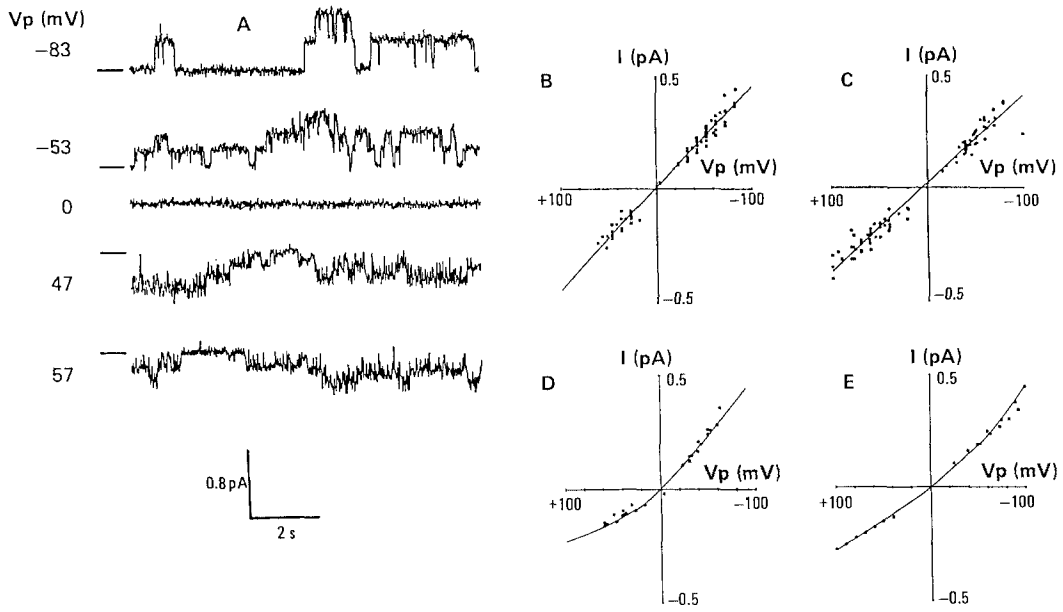

Fig. 1. Single-channel currents in cell-attached membrane patches. (*A*) Current traces from a patch on the apical surface of an epithelial cell in an interlobular rat pancreatic duct. Low pass filtered at 300 Hz. Solutions: bath, Na^+-rich; pipette, K^+-rich. The pipette potential (V_p) is indicated adjacent to each record. In the cell-attached recording configuration, the total voltage across the patch is equal to the membrane potential of the cell minus V_p. In this, and all other figures, an upward deflection from the closed state (horizontal line) represents outward current, and a downward deflection inward current. (*B-E*) Single-channel *I/V* plots. (*B*) Bath, Na^+-rich; pipette, K^+-rich (11 patches). (*C*) Bath, Na^+-rich; pipette, Na^+-rich (14 patches). Regression lines in *B* and *C* were calculated by least-squares analysis. (*D*) Bath, Na^+-rich containing bicarbonate; pipette, K^+-rich (3 patches). (*E*) Bath, Na^+-rich containing bicarbonate; pipette, Na^+-rich (2 patches). Regression lines in *D* and *E* were fitted by eye

Single-Channel Currents from Excised Inside-Out Membrane Patches

Figure 2*A* shows single-channel currents recorded from an inside-out membrane patch excised from the apical surface of an epithelial cell in an isolated interlobular duct. Three active channels were present in this experiment; however, after excision of the patch, channel activity usually declined and frequently disappeared within a few minutes. We only observed activity in inside-out patches if there were active channels already present in the cell-attached patch prior to excision. Prolonged depolarization of quiescent excised patches did not lead to channel activation.

In similarity with cell-attached patches, there was an increase in the number of fast closing events at hyperpolarizing membrane potentials, although these are not clearly resolved in Fig. 2*A*. Open-state probability analysis in inside-out patches was complicated by the often rapid decline in channel activity following patch excision, but did not appear to be voltage dependent. With Na^+-rich, extracellular-type solutions containing 150 mM-chloride on both sides of the membrane, the *I/V* relationship was linear and the single-channel currents reversed at a membrane potential close to 0 mV (Fig. 2*B*). Similar results were obtained when both the inside and outside faces of the patch were bathed in a K^+-rich, intracellular-type solution, and when the inside face was bathed in a K^+-rich solution and the outside face in a Na^+-rich solution. This suggests that the channel is either nonselective or selects for chloride ions over sodium and potassium. The mean single-channel conductance in these cation substitution experiments was 4.1 ± 0.8 pS (n = 15 patches from 13 cells), a value not statistically different from that found in cell-attached patches.

However, unlike the cell-attached situation, the presence of 25 mM-bicarbonate in the bath solution did not affect the *I/V* relationship. Under these conditions, and with a Na^+-rich solution in the pipette, the *I/V* plot was linear, reversed at −2.3 mV (n = 2 patches from 2 cells), and the single-channel conductance was 3.6 pS (n = 2).

Imposing a threefold chloride concentration gradient across the patch by replacing bath chloride with sulphate, but keeping sodium and potassium concentrations constant, shifted the reversal potential by −26 mV (Fig. 2*B*). Since the predicted shift for a channel that only conducts chloride is −26.2 mV, this result confirms a high selectivity for this anion, and indicates that the channel is impermeant to sulphate. The chloride permeability of the chan-

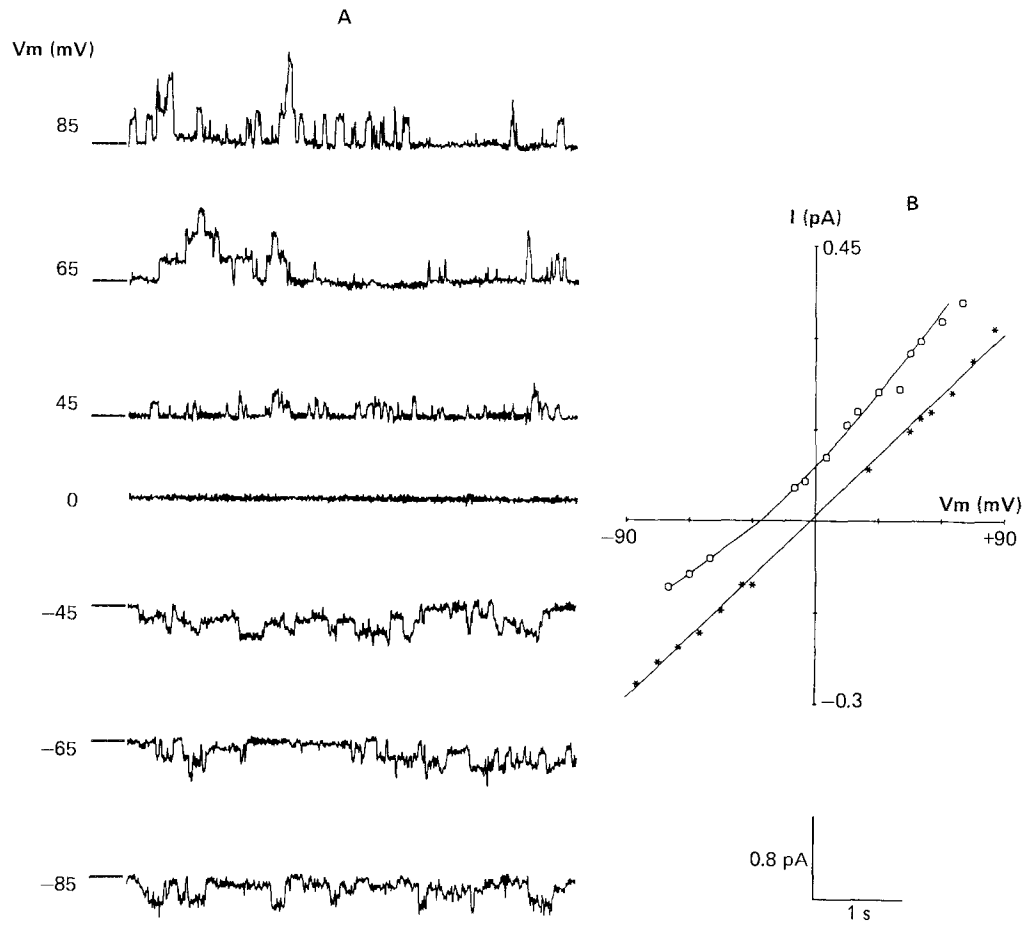

Fig. 2. Single-channel currents recorded from an inside-out patch excised from the apical plasma membrane of an epithelial cell in an interlobular rat pancreatic duct. (*A*) Single-channel current records from an experiment in which both the extracellular (pipette) and intracellular (bath) faces of the membrane patch were bathed in a Na^+-rich solution. Low pass filtered at 200 Hz. The membrane potential (*Vm*) is indicated adjacent to the records. (*B*) Single-channel *I/V* plots. (*) Data from the experiment shown in *A*. (○) Same experiment after a threefold chloride concentration gradient had been created across the patch by replacing bath chloride with sulphate, keeping sodium and potassium concentrations constant

nel, calculated from data obtained in inside-out patches, was 5.8×10^{-15} $cm^3 \cdot sec^{-1}$.

Because we have never observed activity of the chloride channel in cell-attached or excised patches obtained from the basolateral surface of the duct cell, we conclude that it is located only on the apical plasma membrane.

Effect of Secretin on Chloride Channel Activity in Cell-Attached Membrane Patches

The activity of chloride channels in cell-attached patches was increased when cells were exposed to 10 nM-secretin (Fig. 3*A-C*). However, this effect was only observed if active channels were already present on the unstimulated cell. Stimulation with the hormone did not cause the appearance of channel activity in quiescent patches.

Figure 3*A* shows representative single-channel current traces that were recorded over a period of approximately 100 sec and analyzed to obtain the associated current amplitude histograms (Fig. 3*B*). Prior to the addition of secretin (control, Fig. 3*A* and *B*), there were up to three channels open simultaneously in the patch. However, less than two minutes after stimulation with the hormone (secretin 1.75 min, Fig. 3*A* and *B*) there were up to six channels open at the same time. This particular patch contained a cluster of eight channels since this was the maximum number observed to be open simultaneously during the experiment. Binomial analysis of the data also suggested that eight channels were present, and that the number of channels in the patch did not change following secretin stimulation.

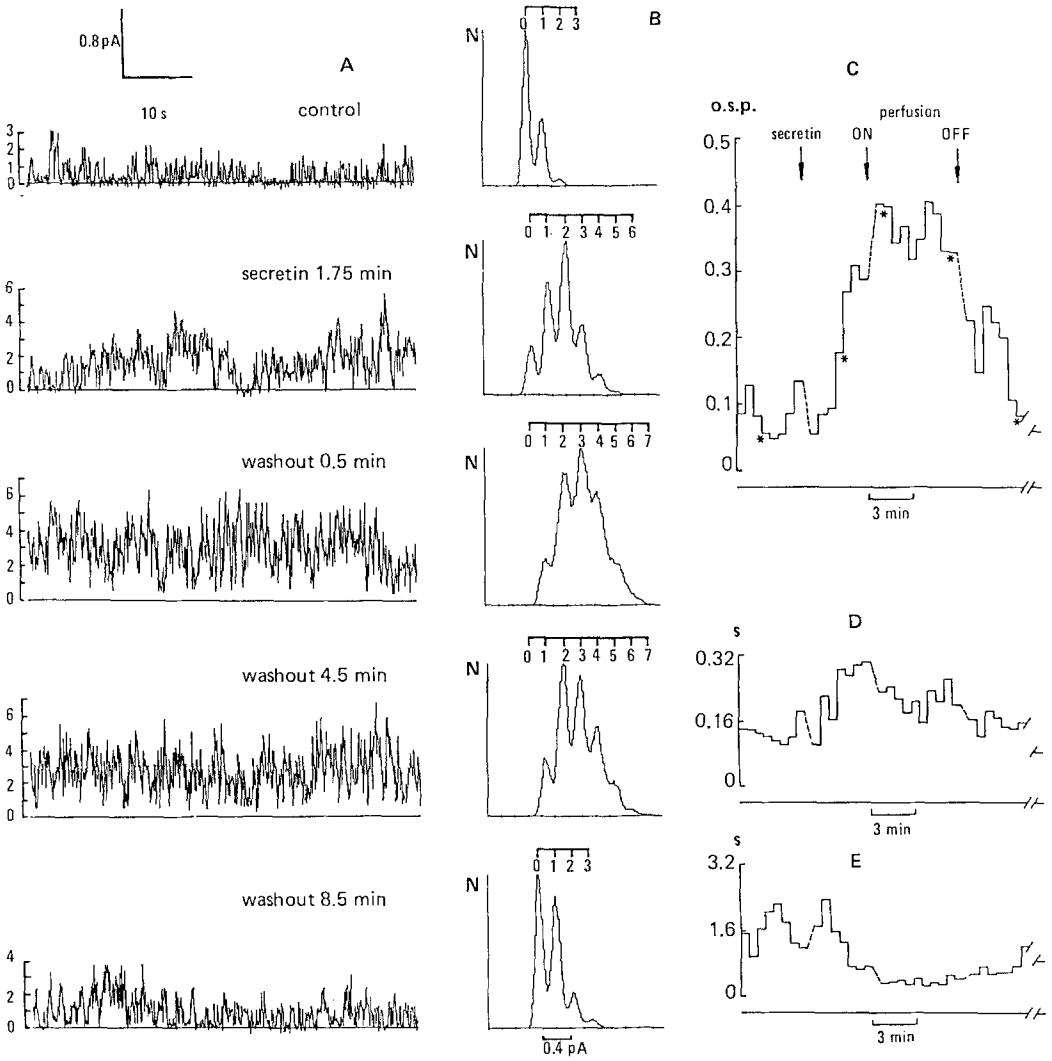

Fig. 3. Regulation of chloride channel activity by secretin. (*A*) Single-channel currents recorded from a cell-attached patch on an isolated pancreatic duct cell before (*control*), during (*secretin 1.75 min*) and after (*washout 0.5 min, 4.5 min, and 8.5 min*) exposure to 10 nM-secretin. The times indicated on each trace refer to the period between addition of secretin to, or washout of secretin from, the tissue bath and the start of each current recording. Low pass filtered at 300 Hz. Solutions, bath, Na^+-rich; pipette, K^+-rich. Vertical scale indicates the number of chloride channels open simultaneously. Because outward currents are easier to resolve (*see* Fig. 1), this patch was voltage clamped at $V_p = -50$ mV. Thus chloride ions will be moving from the recording pipette into the cell. (*B*) Current amplitude histograms derived by analysis of the corresponding tracings. Data sampled at 2.2 kHz. Horizontal scale indicates number of channels open simultaneously. (*C, D, E*) Effects of 10 nM-secretin on the open-state probability (*C*), mean open time (*D*) and mean closed time (*E*) of the chloride channel. Same experiment as *A* and *B*. Arrow *secretin* indicates when the hormone was added to the bath (volume 1.5 ml), and arrows *perfusion ON* and *perfusion OFF* when the perfusion flow (5 ml/min) was switched. Dashed lines indicate access to screened cage, and stars the mid-point of recordings shown in *A*. For illustrative purposes data collected over 4.5 min towards end of experiment has been omitted. Open-state probability, mean open and mean closed times were calculated from data sampled at 5 kHz into 33 sec bins, and assuming a total of eight channels in the patch, which was the maximum number observed to open simultaneously

Washout of secretin from the tissue bath did not cause an immediate reduction in the number of active channels (washout, 0.5 min, Fig. 3*A* and *B*). Usually, 8–10 min were required before the pre-stimulation situation of two to three simultaneously active channels was reached (washout 4.5 min; washout 8.5 min, Fig. 3*A* and *B*).

The open-state probability of the channel over the 5 min prior to addition of secretin was 0.08 (Fig. 3*C*). Exposing the duct cell to the hormone increased this to 0.18 after 1.75 min, and to a maximum value of 0.41 after 4.5 min (Fig. 3*C*). In this particular experiment the hormone was simply added as a bolus to the tissue bath, and not closely

applied to the cell from which the recording was being made. Thus the delay between secretin addition and channel activation will largely represent the time required for diffusion of the hormone to its receptors on the duct cell. The open-state probability remained near the maximum value for 4 min after washout of secretin from the bath (*perfusion ON*, Fig. 3*C*), and then slowly declined to the control value over the next 4 min.

The mean open time of the channel over the period prior to the addition of secretin was 0.135 sec and was increased by the hormone to a maximum value of 0.262 sec (Fig. 3*D*). On the other hand, the unstimulated mean closed time was 1.468 sec and was decreased by secretin to a minimum value of 0.306 sec (Fig. 3*E*). Thus the secretin-induced fivefold increase in open-state probability (Fig. 3*C*) was accompanied by a fivefold decrease in mean closed time and a twofold increase in mean open time of the channel. Similar reversible effects of secretin on chloride activity channel were observed in two other experiments.

Effects of Dibutyryl Cyclic AMP and Forskolin on Chloride Channel Activity in Cell-Attached Membrane Patches

Channel activity in cell-attached patches was also increased by dibutyryl cyclic AMP and forskolin, consistent with the idea that secretin uses cyclic AMP as an intracellular messenger. Like secretin, these agents only had an effect if active channels were already present on the unstimulated cell, and did not cause the appearance of channel activity in quiescent patches.

In the experiment shown in Fig. 4, the control open state probability of the channel was 0.082, and this was increased threefold to 0.249 after exposing the duct cell to 1 μM forskolin and 1 mM dibutyryl cyclic AMP for 1.5 min. Unlike the response to secretin, the effect of these stimulants on channel activity was reversed within 2 min of their washout from the bath (Fig. 4). This particular experiment was rather unusual in that multiple channel openings were not observed, making it likely that there was only one channel present in the patch.

Overall, these experiments, in which we have stimulated cells with either secretin, or dibutyryl cyclic AMP and forskolin for relatively short periods of time during continuous cell-attached recording, suggest that increases in channel activity are not due to the insertion of new channels into the patch, but result from an increase in the open-state probability of channels already present in the membrane.

Effects of Prior Stimulation on the Number of Patches Containing Active Chloride Channels, and on the Number of Channels in Each Patch

Although experiments in which we recorded continuously from duct cells suggested that stimulants did not increase the number of active channels in apical membrane patches, we did observe channel activity more frequently if cells were stimulated prior to giga-seal formation. In these experiments a mixture of secretin (10 nM), dibutyryl cyclic AMP (0.1–1 mM) and forskolin (1 μM) was used as the stimulant, and the length of prior exposure at 21–23°C varied between 5 and 45 min.

With unstimulated cells, we obtained a total of 213 GΩ seals out of 470 attempts in 29 experiments, a sealing rate of 45%. Of these 213 patches, only 22 contained active chloride channels. With stimulated cells, the sealing rate was 59% and we obtained a total of 177 giga-ohm seals out of 301 attempts in 24 experiments. On these 177 patches, 40 contained active chloride channels. Thus, prior stimulation did not markedly affect the sealing rate, but increased the proportion of patches containing active channels from 10 to 23%. This suggests that stimulation prior to giga-seal formation can either cause the insertion of new channels into the apical membrane, and/or the disaggregation of large channel clusters into smaller ones.

We have attempted to determine whether cluster disaggregation is occurring by comparing the number of channels (N) in patches derived from unstimulated cells, with N in patches derived from stimulated cells (Fig. 5). The distribution profile for N in unstimulated cells suggests that there are two populations of patches; one with $N = 1 - 5$, and a second with $N = 6 - 9$ (Fig. 5). In contrast, N was never greater than 5 in patches obtained from stimulated cells (Fig. 5). These results support the idea that disaggregation of channel clusters may occur following stimulation.

Discussion

We have identified a chloride channel on the apical plasma membrane of rat pancreatic duct cells. Because the activity of this channel is controlled by secretin, which is the physiological regulator of pancreatic bicarbonate transport (*see* Case & Argent, 1986), we think it may play an important role in electrogenic bicarbonate secretion.

This view is supported by three other observations. (i) Secretin depolarizes the basolateral membrane of the duct cell and causes a reduction of

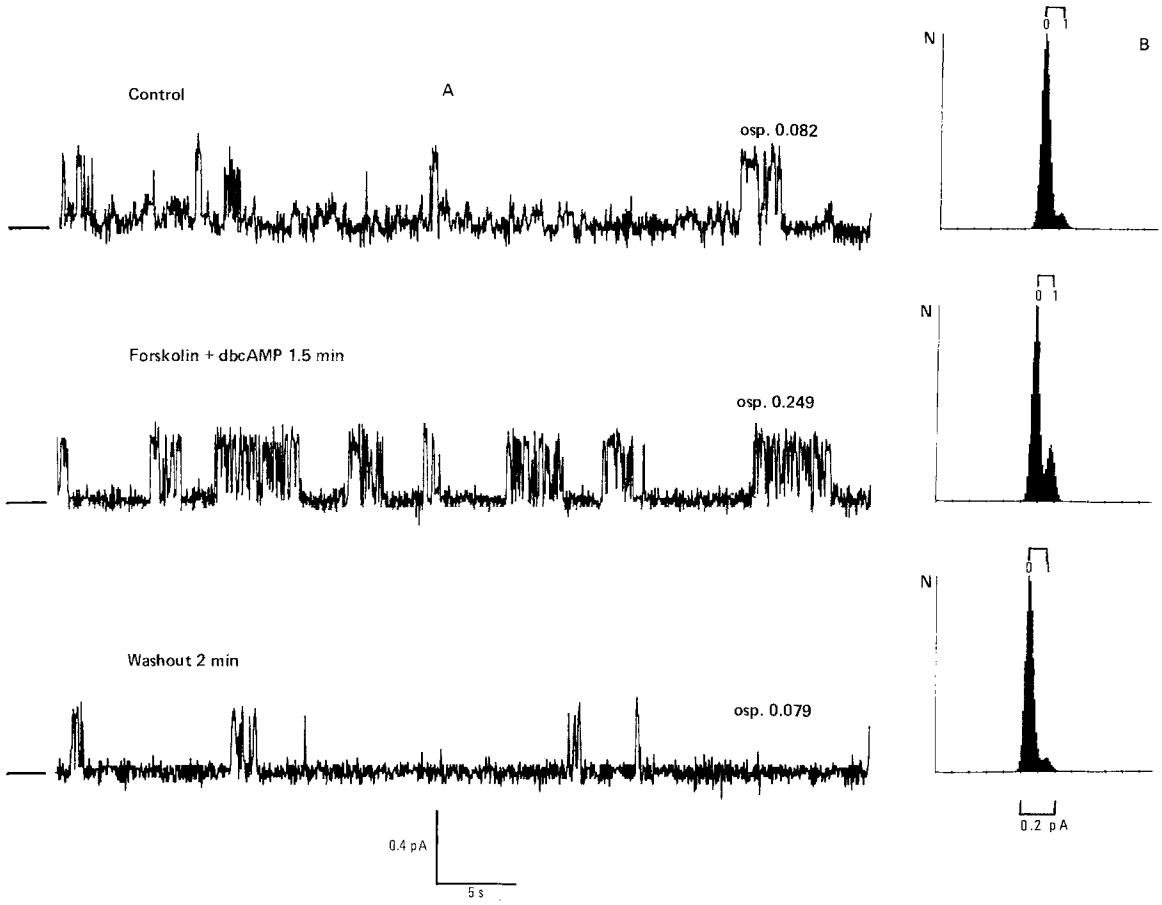

Fig. 4. Regulation of chloride channel activity by dibutyryl cyclic AMP and forskolin. (*A*) Single-channel currents recorded at V_p = −40 mV from a cell-attached patch on the apical surface of an epithelial cell in an intact duct. Both the bath and the recording pipette contained a Na^+-rich solution. Low pass filtered at 300 Hz. Top trace, control; middle trace, 1.5 min after addition of 1 μM forskolin and 1 mM-dibutyryl cyclic AMP to the bath; bottom trace, 2 min after washout of the stimulants. The open-state probability of the channel over the period of these recordings is shown to the right and above each trace. (*B*) Current amplitude histograms derived by analysis of the corresponding tracings. Data sampled at 2.2 kHz

input resistance (Gray, Greenwell & Argent, 1988), both of which could be explained by activation of a chloride conductance on the apical membrane. (ii) A short exposure to the hormone has similar long-lasting effects on channel activation and basolateral membrane depolarization (Gray et al., 1988), and also a prolonged action on bicarbonate secretion from the pancreas (Case, Harper & Scratcherd, 1968). (iii) Dibutyryl cyclic AMP and forskolin also increase channel activity, which is consistent with the idea that secretin uses cyclic AMP as an intracellular messenger (*see* Case & Argent, 1986). This cyclic nucleotide also depolarizes the basolateral membrane of the duct cell, and causes a fall in the fractional resistance of the apical membrane (Novak, 1988*a*,*b*).

The presence of active chloride channels on unstimulated duct cells may reflect the fact that there is a basal secretion of fluid from isolated pancreatic ducts (Argent et al., 1986). Although 10 nM secretin increases fluid secretion from isolated ducts about 14-fold at 37°C (Argent et al., 1986), we found that the same hormone dose only increased channel activity about fivefold (Fig. 3). This difference is probably explained by the patch-clamp experiments being performed at 21–23°C, at which temperature there is a 60–70% inhibition of secretin-stimulated fluid secretion (T. Scratcherd, *personal communication*).

During continuous cell-attached recordings, the effects of secretin, dibutyryl cyclic AMP, and forskolin are best explained by an increase in the open-state probability of channels that are already present in the membrane. This is achieved mainly by a decrease in the time that the channel spends in the closed state, together with a small increase in open time and is presumably controlled by an *A*-kinase-mediated phosphorylation event.

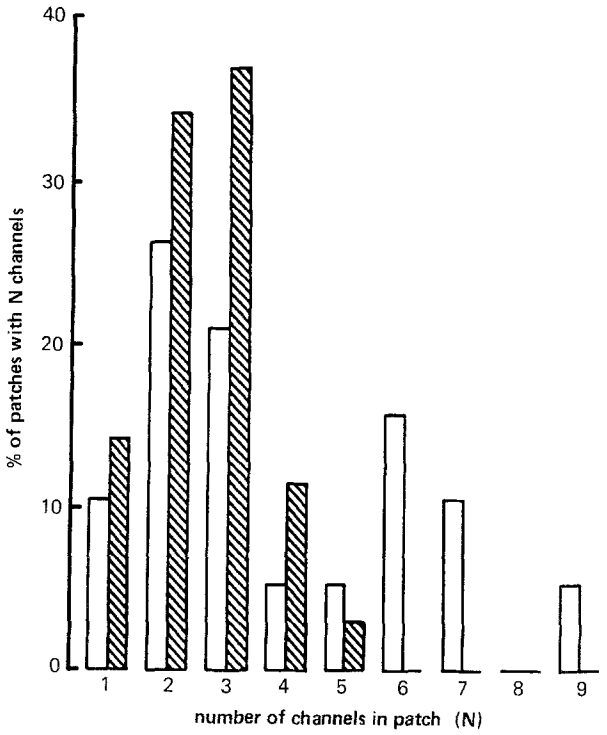

Fig. 5. Number of chloride channels in patches obtained from unstimulated pancreatic duct cells, and from cells stimulated prior to giga-seal formation. The number of channels (N) in a patch was taken as the maximum number of simultaneous single channel current steps observed during the first 45 sec of recording immediately after establishing a giga-seal. Open columns, 19 patches from unstimulated cells. Shaded columns, 35 patches from cells stimulated for between 5 and 45 min prior to giga-seal formation with a mixture of secretin (10 nM), dibutyryl cyclic AMP (0.1–1 mM) and forskolin (1 μM)

However, if duct cells were stimulated prior to obtaining a cell-attached patch, the percentage of patches containing active chloride channels was doubled compared to unstimulated cells. At the same time, the proportion of patches containing between 1 and 5 channels was increased and the proportion containing between 6 and 9 channels reduced. These results suggest that stimulants may also induce the insertion of new channels into the apical membrane, and/or the disaggregation of large channel complexes into smaller ones. However, it remains unclear why, during continuous cell-attached recording, stimulants never caused the appearance of channels in quiescent patches, or a reduction in the number of active channels in a patch. It may be related to the time of exposure to the stimulant, which for technical reasons was never more than a few minutes during continuous recordings, or could reflect mechanical damage to either the plasma membrane, or the cytoskeleton, during giga-seal formation. The induction of calcium channels in invertebrate neurones by 12-O-tetradecanoyl-phorbol-13-acetate is also prevented by the formation of cell-attached patches (Strong et al., 1987).

Recently, the characteristics of Ca^{2+}- and cyclic AMP-activated chloride channels on the apical membranes of a number of chloride secreting epithelial cells have been described (*see* Frizzell, 1987). In airways epithelia (Frizzell et al., 1986*b*; Welsh, 1986; Welsh & Liedtke, 1986), sweat gland coil (Schoumacher, Shoemaker & Frizzell, 1987*a*; Bijman et al., 1988) and colonic epithelia (Frizzell et al., 1986*a*; Hayslett et al., 1987; Reinhardt et al., 1987), the predominant channel is an outward rectifier, which has a slope conductance at 0 mV of about 50 pS. The open-state probability of this channel is voltage dependent, being markedly increased by membrane depolarization. Less commonly a nonrectifying channel with a conductance of 15–30 pS is observed (Frizzell et al., 1986*a,b*; Hayslett et al., 1987). In the shark rectal gland two types of chloride channel have been identified. One is a nonrectifier, with a conductance of 40–50 pS, whose open-state probability is voltage dependent (Gogelein, Schlatter & Greger, 1987). The second type is also a nonrectifier, but has a smaller conductance of 11 pS and an open-state probability only moderately dependent on membrane voltage (Greger, Schlatter & Gogelein, 1987).

All these channels are clearly very different from the one we have identified on the pancreatic duct cell, which has a lower conductance (4 pS), is nonrectifying, and whose open-state probability is unaffected by voltage. In addition, we found no evidence for irreversible activation of the duct cell channel in inside-out patches by prolonged depolarization as has been reported for the 50-pS chloride channel in airways epithelial cells (Shoumacher et al., 1987*b*; Li et al., 1988).

These differences may reflect the fact that the pancreatic duct cell secretes bicarbonate rather than chloride. However, a small conductance anion channel has previously been described on the chloride secreting lacrimal acinar cell (Marty, Tan & Trautmann, 1984). From noise analysis of whole-cell currents, the conductance was estimated at 1–2 pS and the density between 4 and 16 channels per square micrometer of plasma membrane. The whole cell currents were activated by both Ca^{2+} and depolarization, but the channel has not been further characterized or localized to the apical membrane using single-channel recording techniques.

A cellular model of electrogenic bicarbonate secretion that incorporates the regulated chloride channel is shown in Fig. 6. Coupling of the channel to a chloride/bicarbonate exchanger would provide

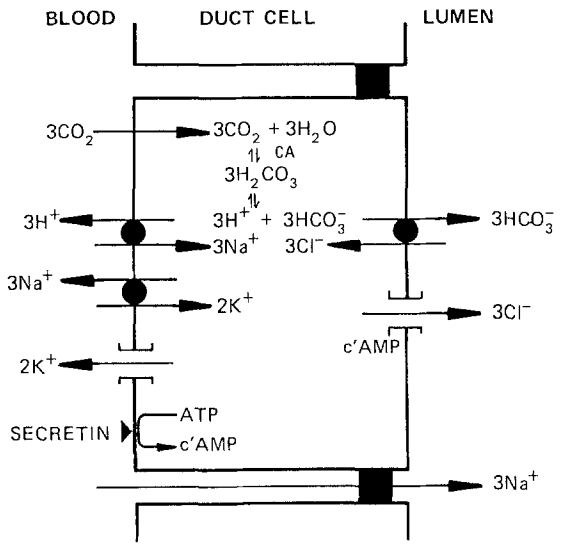

Fig. 6. Model for electrogenic bicarbonate secretion. The model is based on data obtained from pancreatic duct cells, but may also apply to other gastrointestinal epithelia, such as the small intestine (*see* Flemstrom & Garner, 1982). Carbon dioxide diffuses into the duct cell and is hydrated, by carbonic anhydrase (*CA*) (pancreatic duct cell, Kumpulainen & Jalovaara, 1981), to carbonic acid. This dissociates to form a proton and a bicarbonate ion, and the proton is translocated back across the basolateral membrane on a sodium/hydrogen exchanger (pancreatic duct cell, *see* Case & Argent, 1986; Novak & Greger, 1988). Effectively, this is the active transport step for bicarbonate, the energy being derived from the sodium gradient established by the Na^+/K^+ ATPase (pancreatic duct cell, Bundgaard, Moller & Poulsen, 1981; Novak & Greger, 1988; small intestine, Kinne & Kinne-Saffran, 1978). Accumulated bicarbonate then exits across the apical membrane on a chloride/bicarbonate exchanger (pancreatic duct cell, J.I. Gillespie, T. Scratcherd, & B.E. Argent, *unpublished observations;* small intestine, Brown & Turnberg, 1987). The cycling rate of this exchanger depends on the availability of luminal chloride, which in turn would be controlled by the open-state probability of the chloride channel. Because bicarbonate exit at the apical membrane is rheogenic there must be equal current flow across the basolateral membrane during secretion. Two thirds of this current is accounted for by potassium efflux through a voltage-dependent maxi-K^+ channel (pancreatic duct cell, Argent et al., 1987*a;* small intestine, Morris, Gallacher & Lee, 1986), and one third by cycling of the electrogenic sodium pump (pancreatic duct cell, Novak & Greger, 1988). The depolarization (pancreatic duct cell, Gray et al., 1988; Novak, 1988*a,b;* small intestine, Giraldez, Sepulveda & Sheppard, 1988) that results from the stimulant-induced increase in apical chloride conductance would open the K^+ channel. Sodium and potassium would enter the lumen by diffusion through a cation-selective paracellular pathway. This process is driven by an increase in the lumen negative transepithelial potential (pancreatic ducts, Novak, 1988*b;* small intestine, Armstrong, 1987) that will occur when the apical chloride conductance of the epithelial cells increases, and completes the current loop across the epithelium

a mechanism for electrogenic bicarbonate exit across the apical membrane of the duct cell. The cycling rate of the exchanger would depend on the availability of luminal chloride, which in turn would be controlled by the open-state probability of the channel. Alternatively, bicarbonate itself, or a buffer component, could move directly through the channel. We think this is unlikely in the pancreas since bicarbonate secretion is markedly inhibited in the absence of extracellular chloride (Case et al., 1979). In either case, the channel is regulated by stimulants of bicarbonate transport and forms an important control point in the secretory mechanism.

The model predicts that, in the presence of extracellular bicarbonate, intracellular chloride should be above electrochemical equilibrium. An increase in apical membrane chloride conductance would then depolarize the basolateral membrane of the duct cell as measured in microelectrode studies (Gray et al., 1988; Novak, 1988*a,b*), and provide the net increase in chloride efflux required to drive secretion. However, we found that the reversal potential of cell-attached single-channel currents was always close to the resting membrane potential of the cell (Fig. 1), indicating that intracellular chloride was at, or very close to, electrochemical equilibrium. This apparent discrepancy is probably explained by the fact that the microelectrode studies were performed at 37°C, while the patch-clamp experiments described here were carried out at 21–23°C. At room temperature it is likely that intracellular chloride would rapidly fall towards electrochemical equilibrium since anion exchangers are highly temperature dependent (Hoffmann, Simonsen & Sjoholm, 1979; Lowe & Lambert, 1983), and any residual chloride gradient would be dissipated by the resting chloride conductance of the apical membrane, which we detect as channel activity in unstimulated cells.

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