Chloride channels and anion fluxes in a human colonic epithelium (HCA-7)

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1 Colonic epithelial cells, derived from a human adenocarcinoma (HCA-7), were examined by the patch clamp technique.

2 Outwardly rectifying anion (Cl^-) channels were identified in the apical membrane. The conductance was $g_{\text{in}} \approx 26$ pS, $g_{\text{out}} \approx 40$ pS. The open state probability of the channels increased with depolarization and the selectivity for Cl^- over K^+ ($P_{\text{Cl}}/P_{\text{K}}$) was ≈ 7.5 .

3 The channels were sensitive to intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP, 0.1 mM), but not to Ca^{2+} (at concentrations up to 1 mM). At depolarized potentials the channels were blocked by pirenide (1–5 μM) applied intracellularly.

4 HCA-7 monolayers loaded with $^{125}\text{I}^-$ (as a marker for Cl^-) were used to measure I^- efflux and converted to instantaneous rate constants.

5 The rate constant for I^- efflux was increased by forskolin and lysylbradykinin (LBK). The effects of forskolin were not effected by BAPTA (an intracellular calcium chelator). The effects of LBK were inhibited by BAPTA and by Ba^{2+} , indicating that LBK raised intracellular Ca^{2+} (Ca_i) which activates Ca^{2+} -sensitive K-channels, the latter being blocked by Ba^{2+} .

6 Although it cannot be conclusively proved that the outwardly rectifying chloride channels described here are solely or partially responsible for the increased anion efflux or transepithelial chloride secretion, the channels are likely to be more relevant for cyclic AMP-requiring rather than Ca^{2+} -requiring secretagogues.

Keywords: Ion channels; chloride; pirenide; cyclic AMP; lysylbradykinin; forskolin

Introduction

A number of cell lines derived from human adenocarcinoma are important for the study of transepithelial ion transport. This is so because in culture the cells can form polarized epithelial monolayers mimicking a sheet of intact epithelium. Examples are the T84 cell line (Dharmasathaphorn *et al.*, 1984), HT₂₉ cell line (Augerson & Laboisse, 1984) and the HCA-7 cell line, originally described by Kirkland (1985) and used to study chloride secretion (Cuthbert *et al.*, 1985; 1987; Brayden *et al.*, 1989). Thus far, however, evidence for presumed apical chloride channels has been indirect and no direct evidence for increased apical chloride fluxes has been obtained after application of chloride secretagogues. Direct evidence for both of these is provided by the present study.

Methods

Cell culture

HCA-7 cells, derived from a human colonic adenocarcinoma (Kirkland, 1985) were prepared and grown to confluence as described in detail previously (Cuthbert *et al.*, 1985; 1987). Briefly, cells were seeded onto 10 cm² Petri dishes or 6 well (35 mm diameter) plates (Cell Cult, Sterilin Ltd., Hounslow, England) in Dulbecco's Modified Eagle's Medium (Gibco-Europe, Paisley, Scotland) supplemented with glucose (25 mmol l⁻¹) and 10% foetal calf serum (Gibco), kanamycin (100 $\mu\text{g ml}^{-1}$; Bristol Laboratories, Langley, Slough, England) and amphotericin B (2.5 $\mu\text{g ml}^{-1}$; E.R. Squibb, Morton, Cheshire, England). Cells were grown at 37°C in an atmosphere of 95% O₂/5% CO₂ for 5–10 days after seeding.

Patch clamp experiments

Cells grown in the 10 cm² diameter dishes were used. Generally it was easier to patch cells at the edge of confluent areas. If difficulty was experienced in forming seals, cells were 'rounded up' by exposure for 3–5 min at room temperature to a solution containing (mM): NaCl 135, KCl 5, ethylene diamine tetra-acetic acid, disodium salt (EDTA), 0.6 and 0.5 g l⁻¹ trypsin. Patch clamp experiments were conducted using the inside-out configuration of single channel current recording (Hamill *et al.*, 1981). Patch pipettes were made from borosilicate, filamented capillary tubing (1.2 mm o.d., 0.6 mm i.d. (GC120-F10), Clark Electromedical Instruments, Pangbourne, England) and fire-polished to give a pipette resistance of 10–25 M Ω . Solutions were filtered immediately before use with 0.22 μm Acrodisc filters (Gelman Sciences, Ann Arbor, Michigan, U.S.A.). The resistance of the seals was between 50 and 300 G Ω .

Recordings were made with either a List L/M-EPC7 patch clamp amplifier (List Medical, Darmstadt, Germany) or a Dagan 8900 amplifier (Dagan Corporation, Minneapolis, Minnesota, U.S.A.) as described previously (Henderson & Cuthbert, 1991).

Single channel data were analysed on IBM AT-compatible computers using either the pClamp system (Version 5.5.1; Axon Instruments, Inc., Foster City, California, U.S.A.) in combination with a Tecmar Labmaster A/D D/A board, or using the single channel analysis programme PAT (V 6.2) (Dempster, 1988) in combination with a Data Translation DT 2801A A/D D/A board (Data Translation, Inc., Marlborough, Massachusetts, U.S.A.). Current amplitudes were determined from single-point histograms and single channel conductances derived from current-voltage relationships obtained by recording amplitude histograms at a number of different holding potential (V_h) values. The open-state probability was calculated from peak areas of amplitude histog-

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rams using the areas of Gaussian distributions fitted to the data (Colquhoun & Sigworth, 1983). Open and closed time distributions of single channel records were determined off-line using analysis programmes incorporating a 50% threshold crossing parameter to detect events. Stretches of data were replayed at the recorded speed, filtered at a bandwidth of 1 kHz (-3 dB) and digitally sampled at 20 μ s intervals. Data are represented as mean $\pm$ standard error of mean (s.e.mean).

To check the selectivity of chloride channels two approaches were used to change the Cl^- concentration bathing the patch. In the first, the pipette solution contained (mM): HEPES-Na (pH 7.4) 10, KCl 145, EGTA (ethyleneglycol-*bis*-(β aminoethyl ether) N,N,N',N'-tetra-acetic acid) 1 and CaCl_2 (adjusted to give 1 μM free Ca^{2+} activity). Free Ca^{2+} activity was calculated from the binding constants for EGTA given by Fabiato & Fabiato (1979). The bath (in contact with the cytosolic face of the patch) contained (mM): NaCl 140, KCl 5, HEPES-Na (pH 7.4) 10, EGTA 1 and Ca^{2+} 1 μM . To reduce the Cl^- concentration the bath solution was replaced with one containing (mM): sucrose 200, NaCl 47, KCl 1.7, HEPES-Na (pH 7.4) 10, EGTA 1 and Ca^{2+} 1 μM . For the second, selectivity for anions was determined with non-isosmotic solutions of NaCl and KCl in the pipette and bath, with gradients for either KCl or NaCl. Solutions containing either 70, 140 or 210 mM NaCl or KCl were used to provide ion gradients across the membrane. The solutions also contained (mM): Na-HEPES (pH 7.4) 10, EGTA 1 and Ca^{2+} at 1 mM or 1 μM . Full details of specific experimental conditions are given in the Results section. Both approaches provided gradients across the membrane for K^+ , Na^+ and Cl^- . To prevent junction potentials distorting the data when the bath Cl^- composition was changed, the amplifier head stage indifferent electrode was connected to the bath via a 1.5% agar bridge having the same ionic composition as the pipette solution (but without Ca^{2+}).

¹²⁵Iodide efflux from HCA-7 monolayers

HCA-7 cells were plated out into 6 well (35 mm diameter) plates and incubated at 37°C with the same medium and conditions as used for the patch clamp experiments. The plates were used at 5–6 days when the epithelia were 50–70% confluent.

To load the cells with ¹²⁵I⁻ the monolayers were washed, three times with efflux buffer and then exposed to the buffer containing 2.5 $\mu\text{Ci ml}^{-1}$ for 90 min at 37°C. At the end of the loading period the monolayers were again washed, three times, with efflux buffer ($t = 0$). Efflux of ¹²⁵I⁻ was measured every 30 s by rapidly removing efflux buffer (3 ml) and adding a further aliquot for a total period of 10 min. At the end of 10 min, 3 ml 0.1 N HNO_3 was added and left for 30 min, to remove the remaining tracer. To determine the effect of a secretagogue this was added to the efflux buffer at $t = 3.5$ min until the end of the experiment. The efflux buffer had the following composition (mM): NaCl 135, CaCl_2 1.2, MgCl_2 1.2, K_2HPO_4 2.4, KH_2PO_4 0.6, glucose 10 and HEPES 10 (pH 7.2). The method used is essentially similar to that described by Venglarik *et al.* (1990) and Clancy *et al.* (1990). After counting the samples, with allowance for background, the data were processed to give instantaneous rate constants. Briefly, this was achieved as follows. The total counts released were summed with the residual counts left in the tissue. Thus the fraction of counts remaining in the tissue after $t = 0$ could be plotted with respect to time. If no drug treatment was applied washout was exponential. If a drug increasing or reducing efflux was added, the washout profile changed to one with a greater or lesser slope. Calculation of instantaneous rate constants assumes that the fractional content of isotope between any two adjacent time points represents a segment of an exponential decay curve. The rate constant is then, simply, the reciprocal of the time for the content to fall to 1/e of the value obtaining at the beginning

of time segment. A computer programme requiring only the counts released in each interval and the residual counts was devised to process the data.

BAPTA-AM was used to chelate intracellular Ca^{2+} ions in some experiments. To do this the acetoxymethyl ester was dissolved in dimethylsulphoxide (DMSO), diluted in buffer and incubated with the epithelial monolayers for 90 min at a final concentration of 5 μM . Subsequent washing removed excess BAPTA-AM while the intracellularly released BAPTA was trapped in the cells. Cells were loaded with ¹²⁵I⁻ at the same time as being exposed to BAPTA-AM.

When the effects of Ba^{2+} (1 mM) were investigated, this ion was included in both the loading and efflux solution. However, the composition of the solutions was changed to prevent precipitation as follows. Phosphates were replaced with KCl, 3 mM, and the HEPES concentration was increased to 15 mM.

Both BAPTA-AM and Fura2-AM were purchased from Molecular Probes, Eugene, Oregon, U.S.A.

Results

We have recorded single channel currents from inside-out patches of HCA-7 cells. Several different channel types were seen, but the predominant anion-selective channel was characterized by having an outwardly-rectifying current/voltage relationship. The channel was not evident at a V_h of 0 mV immediately upon excision of the patch, but became active once the membrane had been depolarized to $\approx +40$ mV. Figure 1 shows the current/voltage relationship, pooled for ten patches where $[\text{Cl}^-]$ was altered iso-osmotically by replacement with sucrose.

With symmetrical high Cl^- in bath and pipette the inward conductance (g_{in} ; measured at the point on the plot corresponding to -39.2 mV) was 26.8 ± 5.8 pS ($n = 7$), and the outward conductance (g_{out} ; measured at $+32$ mV) was 40.1 ± 5.1 pS ($n = 10$). Little effect was seen on the outward current (representing Cl^- movement into the cell) when the bath $[\text{Cl}^-]$ was reduced by one third, but the inward current

Figure 1 Current-voltage relationship produced from ten separate channels in ten different patches. The numbers adjacent to the points show the number of observations at each point. Cl^- was iso-osmotically replaced with sucrose. (○), Symmetrical solutions containing 145 mM Cl^- ; (●), bath solution containing 48.7 mM Cl^- ; (◇), represents the equilibrium potential for Cl^- . For full details of the composition of the solutions see Methods.

diminished as one would expect for a channel showing selectivity for Cl⁻. The reversal potential (V_{rev}) of $\approx +20$ mV also moved towards the equilibrium potential for Cl⁻ ≈ -26 mV (Table 1). The selectivity for Cl⁻ over K⁺ can be calculated using the equation:

$$E_{rev} = -\frac{RT}{F} \cdot \ln \frac{P_K + [K^+]_o + P_{Cl} - [Cl^-]_i}{P_K + [K^+]_i + P_{Cl} - [Cl^-]_o}$$

Using the value for E_{rev} of ≈ -20 mV obtained from Figure 1 gives a selectivity for Cl⁻ over K⁺ (P_{Cl}/P_K) of ≈ 7.5 .

Figure 2 shows single channel recordings made in symmetrical high [Cl⁻] solutions and the relationship between single channel open-state probability (P_o) and V_h . There were two channels present in this patch and P_o increases with depolarizing potentials (Figure 2a). As reflected in Figure 2b the channels show rapid openings and closures. This was reinforced by the single channel open and closed dwell time histograms (data not shown). The open events could be fitted with a single exponential, while the closed events were best fitted by two exponentials. Raising the bath (intracellular) calcium concentration from 1 μ M to 1 mM produced no significant change in P_o over the range of potentials studied.

The channel exhibited similar properties when the chloride gradient was imposed in the opposite way, i.e. from the bath to pipette, the direction in which chloride ions will flow during transepithelial ion secretion. The results from a series of experiments with different gradients, but with no correction for tonicity, are given in Table 1. The current-voltage curve is shifted in the opposite direction towards E_{Cl} when the gradient is reversed, and the shape of the curve is altered to produce a more linear relationship, reflecting Goldman rectification produced by the Cl⁻ gradient (Figure 3). Again the relationship of P_o to V_h with symmetrical solutions is as seen earlier with P_o increasing at depolarizing potentials.

The loop diuretic, piretanide, at high concentration (0.2 mM) inhibits chloride secretion on application to the basolateral side of intact epithelial monolayers (Cuthbert *et al.*, 1985). Similarly, it blocks secretion both apically and basolaterally in HCA-7 Colony 1 monolayers (Cuthbert *et al.*, 1987). It was surprising to find that piretanide could block chloride channels in patches from HCA-7 cells when applied to the cytosolic face in low concentrations (1–5 μ M).

Figure 4 illustrates one of three similar experiments and shows the effects of piretanide at 1 and 3 μ M, on an inside-out patch in symmetrical high chloride solutions. Channel inhibition is shown and was analysed by measuring open and closed dwell time histograms (data not shown). Before piretanide the data were best fitted by one exponential for the open times and two for the closed times. After piretanide, 1 μ M, the open time histogram was now best fitted with two exponentials, both with time constants shorter than during the control period. The closed time histogram remained virtually unchanged in the presence of piretanide and furthermore the open state probability (P_o) was reduced by 48%

Figure 2 (a), Relationship between single channel open probability (P_o) and holding potential (V_h) for single channel. (b), Single channel records obtained at V_h values of +40 mV (upper) and -50 mV (lower), reflecting the P_o shown in (a). There were two channels active in the patch and the closed levels are shown by the arrows.

Figure 3 (a) Current-voltage relationship for a single channel, with gradient causing positive reversal potential: (●) symmetrical 140 mM NaCl in bath and pipette; (▽) a different patch with 70 mM NaCl in the pipette. (b), Relationship between P_o and V_h for symmetrical solutions shown in (a).

Table 1 Data for channels recorded with differing Cl⁻ and cation gradients

Pipette solution	Bath solution	Symmetrical bath and pipette solutions		[Cl ⁻] gradient	
		E_{in}	E_{out}	E_{Cl}	E_{rev}
70 NaCl	140 NaCl	—	—	+18	+18
210 NaCl	140 NaCl	—	—	-10	-8
140 NaCl	210 NaCl	31	55	+10	+8
140 NaCl	70 NaCl	23	57	-18	-10
140 NaCl	140 KCl *	24 ± 2	40 ± 1	—	—
140 NaCl	37 NaCl**	27 ± 6	40 ± 5	-26	-20

For the symmetrical condition the bath solution was adjusted to be the same as in the pipette. All the solutions given above contained 1 mM EGTA, 1 μ M Ca²⁺ and 10 mM HEPES buffered to pH 7.4. The data are for single patches unless otherwise stated (* $n = 7$, ** $n = 10$).

Figure 4 Effect of pirenthane (Pir) on single channel behaviour. V_h +40 mV. An upward deflection indicates channel opening.

(Table 2). With 3 μ M pirenthane the amplitude of the single channel events was reduced during periods of high frequency flicker block, due to frequency limitation of recording (Figure 4). Note, too, the effects of pirenthane were easily reversible. Changing the potential from +40 mV to -40 mV in the presence of pirenthane removed the high frequency flicker block, which was restored on returning to +40 mV.

When pirenthane at concentrations of 3–5 μ M was applied to patches there were long periods of no activity between the high frequency bursts, so that recordings many minutes long were required for analysis. This was achieved in another experiment in which 3 μ M pirenthane was used (Table 2). One open time and two closed times were evident before pirenthane and the closed times were little changed by its addition. The blocker reduced the open time by over 80%, but it was not possible to resolve the very brief open time. P_o was reduced 61% in this instance. It is noted that the control open times were variable over a fourfold range in different patches. Analysis of these data was made during periods of channel activity. In the presence of pirenthane it is theoretically possible to measure a third closed time representing long periods of inactivity occurring between the bursts. However, hours of recording in a single patch would be required to collect sufficient events for exponential fitting.

In some isolated inside-out patches in which single chloride channels had been activated by depolarization, after a period of time (10–20 min), the single channel activity ceased and could not be re-elicited by repeated depolarization. To examine if the quiescent channels could be reactivated by adenosine 3':5'-cyclic monophosphate (cyclic AMP) the nucleotide was applied to the cytosolic face. After a delay due to diffusion, cyclic AMP (0.1 mM) in the presence of Ca^{2+} , 1 μ M produced a rapid activation of the chloride channel. The result shown in Figure 5 is typical of four experiments. In this example the mean open time before the patch became quiescent was 11.19 ms and the mean closed times were 0.35 and 3.18 ms. These closed times are close to those given in Table 2, while the open time lies between the previously given values. P_o was 0.86 before the patch became quiescent. P_o was, of course, zero during the quiescent phase but cyclic AMP was able to restore the pattern of channel activity to values almost identical to those seen initially (open time, 12.93 ms; closed times 0.28 and 2.05 ms; P_o = 0.87).

Thus far we have shown that the apical membranes of HCA-7 cells have outwardly rectifying chloride channels, sensitive to cyclic AMP and blocked by pirenthane applied to the cytosolic face. To examine the question whether the flux of chloride across the apical face was increased by agents which increase intracellular cyclic AMP we used the radioiodide efflux method described under Methods.

Forskolin, an activator of adenylate cyclase increases chloride secretion in HCA-7 monolayers and also increases tissue cyclic AMP (Cuthbert *et al.*, 1987). Forskolin (10 μ M) increased the rate constant for I^- efflux from HCA-7 cells (Figure 6). The presence of BAPTA within the cell did not reduce the effect of forskolin on increased I^- efflux.

In contrast to the sustained secretory response to forskolin, lysylbradykinin (LBK) produces a transient, rapidly desensitizing chloride secretory response which is Ca^{2+} -dependent (Cuthbert *et al.*, 1987; Pickles & Cuthbert, 1991). LBK, applied apically, also produced a transient increase in I^- efflux in HCA-7 monolayers (Figure 7). However, preincubation with Ba^{2+} or with BAPTA-AM abolished the response to the kinin.

Figure 5 Effect of 100 μ M cyclic AMP on channel activity. V_h +40 mV. Cyclic AMP was added at the point indicated by the arrow resulting in activation of the channel.

Table 2 Effect of pirenthane on time constants for channel opening and closing by fitting dwell times

Conditions	Open times τ (ms)	Closed times τ (ms)	P_o	Number of events
Control V_h = +40 mV Symmetrical Cl^- , 140 mM	14.92	0.480, 2.37	0.61	1500
Pirenthane, 1 μ M, V_h = +40 mV Symmetrical Cl^- , 140 mM	0.85, 3.93	0.95, 2.49	0.32	1459
Control V_h = +40 mV Symmetrical Cl^- , 140 mM	4.31	0.42, 1.17	0.49	4051
Pirenthane, 3 μ M V_h = +40 mV Symmetrical Cl^- , 140 mM	NR, 0.7	0.51, 1.92	0.19	3500

Time constants derived from best-fit exponentials to histograms obtained from the number of events indicated at a bin-width of 0.75 ms. NR indicates not resolved due to frequency limitation of the recording. Open time, closed times and P_o values were measured during periods of channel activity.

Figure 6 Effects of forskolin, 10 μ M, on the rate constant for efflux from HCA-7 monolayers. Each point shows mean for 6 measurements with s.e. mean indicated by vertical bars; (●) data from cells incubated with BAPTA-AM during the 125 I⁻-loading period.

Figure 7 (a) As for Figure 6 except lysylbradykinin LBK, (0.1 μ M) was applied as indicated. (●) Results with tissues preincubated with BAPTA-AM. (b) Effect of LBK (0.1 μ M) on I⁻ efflux in the absence (○), and presence (●), of Ba²⁺, 1 mM. Each point shows mean for 12 measurements; s.e. mean indicated by vertical bars.

Discussion

The outwardly rectifying anion-selective channels found in HCA-7 colonic epithelial cells are similar to outward-rectifying channels described in other epithelia, for example, *Necturus* enterocytes (Giraldez *et al.*, 1989), primary cultures and a cell line derived from the human pancreas (Gray *et al.*, 1989; Hanrahan & Tabcharani, 1990), canine tracheal epithelium (Welsh, 1986) and the apical membrane of the HT₂₉ and T84 lines (Hayslett *et al.*, 1987; Halm *et al.*, 1988) also, like HCA-7, derived from human adenocarcinoma. The conductance values of the present study ($g_{in} \approx 25$ pS; $g_{out} \approx 40$ pS) (Table 1) accord well with those seen in HT₂₉ and T84 cells (g_{in} and g_{out} approximately 32 and 50 pS, and 24 and 45 pS respectively for the two cell types). The relation of V_h to P_o is also similar in all three cell types, depolarization increasing the open probability. It is most likely that the anion-selective channels are in the apical membranes of the epithelial cells since in culture they grow with the apical surface uppermost and the basolateral face applied closely to the substrate.

As has been shown with other outward rectifying chloride channels (Schoumacher *et al.*, 1987; Li *et al.*, 1988) the channels in HCA-7 cells were cyclic AMP-selective. However, in T84 cells activated by cyclic AMP and with whole cell recording a linear current-voltage relationship is seen, while an outwardly rectifying current results from an increase in Ca_i (Cliff & Frizzell, 1990). This means that chloride secretion in T84 epithelia, and presumably HCA-7 tissues, is complex and that the outwardly rectifying channel may be only one type involved in chloride secretion. For example, low conductance (~ 10 pS) Cl⁻ channels with different properties have been seen in other epithelial tissues (Gray *et al.*, 1988).

In HCA-7 epithelia, forskolin or vasoactive intestinal peptide, both of which increase cyclic AMP, also cause chloride secretion (Cuthbert *et al.*, 1987). Here we show that forskolin also increases the rate of I⁻ efflux, as a marker of Cl⁻, from the epithelium. The question which cannot be answered by the present experiments is whether the increased efflux is exclusively or only partially through the outward rectifying channels. By use of short circuit current measurements in HCA-7 epithelia it was shown that the calcium ionophore A23187 and lysylbradykinin (LBK) also increased chloride secretion (Cuthbert *et al.*, 1987). Since LBK does not stimulate prostaglandin formation in HCA-7 cells it was considered that the peptide acted through an increase in Ca_i . Here, we have seen no evidence of channel activation when the cytosolic face of patches were exposed to Ca²⁺. We have shown elsewhere that LBK is unlikely to raise the Ca_i value to above 1 μ M in these colonic epithelia (Pickles & Cuthbert, 1991). It must be concluded that agents which increase Ca_i must act indirectly to increase secretion, most likely by activation of Ca²⁺-sensitive maxi-K channels in the basolateral membrane. These channels are blocked by Ba²⁺, which was shown to inhibit the effects of LBK on I⁻ efflux.

The consequence of activation of basolateral K⁺ channels would be to hyperpolarize both the basolateral and apical membranes of the cells; a situation which greatly reduces the open state probability of the Cl⁻ channels. Thus in HCA-7 monolayers it is likely that the channels we have described are more important for cyclic AMP-mediated secretion than for Ca_i-mediated secretion.

It is of interest that the classical loop diuretic, piretanide, an inhibitor of NaK 2 Cl cotransport, is able to block chloride channels when applied to the cytosolic face at low concentration. Concentrations in the millimolar range are required to inhibit chloride secretion when applied to the basolateral NaK2Cl transporter (Cuthbert *et al.*, 1985). However in intact epithelia with the apical membrane at ≈ -40 mV it is unlikely that piretanide would affect the channels even if it could penetrate the cells, since the block was most apparent at depolarized potentials. Another loop

diuretic, furosemide, has also been shown to inhibit Ca^{2+} -dependent chloride currents in rat lacrimal gland cells (Evans *et al.*, 1986) and more recently both furosemide and

bumetanide were shown to inhibit chloride channel fluxes in vesicles prepared from kidney cortex (Pope *et al.*, 1991).

Support from the Wellcome Trust is gratefully acknowledged.

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(Received October 23, 1991)

Revised January 14, 1992

Accepted January 20, 1992)