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Phenanthrolines – a new class of CFTR chloride channel openers

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1 A number of phenanthrolines and benzoquinolines were examined for their ability to activate epithelial chloride secretion by measuring short circuit current (SCC) using the mouse colon epithelium. 1,10 phenanthroline stimulated electrogenic chloride secretion with an EC_{50} of 612 ± 10 μM and a Hill slope of 4.9 ± 0.3. A similar pharmacology was demonstrated by both 1,7 and 4,7 phenanthrolines, 7,8 benzoquinoline and phenanthridine.

2 Evidence that the increase in SCC caused by 1,10 phenanthroline was due to chloride secretion is based upon (a) inhibition of the current by furosemide, (b) failure of cystic fibrosis (CF) colons to respond and (c) an associated net flux of $^{36}Cl^-$.

3 1,10 Phenanthroline affected neither the generation of cyclic AMP or the concentration of intracellular Ca^{2+} in colonic epithelial cells.

4 1,10 phenanthroline affected the chloride conductance of the apical membrane, as shown by an increase in chloride current in 'apical membrane only' preparations in the presence of an apical to basolateral chloride gradient. The increase in chloride current was inhibited by 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and was not present in CF colons.

5 Additionally, 1,10 phenanthroline activated basolateral K^+ channels, both Ca^{2+}- and cyclic AMP-sensitive channels, as shown by inhibitor studies with charybdotoxin (ChTX) and XE991, and after the apical membrane was permeabilized with nystatin.

6 The phenanthrolines and benzoquinolines described here, with dual actions affecting CFTR and basolateral K^+ channels, may constitute useful lead compounds for adjunct therapy in CF.
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Keywords: CFTR; cystic fibrosis; chloride channel openers; phenanthrolines; 7,8 benzoquinoline; phenanthridine; potassium channels; cyclic AMP; intracellular Ca^{2+}

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ChTX, charybdotoxin; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; P_o, open state probability; SCC, short circuit current

Introduction

Increased interest in agents affecting epithelial chloride channels arose after it was shown that the cystic fibrosis transmembrane conductance regulator (CFTR) is also a chloride channel in many epithelial membranes (Anderson *et al*., 1991). Not only have blockers of CFTR been described but considerable interest has also focused on CFTR chloride channel openers (Schultz *et al*., 1999). The most common mutation in cystic fibrosis (CF) is ΔF508, in which phenylalanine is absent from position 508. This mutant protein (ΔF508CFTR) can function as a chloride channel (Drumm *et al*., 1991), however only a minute fraction of this protein is transported to the apical domain of epithelial cells (Ward & Kopito, 1994). Instead the quality control mechanisms of the cell cause the mutant protein to be degraded in the proteasome. Therefore attempts are being made to increase the delivery of ΔF508 CFTR to the membrane (Brodsky, 2001; Fischer *et al*., 2001; Fuller & Cuthbert, 2000). Thus agents which open CFTR channels are of interest, particularly so if they also activate ΔF508 CFTR.

The present classes of CFTR openers are as follows; phosphatase inhibitors (Becq *et al*., 1998), xanthines, particularly CPX (Guay-Broder *et al*., 1995), benzimidazolones such as EBIO (Devor *et al*., 1996; Cuthbert *et al*., 1999), flavones, for example genistein (Illek *et al*., 1995), benzoxazolones like chlorzoxazone (Singh *et al*., 2000) and finally the MPB compounds described by Becq *et al*. (1999).

The purpose of this study was to further investigate the actions of 1,10 phenanthroline on chloride secretion in the mouse colon following the observations made by Duszyk *et al*. (1999) that 1,10 phenanthroline, a powerful inhibitor of matrix metalloproteases (MMPs) activated a chloride conductance, measured as whole cell current, in human airway epithelial cells, Calu-3. No effect was found in airway cells lacking CFTR, namely A549 cells. Furthermore, an antibody to MMP2 caused a similar effect in Calu-3 cells as did 1,10 phenanthroline, suggesting metalloproteinase inhibition was responsible. A further aim was to explore structure activity relations in the phenanthroline isomers and to ask if both heterocyclic nitrogens in the tricyclic aromatic molecules were necessary for the effect. From various experimental approaches we conclude that the phenanthrolines and related structures constitute a new class of agents that stimulate chloride secretion.

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Methods

Short circuit current recording

Mice (Balb C) were used throughout and were killed by CO_2 narcosis. CF mice, either CF null (*Cftr*^{tm1Cam}) or CF ΔF508 (*Cftr*^{tm2Cam}) were used on a few occasions, the procedures being identical to those for wild type animals. Methods for recording the short circuit current (SCC) in murine epithelia have been described before (Cuthbert *et al*., 1999). Briefly, for colonic epithelia, the muscle layers were dissected away, under a microscope, from small lengths (0.75 cm) of colon, opened by a longitudinal incision. They were mounted in Ussing chambers with a window area of 20 mm^2 and voltage clamped using a WPI Instruments Dual Voltage Clamp (Sarasota, FL, U.S.A.) and the data collected and stored using a PowerLab 8sp series (ADInstruments. NSW 2154, Australia). SCC was recorded continuously throughout the experiments and voltage compensation was made for the IR drop between the voltage sensing electrodes, placed close to the epithelium. The tissues were bathed on each side with 20 ml Krebs Henseleit Solution (KHS), warmed to 37°C and continuously circulated using a gas lift, with 95%O_2/5%CO_2. The only unusual feature was that in most experiments the apical bathing solution contained amiloride, 100 μM and Ba^{2+}, 5 mM. This was to eliminate electrogenic sodium absorption and to severely reduce potassium secretion. The protocol used for the nasal epithelium was similar, except the window area of the Ussing chambers was only 1.8 mm^2 and amiloride/Ba^{2+} was not added. The nasal epithelium was used without further dissection, once it had been taken from the nasal cavity.

Apical membrane only preparations were prepared in exactly the same way as descibed above except the basolateral (serosal) bathing solution was modified, potassium gluconate replacing sodium chloride in the KHS solution. This procedure makes the basolateral membrane electrically transparent (Cuthbert, 2001). Epithelia mounted in this way have an inward chloride gradient and an outward potassium gradient. A further modification in this study was to also replace the sodium chloride in the apical solution with potassium chloride. In this situation the only significant gradient was an inward chloride gradient. The composition of the bathing solutions used were as follows (in mM): KHS, NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose 11.1. In the 'apical membrane only' preparations NaCl was replaced with K gluconate and the $CaCl_2$ concentration was doubled for the basolateral bathing solution (Cuthbert, 2001). When high K was also added apically the NaCl in KHS was simply replaced by KCl. Both solutions were bubbled with 95%O_2/5%CO_2.

Apical membrane permeabilization

Nystatin (180 μg ml^{-1}) was added to the apical solution of short circuited, colonic preparations under SCC conditions and in the presence of an apical to basolateral potassium gradient, as described elsewhere (Cuthbert *et al*., 1999). Permeabilization makes the apical membrane permeable to small cations and causes an increase in SCC due to the K^+ gradient. Drugs affecting the basolateral K^+ channels either increase (openers) or reduce (blockers) the nystatin induced current. In these experiments the apical bathing solution had the following composition (mM); K gluconate 120, $CaCl_2$ 4, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 3.3, K_2HPO_4 0.8 and glucose 10. The basolateral bathing solution was identical except that sodium gluconate replaced potassium gluconate. The solutions were bubbled with 95%O_2/5%CO_2.

Measurement of ^{36}Cl fluxes

To measure chloride flux from the basolateral to apical side colonic epithelia were short circuited as described above. Two to three μCi of ^{36}Cl was added to the basolateral side and 30 min allowed for equilibration. At time equal to zero two 1 ml samples were taken from the apical side and replaced with fresh KHS. The same was repeated at 30 and 60 min, except the drug under investigation was present during the last 30 min. Basolateral samples gave the specific activity for chloride and allowed calculation of the change in flux caused by the drug. This change was compared to the change in the SCC response, calculated from the integrals of the SCC versus time records. ^{36}Cl fluxes in the apical to basolateral direction were measured in exactly the same fashion with the radioisotope added to the apical bathing solution.

cyclic AMP radioimmunoassay

A radioimmunoassay procedure was used in which the samples were acetylated before analysis (Harper & Brooker, 1975). Colonic epithelia, 20 mm^2, prepared as for SCC recording, were exposed either to phenanthroline, 1 mM or to forskolin, 10 μM for 10 min in 50 μl of KHS, either with or without IBMX, 100 μM, at 37°C. Acetic anhydride, 50 μl (final concentration 5 mM) was added to stop the reaction. The whole was then frozen and thawed three times to disrupt the cells, using solid CO_2. Aliquots were taken for protein estimation and the remainder was boiled for 5 min, centrifuged at 20,000 × *g* and used for immunoassay. Each measurement was the average of triplicate determinations.

Measurement of intracellular Ca^{2+} concentration

$Ca^{2+}{}_i$ was measured in isolated colonic crypts using Fluo-4 as the reporter molecule. A 4–5 cm long piece of mouse colon was everted, tied at both ends and filled with low calcium solution (LCS) *via* a syringe (Siemer & Gogelein, 1992). The distended and everted colon was submersed in low calcium solution at 37°C for 5 min with gentle shaking. Low calcium solution (LCS) contained the following (mM): NaCl 127, KCl 5, $MgCl_2$ 1, glucose 5, Na pyruvate 5, HEPES 10, EDTA 5, bovine serum albumin 1%. This solution had a pH of 7.4 when bubbled with O_2. At the end of 5 min the colon preparation was shaken vigorously for 2 min, the tissue discarded and the crypt suspension separated by gentle centrifugation. The crypts were resuspended in DMEM and loaded with Fluo 4-AM, 5 μM, for 60 min. Further centrifugation and washing in normal medium (identical to LCS except no EDTA was present and $CaCl_2$, 2.0 mM was added), followed by a 30 min de-esterification period, gave the suspension used for fluorescence measurements. Emission fluorescence was measured at 516 nm, with excitation at 494 nm, each at a bandwidth of 5 nm.

We were not able to use a ratiometric method, as with Fura 2, to determine intracellular $Ca^{2+}{}_i$ as 1,10 phenanthroline showed strong absorption at wavelengths up to 350 nm. We have not attempted to convert the measurements to $Ca^{2+}{}_i$, but have simply compared the fluorescence increases in response to ATP, an agent increasing $Ca^{2+}{}_i$ *via* P2Y2 receptors, with those to phenanthroline.

The following were obtained from Sigma-Aldrich Co Ltd. (Poole, Dorset, U.K.): amiloride, 7,8 benzoquinoline, charybdotoxin, furosemide, isobutylmethylxanthine (IBMX), nystatin, phenanthridine, 1,10;1,7 and 4,7 phenanthroline and pyrido(1,2-A)quinolinylium. Forskolin was obtained from Calbiochem, Fluo4-AM from Molecular Probes Europe BV (Leiden, The Netherlands) and ^{36}Cl from Pharmacia Biotech U.K. Ltd. XE991 was a generous gift from Dr B.S. Brown.

Results

Effects of 1,10 phenanthroline on SCC

Applied to the isolated, murine, colonic epithelium 1,10 phenanthroline produces a sustained increase in SCC, that can be rapidly terminated by addition of furosemide or by washing away the drug. Addition of 1 mM 1,10 phenanthroline increased the basal current from 45.5 ± 8.5 to 156.5 ± 22.5 μA cm^{-2} that returned to 58.0 ± 11.0 μA cm^{-2} (all $n = 5$) after washing. The SCCs before and after 1,10 phenanthroline were not significantly different. In the following studies using SCC measurements amiloride, 100 μM and Ba^{2+}, 5 mM were present in the apical bathing solution for the reasons given in the Methods section. Addition of 1,10 phenanthroline, 1 mM with amiloride and Ba^{2+} present produced a SCC increase of 134.4 ± 6.9 μA cm^{-2} ($n = 18$). Figure 1 illustrates the cumulative addition of 1,10 phenanthroline to a short circuited epithelium and also shows that furosemide quickly restores the current to near baseline values. In four experiments, similar to that of Figure 1, SCC increased by 202.8 ± 4.5 μA cm^{-2} after the concentration of 1,10 phenanthroline had reached 2 mM. Furosemide, 1 mM, reduced this increase to only 14.1 ± 4.2 μA cm^{-2} representing 93% inhibition. From the direction of the current change caused by 1,10 phenanthroline, plus the presence of amiloride and Ba^{2+} in the apical solution and the inhibition of the responses by furosemide, the indications are that 1,10 phenanthroline causes an increase in electrogenic chloride secretion. This conclusion was strengthened when 1,10 phenanthroline, 1 mM, was applied to CF colonic epithelia under the same conditions, using tissues from both null mice (*Cftr*^{tm1Cam}) and those with the ΔF508 mutation (*Cftr*^{tm2Cam}) (see Table 1). No SCC increase occurred in CF colons with 1,10 phenanthroline, indicating that chloride secretion was no longer possible, as expected in tissues without CFTR (Cuthbert *et al*., 1994).

1,10 Phenanthroline is a very powerful chelator of metal ions, particularly Zn^{2+}, with affinity constants in the region of 10^{14} M^{-1} (Auld, 1995). This makes it a powerful inhibitor of Zn^{2+} metalloproteases, some of which are associated with cell membranes. This property, together with the rather slow onset of the effects on SCC, suggested that removal of Zn^{2+} from surface metalloproteases may be responsible for its pharmacological effects, especially in view of the earlier literature (Duszyk *et al*., 1999). To test this hypothesis tissues were exposed to a low concentration of 1,10 phenanthroline (100 μM) for 30 min, sufficient time for the surface metalloproteases to be inhibited. After 30 min the concentration of 1,10 phenanthroline was raised to 1 mM. 1,10 Phenanthroline (100 μM) caused a sustained increase in SCC of 7.8 ± 3.6 μA cm^{-2} and after 30 min when the concentration was increased to 1 mM a further increase of 120.6 ± 16.9 μA cm^{-2} (both $n = 8$) was recorded. Thus it appeared unlikely that metalloproteases are involved in the chloride secretory response. This view is strengthened by the subsequent finding that non-chelating phenanthrolines have a similar pharmacology.

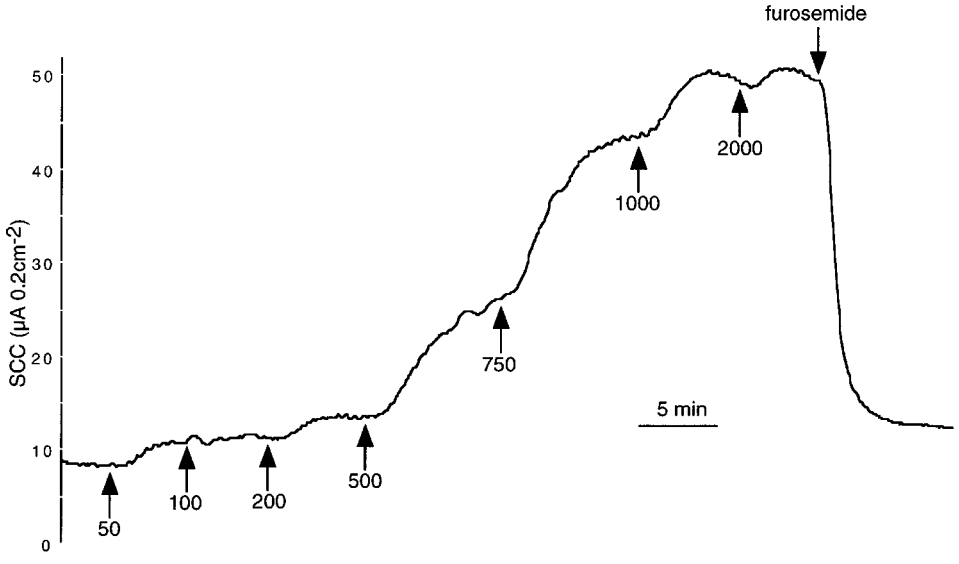

Figure 1 Effect of 1,10 phenanthroline on SCC in mouse colon epithelium. The epithelium was bathed in KHS with amiloride, 100 μM and Ba^{2+}, 5 mM in the apical bathing solution. Cumulative addition of 1,10 phenanthroline to both sides of the epithelium (in μM) is indicated. Furosemide, 1 mM, was added basolaterally after the maximal response had been reached.

Effects of phenanthroline isomers

Phenanthroline can exist in three isomeric forms, namely 1,10; 1,7 and 4,7 and thus far all experiments have been made with the 1,10 isomer. Unlike 1,10 phenanthroline the other isomers are not metal chelators since the nitrogens are unsuitably placed. However both 1,7 and 4,7 phenanthrolines proved active in increasing SCC when applied to colonic epithelia. The concentration response curves for all three isomers are shown in Figure 2 and were fitted according to the equation:

$$Y = SCC_{min} + \Delta SCC_{max}/1 + [EC_{50}/D]^n \quad (1)$$

where Y = SCC, D = drug concentration and *n* is the Hill slope. Table 2 gives the data from curve fitting for the three phenanthroline isomers. It is found that 4,7 phenanthroline is less potent than either 1,10 or 1,7 phenanthroline, but all have Hill slopes greater than 1.

Effect of 1,10 phenanthroline on unidirectional chloride fluxes

A direct way to show that 1,10 phenanthroline increases chloride secretion is to measure chloride movement using $^{36}Cl^-$. $^{36}Cl^-$ fluxes were measured in both the basolateral to apical and in the apical to basolateral directions. The change in chloride movement in each direction in response to 1,10 phenanthroline was calculated from the specific activity and expressed as a percentage of the SCC response to phenanthroline, obtained by integrating the area under the SCC-time record. The experiments were unpaired, that is each preparation was used to measure flux in only one direction. The data are given in Table 3. It is found that phenanthroline increases flux in the basolateral to apical direction by 72.9% of the SCC, while the backflux is reduced by phenanthroline by 84.7% of the SCC. The net flux therefore corresponds to 157% of that predicted by the SCC.

Table 1 Effect of phenanthroline, 1 mM, on SCC

Tissue type	ΔSCC (μA cm^{-2})	n
Wild type	134.4±6.9	18
Null (*Cftr^{tm1Cam}*)	2.4±0.7	4
ΔF508 (*Cftr^{tm2Cam}*)	1.5±2.0	6

Amiloride, 100 μM and Ba^{2+}, 5 mM were present in apical solution in all experiments.

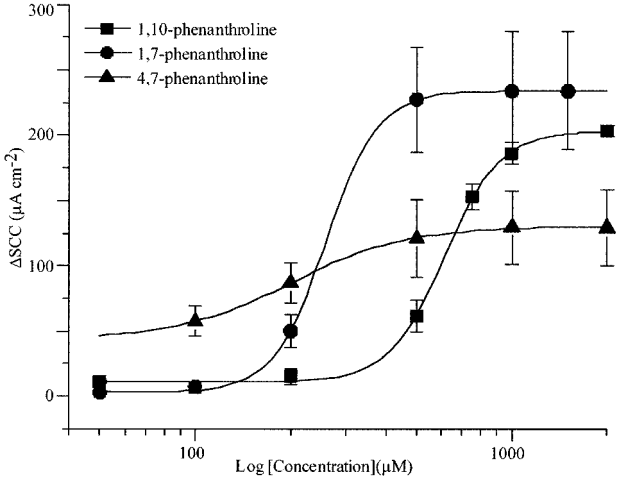

Figure 2 Concentration response curves for 1,10; 1,7 and 4,7 phenanthrolines. Cumulative additions of the phenanthrolines were made to epithelia in the presence of amiloride, 100 μM and Ba^{2+}, 5 mM in the apical bathing solution. Mean values±standard errors (all *n* = 4) are shown. The curves were fitted using the equation

$$Y = SCC_{min} + \Delta SCC_{max}/1 + [EC_{50}/D]^n \quad (3)$$

where Y = SCC, D = drug concentration and *n* is the Hill slope.

Effects of 1,10 phenanthroline on CFTR chloride channels and basolateral K^+ channels

Maintained chloride secretion, as with phenanthrolines, often requires co-ordinate actions on both apical and basolateral membranes. For example an agent opening both apical CFTR chloride channels as well as basolateral potassium channels will be more effective than one having only one of these actions. Thus sets of experiments were devised to examine (a) does 1,10 phenanthroline activate CFTR? and (b) does 1,10 phenanthroline activate basolateral K^+ channels? To answer the first of these questions the basolateral membrane was effectively eliminated by the method described recently (Cuthbert, 2001) in which the basolateral epithelial surface is depolarized with a high K^+ solution containing impermeant anions, while the apical surface is bathed in KHS containing amiloride and Ba^{2+} ions. Using this configuration there is an inward chloride gradient through the apical membrane. Agents increasing P_o for CFTR cause an increase in negative SCC. Figure 3 shows results of single experiment where the basolateral side was depolarized with a potassium gluconate solution (see Methods). An increase in negative SCC was seen with 1,10 phenanthroline (1 mM) indicating an

Table 2 Kinetic parameters for the effects of phenthrolines on SCC in colonic epithelia

Agent	*EC_{50}* (μM)	*ΔSCC_{max}* (μA cm^{-2})	*Hill slope*
1,10 Phenanthroline	612±10.0	204±3.0	4.9±0.3
1,7 Phenanthroline	259±6.0	235±2.0	5.2±0.4
4,7 Phenanthroline	201±11.0	131±1.0	2.4±0.3

Table 3 $^{38}Cl^-$ fluxes in intact epithelia in response to 1,10 phenanthroline, 1 mM

(a) Basolateral to apical fluxes

Additions	*J_{S-M}*	*$\mu Eq\ cm^{-2}\ h^{-1}$ ΔSCC*	*$\%\Delta SCC$*	n
None	2.2	3.9	56.4	2
Amiloride/Ba^{2+} apical	4.2	5.2	80.8	4
All experiments	3.50±0.65	4.80±0.71	72.9	6

(b) Apical to basolateral fluxes

Additions	*J_{M-S}*	*$\mu Eq\ cm^{-2}\ h^{-1}$ ΔSCC*	*$\%\Delta SCC$*	n
Amiloride/Ba^{2+} apical	−2.44±1.08	2.88±0.40	−84.7	6

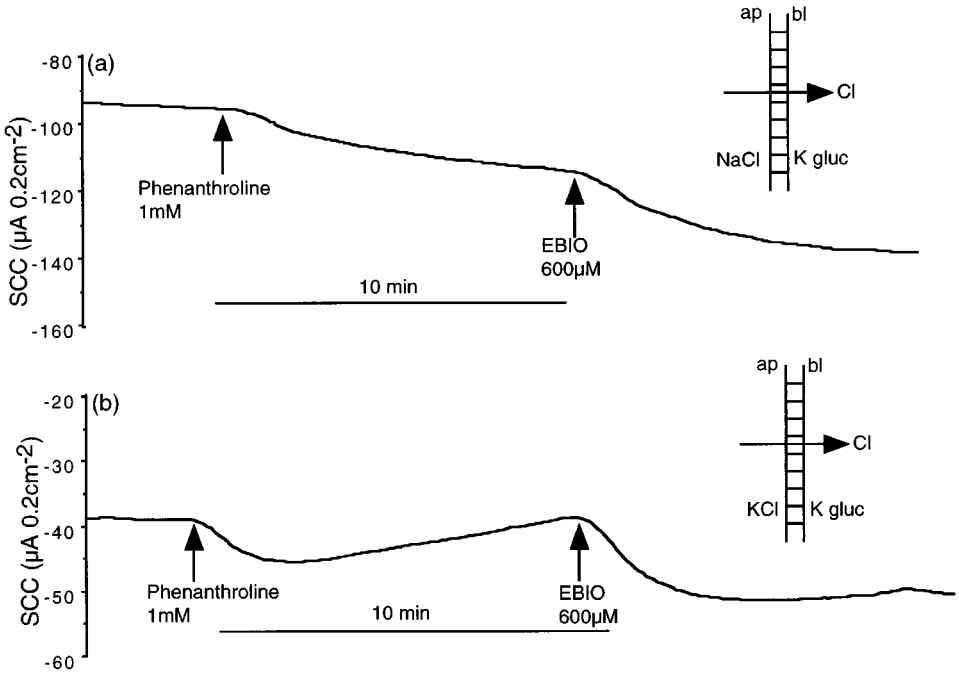

Figure 3 Chloride currents in depolarized mouse colonic epithelia. Epithelia were depolarized on the basolateral side with potassium gluconate while the apical solution was KHS in (a) or KCl solution in (b). 1,10 Phenanthroline, 1 mM was added to both sides as indicated, followed by EBIO, 600 μM. The insets show the direction of the chloride currents. ap indicates apical and bl indicates basolateral.

increase in chloride conductance. The increase in negative SCC with 1,10 phenanthroline (1 mM) was 65.1 ± 14.9 μA cm^{-2} ($n=7$) (Figure 4). Evidence that these responses to 1,10 phenanthroline were not the result of leak currents, due to the gradients, was gained from further experiments. In the first, after amiloride had been added, subsequent addition of Ba^{2+}, 5 mM, reduced the negative SCC by $+130.2 \pm 13.7$ μA cm^{-2}. When 1,10 phenanthroline, 1 mM, was added a change of -41.1 ± 5.8 μA cm^{-2} was recorded that was reversed by NPPB, 100 μM, by $+33.6 \pm 15.1$ μA cm^{-2} (all $n=3$). Using CF colons and the same protocol Ba^{2+} 5 mM, reduced the negative SCC by $+75.5 \pm 3.0$ μA cm^{-2} and 1,10 phenanthroline, 1 mM, changed the current by $+5 \pm 8.45$ μA cm^{-2} (both $n=3$), a value not significantly different from zero, but significantly different from either value for 1,10 phenanthroline in wild type colons ($P<0.02$). Finally, the apical bathing solution was changed to one containing high K^+, such that the only gradient that exists is an apical to basolateral chloride gradient. Phenanthroline was still able to cause a transient outward chloride current in these circumstances, as also shown in Figure 3. The known chloride channel opener, EBIO, added after phenanthroline was able to further increase the negative SCC (Figures 3 and 4). In summary, it appears that 1,10 phenanthroline increases the chloride conductance in 'apical membrane only' colons, a property not shared by CF colons, strongly suggesting that CFTR is essential for the response.

Turning to the second question 'does phenanthroline open basolateral K^+ channels?' two different approaches were used; first the effects of K^+ channel blockers were considered and, secondly, the effects of phenanthroline on K^+ currents, after the apical membrane had been permeabilized with nystatin, were investigated.

The data from four sets of experiments in which K^+ channel blockers were used is given in Figure 5. Throughout 1,10 phenanthroline, 1 mM, was used to generate a standard response. Addition of either ChTX, 50 nM, a blocker of intermediate conductance Ca^{2+}-sensitive K^+ channels (KCNN4) or addition of XE991, 30 μM, a blocker of cyclic AMP-sensitive K^+ channels (KCNQ1/KCNE3) (MacVinish *et al*., 2001) caused a significant reduction of the responses to 1,10 phenanthroline (Figure 5a,b) that was further reduced with the second blocking agent. If one of the inhibitors was added before phenanthroline there was little difference in the responses obtained to phenanthroline (Figure 5c,d), indeed ANOVA analysis showed that that none of the phenanthroline responses were significantly different from each other in the four sets of experiments (Figure 5a–d) ($F=0.52$, $P=0.67$). This suggests that phenanthroline recruits both types of K^+-channel to support chloride secretion, but just one can drive the membrane potential sufficiently towards E_K to give the maximal effect on chloride secretion. Thus channels can be recruited singly by adding one of the blocking agents ahead of phenanthroline. For example, if XE991 is added before phenanthroline (Figure 5c) the response (115.7 ± 17.7 μA cm^{-2}) is similar to that obtained in its absence (Figure 5a) (126.5 ± 11.5 μA cm^{-2}), but this makes a significant difference to the subsequent response to ChTX. ChTX causes 61.0% inhibition of the phenanthroline response in Figure 5c, but only inhibits by 23.3% in Figure 5a, the residual currents in these two (44.8 ± 6.2 μA cm^{-2} versus 97.0 ± 12.0 μA cm^{-2}) being significantly different ($P<0.01$). Similar arguments can be made for the pre-addition of ChTX on the responses to XE991, which causes 43.0% inhibition in Figure 5d, but only 23.0% inhibition in Figure

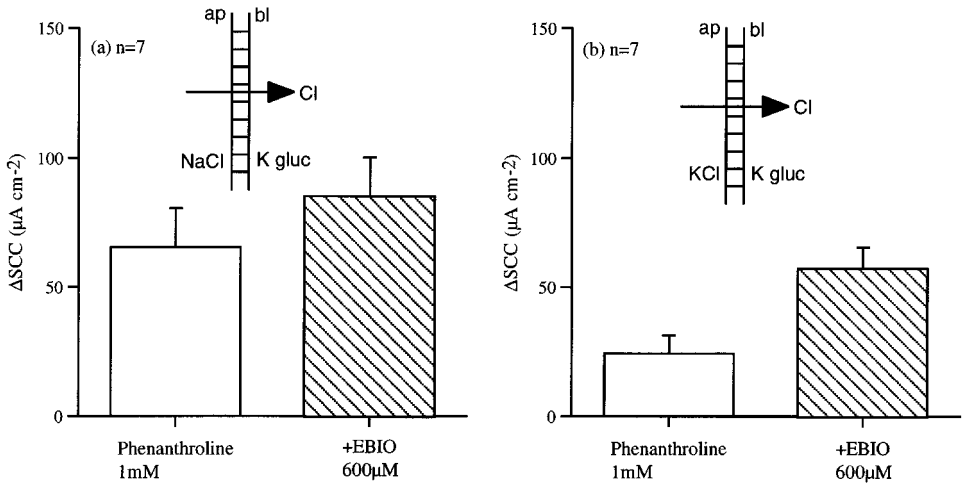

Figure 4 SCC changes in depolarized colonic epithelia after phenanthroline and EBIO. Consolidated data from experiments of the type shown in Figure 3 where *n* values indicate the number of experiments.

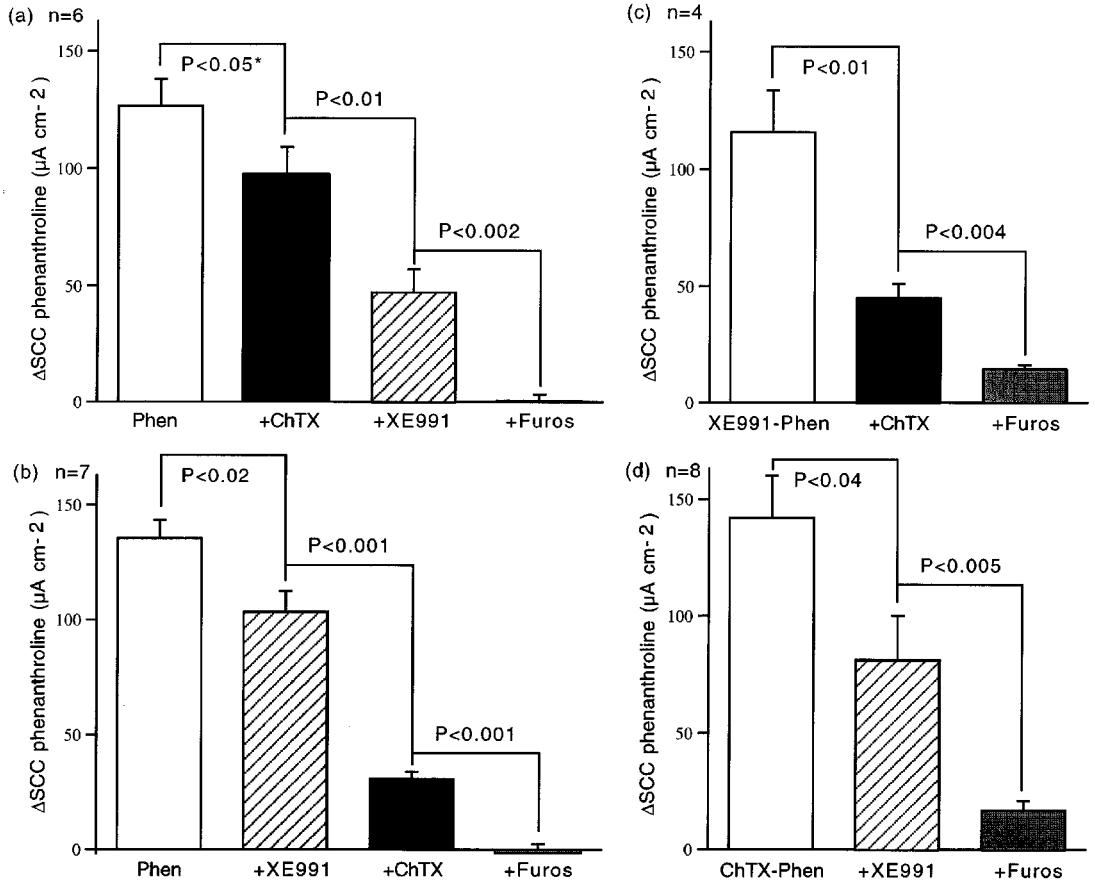

Figure 5 Effects of K^+ channel blockers on the effects of phenanthroline. 1,10 Phenanthroline, 1 mM (phen) was used throughout to increase SCC. After the current had stabilized either ChTX, 50 nM followed by XE991, 30 μM was added (a) or the order of the addition was reversed (b). In (c) XE991 and in (d) ChTX were added before phenanthroline. Furosemide, 1 mM (Furos) was added basolaterally at the end of each experiment. The mean values $\pm$ s.e. are indicated, together with the number of experiments. *P* values are indicated on the diagram were determined with an unpaired *t*-test except where an asterisk is shown, when a Mann-Witney non-parametric comparison was used.

5b. In this instance the residual currents were not significantly different. Thus the case for the recruitment of ChTX-sensitive K^+-channels is strong, while the case for XE991-sensitive K^+-channels is less so, and is probably confounded by the constitutively open nature of these channels (Schroeder *et al.*, 2000).

Nystatin permeabilization of the apical membrane, in the presence of an apical to basolateral K^+ gradient allowed the effects of phenanthroline to be examined directly on the K^+-current. Figure 6 shows there is an increase in SCC when 1,10 phenanthroline is applied to nystatin treated tissues, the increase being sensitive to both XE991 and to ChTX. The SCC increase caused by 1,10 phenanthroline, 1 mM in nystatin treated tissues was 123.3 ± 37 μA cm^{-2} and ChTX reversed this by -161.3 ± 70.3 μA cm^{-2} (both $n=3$). Similarly, XE991,30 μM reversed the SCC by -169.7 ± 23.2 μA cm^{-2} after phenanthroline caused an increase of 50.7 ± 2.7 μA cm^{-2} (Both $n=3$). Note that the reversal caused by XE991 far exceeds the increase in K^+ current, supporting the view that cyclic AMP-sensitive K^+-channels are constitutively open. Thus both the use of K^+ channel blockers and nystatin permeabilization studies suggest that phenanthrolines are K^+ channel openers, at least for the ChTX-sensitive channel.

Effect of 1,10 phenanthroline on second messenger systems

As the last section indicates phenanthrolines have effects on both Ca^{2+} and cyclic AMP sensitive K^+ channels it is important to know if the effects are *via* second messenger systems. Cyclic AMP concentrations were measured by radioimmunoassay following exposure of epithelia to either 1,10 phenanthroline or to forskolin in the presence or absence of isobutylmethylxanthine (IBMX). The cyclic AMP content of the tissues plus that released into the medium were measured. Table 4 shows that 1,10 phenanthroline was without effect on adenylate cyclase.

To measure if phenanthrolines caused release of intracellular Ca^{2+} we used Fluo 4 as reporter molecule and challenged isolated colonic crypts with ATP and 1,10 phenanthroline. No discernable response was seen with phenanthroline, in contrast to ATP (Figure 7). A total of six experiments were made like the two illustrated in Figure 7 in which ATP was added first in three and second in the others. Integrating the peaks resulting from the drug additions gave values for the fluorescence increase in response to ATP, 100 μM of 338.3 ± 84.7 (arbitrary units), while phenanthroline, 1 mM gave a value of 2.5 ± 2.4 ($P<0.003$, $n=6$).

Effects of benzoquinolines on colonic SCC

The phenanthrolines all have two nitrogens within the phenanthrene ring structure and examination of space filling models indicated all are flat planar structures. To determine if both nitrogens were essential the activities of a number of derivatives with single ring nitrogens were examined. They were 7,8 benzoquinoline, phenanthridine and pyrido(1,2-A) quinolinylium, the latter being the nucleus of compounds such as MPB-07 and MPB-27, which are known to be CFTR chloride channel openers (Becq *et al.*, 1999). In addition phenanthrene, without any nitrogens, was examined for activity. This latter was found to be inactive on colonic epithelia. Addition of phenanthrene, cumulatively up to 2 mM, changed the basal SCC from 48.5 ± 9.0 μA cm^{-2} to

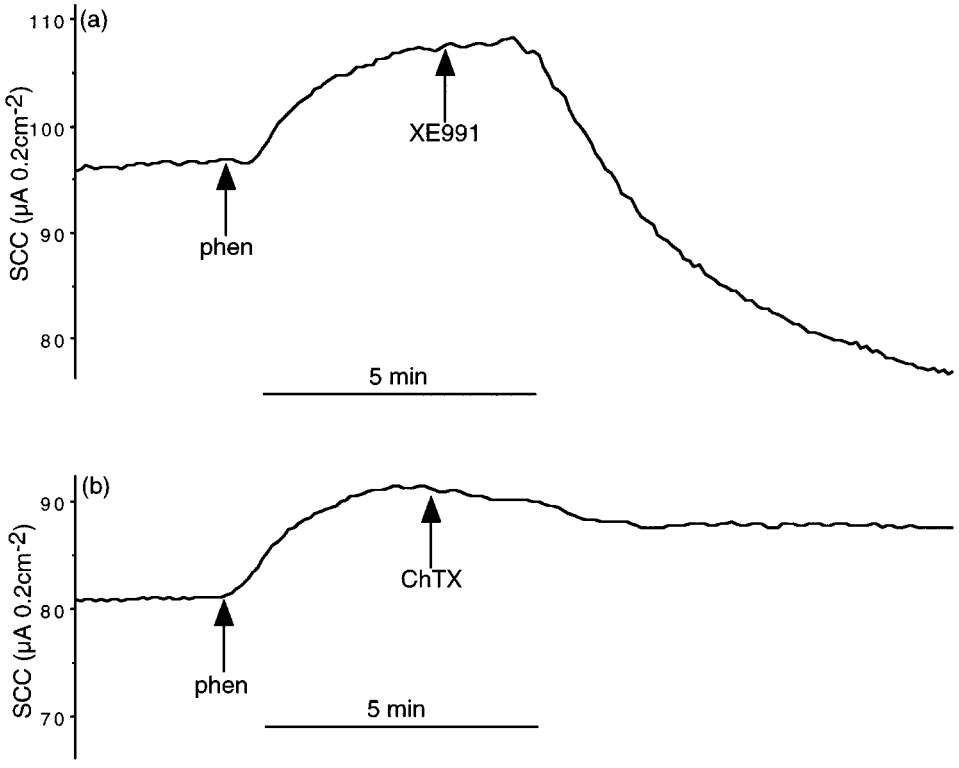

Figure 6 Effects of phenanthroline on nystatin permeabilized colonic epithelia. Epithelia were subjected to an apical to basolateral K^+ gradient and the apical surface was exposed to nystatin (180 μg ml^{-1}). The SCC eventually stabilized at high values after which 1,10 phenanthroline (phen), 1 mM was added. The phenanthroline induced current was sensitive to ChTX, 50 nM and XE991, 30 μM.

Table 4 Effect of forskolin and 1,10 phenanthroline on cyclic AMP in colonic epithelia

Agent	*Without IBMX* (*cyclic AMP pmol mg protein^{-1}*) n=4	P	*With IBMX* (*cyclic AMP pmol mg protein^{-1}*) n=4	P
Basal	1.04±0.16		3.04±0.56	
1,10 phenanthroline (1 mM)	1.91±0.42	NS	3.55±0.52	NS
Forskolin (10 μM)	4.14±0.87	<0.02	16.31±2.32	<0.002

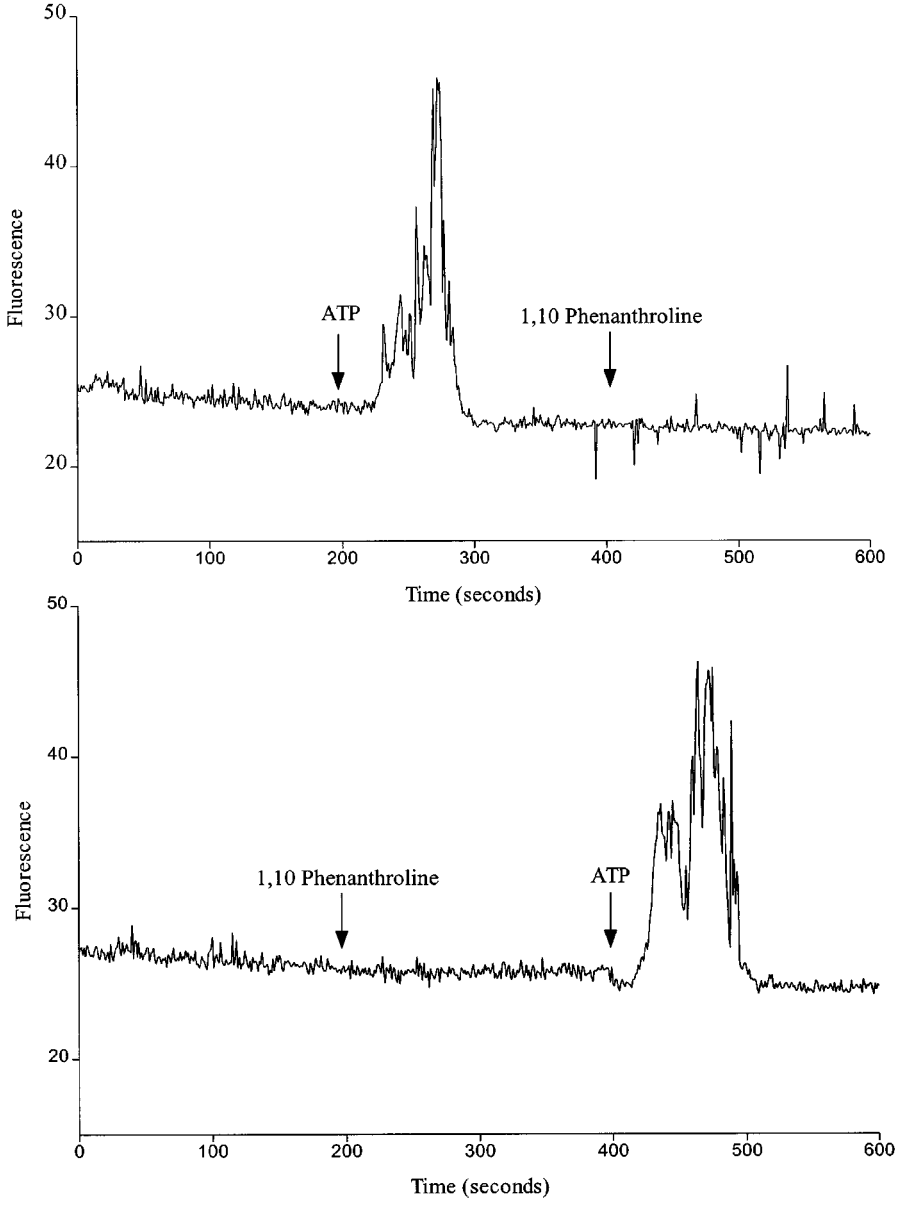

Figure 7 Fluorescence in colonic crypt suspensions loaded with Fluo 4. A colonic crypt preparation, loaded with Fluo 4, was divided into two. One half was exposed first to ATP, 100 μM and then to 1,10 phenanthroline, 1 mM. The other half received the same drugs in reverse order. Emission fluorescence (in arbitrary units) was measured at 516 nm with excitation at 494 nm.

57.0±7.5 μA cm^{-2}, an increase of only 8.5±4.7 μA cm^{-2} (all $n=4$) which was not significant. In the presence of phenanthrene, 2 mM, forskolin, 10 μM, caused a SCC increase of 256.0±25.5 μA cm^{-2} ($n=4$), indicating that phenanthrene had no adverse effect on the tissues' ability to secrete chloride, even at high concentration. The three single nitrogen compounds all showed activity, but the activity of pyrido (1,2-A) quinolinylium was low, at least at the concentrations that could be tested (Figure 8). Only incomplete concentration response curves for phenanthridine and pyrido (1,2-A) quinolinylium were obtained, because of limitations of solubility or availability of the materials. A

virtually complete response curve was obtained for 7,8 benzoquinoline and fitting the data to the equation

$$Y = SCC_{min} + \Delta SCC_{max}/1 + [EC_{50}/D]^{n} \quad (2)$$

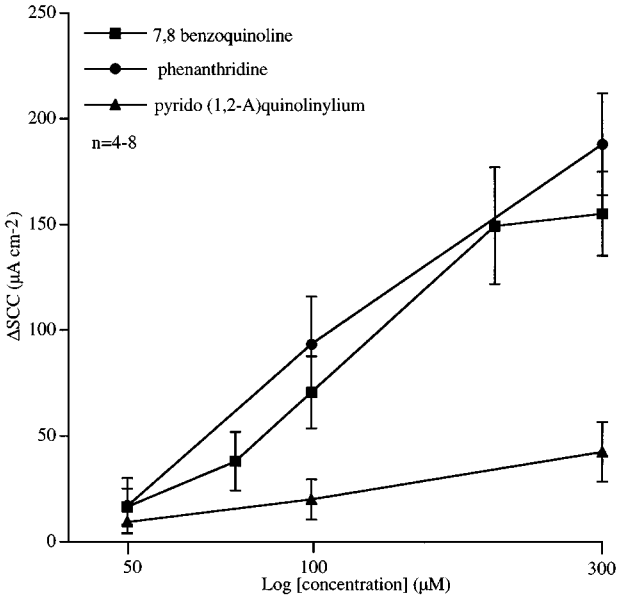

Figure 8 Concentration response curves for 7,8 benzoquinoline, phenanthridine and pyrido (1,2-A) quinolinylium. Epithelia were exposed to amiloride, 100 μM and Ba^{2+} 5 mM on the apical side. The three agents were then added cumulatively to both sides of the tissues.

where Y = SCC, D = drug concentration and n is the Hill slope, gave values of 145.5 ± 1.0 μA cm^{-2} for the maximal response, 109.1 ± 2.7 μM for the EC_{50} and a Hill slope of 4.3 ± 0.6. Thus 7,8 benzoquinoline has an apparent affinity greater than for the compounds containing two nitrogen atoms. The concentration response curves were extremely steep as shown in Figure 9. In Figure 9a there was little or no response at 50 μM, yet at 100 μM the response was maximal whereas in Figure 9b graded concentrations up to 300 μM were required to reach the maximal response. Nevertheless both preparations had concentration response relationships covering just one third of an order of magnitude. The actions of 7,8 benzoquinoline were almost completely inhibited by furosemide (Figure 9) indicating that their overall effect was to cause chloride secretion.

Effect of phenanthroline on airway epithelia

There is a particular interest in finding compounds that increase chloride secretion in airway epithelia in relation to cystic fibrosis. In earlier studies we have found that compounds that either increase or inhibit chloride secretion in the colon have relatively modest effects in airway epithelium (MacVinish *et al*., 1998). The effects of 1,10 phenanthroline on murine nasal epithelium were measured using procedures described elsewhere (MacVinish *et al*., 1998). 1,10 phenanthroline, 1 mM, had a modest effect compared to that on the colon and, furthermore the response was not maintained. After the effect had declined to zero forskolin was able to produce a maintained response of greater magnitude (Table 5).

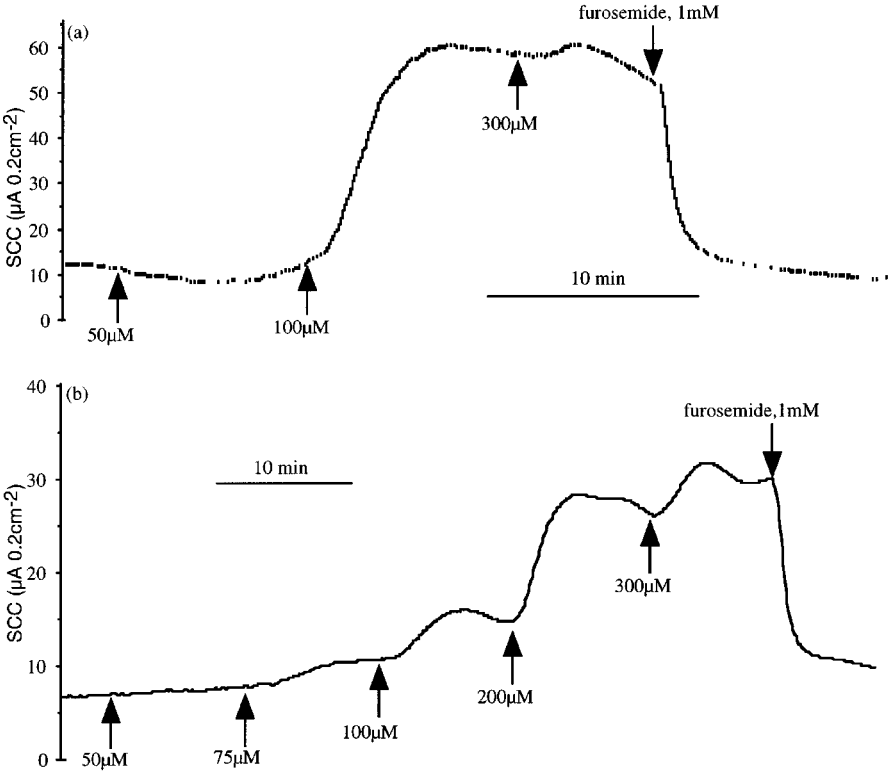

Figure 9 Examples of the effects of 7,8 benzoquinoline on SCC in colonic epithelia. In (a) no response is seen with 50 μM, yet the response is maximal at 100 μM. In (b) more graded responses are seen. In both (a) and (b) the responses are inhibited by furosemide, 1 mM, applied basolaterally.

Discussion

The majority of the experiments described in this study have been made with 1,10 phenanthroline and the results have indicated that this agent increases electrogenic chloride secretion in colonic epithelia. We assume the other compounds studied have a similar mode of action, but appreciate that the evidence for this is only partial, being based on structural similarities, common characteristics of action, such as a large Hill coefficient and the effectiveness of inhibitors. The structures investigated are shown in Figure 10. All are planar molecules with little to distinguish them, except the charge distribution associated with nitrogen.

The evidence that these structures stimulate chloride secretion is as follows: (a) the nature and direction of the SCC and its inhibtion by furosemide, (b) the association of the SCC response with increased net Cl^- flux, (c) absence of responses in CF colons and (d) the demonstration of outward chloride currents in depolarized colons, especially when the only concentration gradient was due to Cl^-. In regard to (c) the net flux of Cl^- was greater than predicted by the SCC indicating that other ions must contribute to reduce the SCC or alternatively some Cl^- movement is by non-electrogenic processes. The chloride currents in depolarized colons are unlikely to result from an increase in leak current due to phenanthroline, since they were not found with CF colons and were reversed by NPPB, a non-specific CFTR blocking agent (Cuthbert, 2001). No second messenger effects involving either cyclic AMP or $Ca^{2+}{}_i$ were discovered indicating that the phenanthrolines are unlikely to act in ways similar to common physiological agonists. We considered the possibility that phenanthrolines produce their effects by inhibiting metalloproteases, as suggested earlier (Duszyk *et al*., 1999). This hypothesis is no longer tenable, as non-chelating phenanthrolines were similarly active. However many of the compounds studied, including tricyclic aromatic compounds with a single heterocyclic nitrogen can form co-ordinate complexes with heavy metal ions (Agarwal & Gupta, 1987), so metallic ion involvement cannot be completely eliminated.

All of the compounds investigated have large EC_{50} values, being in range the 0.1–0.6 mM. These values are similar to chloride channel openers of other classes, such as EBIO and chlorzoxazone, which also have actions at the basolateral side of epithelia (Devor *et al*., 1996). All four compounds for which complete concentration response curves were obtained showed Hill coefficients in the range 2–6, a feature also found with EBIO (Cuthbert *et al*., 1999). The high values for the Hill slope found in this study cannot be taken to mean there is a co-operative interaction between CFTR and phenanthrolines. The steep concentration response relationships might equally well arise from dual action on, say CFTR and basolateral K^+ channels, one activity amplifying the effectiveness of the other.

Pyrido (1,2-A) quinolinylium proved to be the least potent of the compounds we examined. This was surprising as it is the nucleus of a series of substituted benzo [c] quinolizinium compounds produced by Becq *et al*. (1999), such as MPB-07 and MPB-27, which are CFTR activators. MPB-07 and MPB-27 are required at concentrations of 200–500 μM to produce substantial effects not unlike the EC_{50} values for the phenanthrolines. Nevertheless from a comparison of the Becq data with our data on pyrido (1,2-A) quinolinylium we estimate that the introduction of substituent groups produces a substantial increase in potency. The possibilty exists, therefore, that substituents added to the phenanthroline or benzo-quinolinium nucleus might yield far more potent compounds. However, the MPB compounds may act in a different way to those examined here. For example we fitted the concentration response curve (Figure 8b) given by Becq *et al*. (1999) and found that the Hill slope was close to 1. Further, of the six compounds we have examined pyrido (1,2-A) quinolinylium is the only one that bears a charge.

From this study it is evident phenanthrolines have more than one action. We have shown that 1,10 phenanthroline also activates basolateral K^+ channels. The evidence for this is: (a) selective inhibitors of the Ca^{2+} activated K^+ channels (charybdotoxin) and cyclic AMP sensitive K^+ channels (XE991) reduce the SCC response to 1,10 phenanthroline. This suggests that it opens these channels, hyperpolarizing the cells, so increasing the electrical gradient for chloride exit across the apical face of the epithelium. and (b) permeabilization of the apical face, in the presence of a K^+ gradient, showed that 1,10 phenanthroline increased the potassium current through the basolateral membrane.

However the arguments for activation of ChTX-sensitive K^+-channels are clearer, as the cyclic AMP-sensitive channels are already partially active without stimulation (Schroeder *et al*., 2000). As 1,10 phenanthroline had no effect on adenylate cyclase activity the effect on cyclic AMP sensitive K^+ channels, and indeed on CFTR, is not *via* the normal second messenger mechanisms. Similarly the lack of effect of 1,10 phenanthroline on $Ca^{2+}{}_i$ indicates too that an alternative to the normal second messenger mechanism is involved. How do the phenanthrolines and benzoquinolines increase chloride secretion in the colon? The detailed mechanisms for known chloride channel openers has been recently reviewed (Schultz *et al*., 1999) and a plethora of different mechanisms has been proposed. For example, for xanthines an increase in $Ca^{2+}{}_i$, inhibition of phosphodiesterases and phosphatase inhibition

Table 5 Effect of 1,10 phenanthroline and forskolin on SCC in nasal epithelia

ΔSCC (μA cm^{-2})			
1,10 Phenanthroline, 1 mM		*Forskolin* 10 μM	n
11.3±3.6 μA cm^{-2}	5.2±1.7 min	48.4±17.9 μA cm^{-2}	4

Both the extent and the duration are given for the phenanthroline responses.

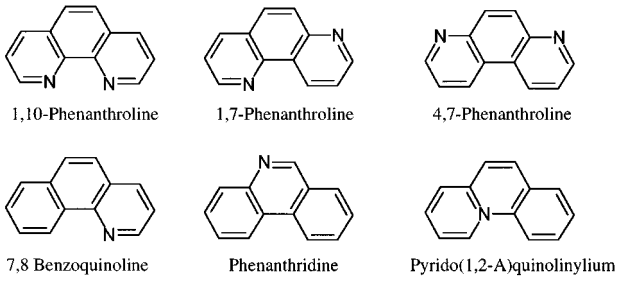

Figure 10 Chemical structures of the compounds used in the study.

are all proposed mechanisms of action for their chloride secretory activity. It would seem that the first two mechanisms are eliminated for the phenanthrolines, while phosphatase inhibition must remain a possibility. Note too that the three possible mechanisms for xanthines are all indirect, for example, phosphatase inhibition is expected to preserve the active, phosphorylated state of CFTR. Flavones, such as genistein have been reported to act directly on the CFTR channel, while other evidence suggests that inhibition of tyrosine kinases or of phosphatases are involved. It seems clear that genistein can enhance submaximally cyclic AMP stimulated chloride secretion (Illek *et al*., 1996) and enhances cyclic AMP production (Sears *et al*., 1995). Perhaps the effects of genistein on different chloride secretory systems depends on the level of background activity of adenylate cyclase or other signalling systems. The benzimidazolone, NS004, was reported by Gribkoff *et al*. (1994) to directly activate CFTR and ΔF508CFTR, but subsequent work suggested that CFTR needed to be in a permissive state for activation in inside-out membrane patches. Importantly, NS004, had little effect on chloride secretion in intact epithelia (Devor *et al*., 1996) stressing how important co-ordinate activity at the apical and basolateral membranes is necessary to obtain transepithelial chloride transport, as found with another benzimidazolone, such as EBIO (Cuthbert *et al*., 1999). As with NS004, the psoralen, 8-methoxypsoralen (8-MOP), failed to induce chloride secretion in T84 monolayers (Devor *et al*., 1997) unless other agonists were used to activate basolateral K^+-channels. Although the evidence points to a direct effect of 8-MOP on CFTR the failure to activate CFTR on inside-out patches (quoted by Schultz *et al*., 1999) may indicate that CFTR needs to be in the appropriate state engendered by intracellular mechanisms. The data with the phenanthrolines support the concept, that activity at both membranes is required for chloride secretory activity. This brief summary of the actions of known CFTR chloride channel openers reveals that there is no uncontested evidence for a direct effect on CFTR for any of the present CFTR channels openers. While the activation of CFTR on inside-out membrane patches limits the possible targets, it seems that the channels may need to be primed to a permissive state for activation to occur.

In summary, evidence shows that 1,10 phenanthroline activates epithelial K^+ channels as well as CFTR. Generally compounds with a precise single action are useful starting points for the development of therapeutic agents. However, in this instance the combination of CFTR and K^+ channel targets makes phenanthrolines more active as epithelial chloride secretagogues. Whether their actions at the two sites are direct or involve other cellular mechanisms is unclear, and different experimental approaches will be needed to establish the site of action. It will be important to learn if these compounds, or their derivatives, can activate membrane located ΔF508 CFTR and therefore be of potential use, if the mutant protein can be chaperoned to the apical membrane.

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