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4-Chloro-benzo[*F*]isoquinoline (CBIQ) activates CFTR chloride channels and KCNN4 potassium channels in Calu-3 human airway epithelial cells

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1 Calu-3 cells have been used to investigate the actions of 4-chloro-benzo[*F*]isoquinoline (CBIQ) on short-circuit current (SCC) in monolayers, whole-cell recording from single cells and by patch clamping.

2 CBIQ caused a sustained, reversible and repeatable increase in SCC in Calu-3 monolayers with an EC_{50} of 4.0 μM. Simultaneous measurements of SCC and isotopic fluxes of $^{36}Cl^-$ showed that CBIQ caused electrogenic chloride secretion.

3 Apical membrane permeabilisation to allow recording of basolateral membrane conductance in the presence of a K^+ gradient suggested that CBIQ activated the intermediate-conductance calcium-sensitive K^+-channel (KCNN4). Permeabilisation of the basolateral membranes of epithelial monolayers in the presence of a Cl^- gradient suggested that CBIQ activated the Cl^--channel CFTR in the apical membrane.

4 Whole-cell recording in the absence of ATP/GTP of Calu-3 cells showed that CBIQ generated an inwardly rectifying current sensitive to clotrimazole. In the presence of the nucleotides, a more complex I/V relation was found that was partially sensitive to glibenclamide. The data are consistent with the presence of both KCNN4 and CFTR in Calu-3.

5 Isolated inside-out patches from Calu-3 cells revealed clotrimazole-sensitive channels with a conductance of 12 pS at positive potentials after activation with CBIQ and demonstrating inwardly rectifying properties, consistent with the known properties of KCNN4. Cell-attached patches showed single channel events with a conductance of 7 pS and a linear I/V relation that were further activated by CBIQ by an increase in open state probability, consistent with known properties of CFTR. It is concluded that CBIQ activates CFTR and KCNN4 ion channels in Calu-3 cells.
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Abbreviations: cAMP, cyclic adenosine monophosphate; CBIQ, 4-chloro-benzo[*F*]isoquinoline; CFTR, cystic fibrosis transmembrane conductance regulator; ChTX, charybdotoxin chromanol 293B, *trans*-6-cyano-4-4(*N*-ethylsuphonyl-*N*-methylamino)-3-hydroxy-2,2-dimethyl-chromane; DPC, diphenylamine carboxylate; EBIO, 1-ethyl-2-benzimidazolone; EC_{50}, concentration causing 50% of the maximal response; FRT, Fischer rat thyroid cells; IBMX, isobutylmethylxanthine; KCNN4, intermediate-conductance calcium-sensitive potassium channel

Introduction

There is intense interest in compounds that activate the cystic fibrosis transmembrane conductance regulator, CFTR, which may be useful in developing a therapeutic strategy for cystic fibrosis. CFTR protein is located in the apical domain of many types of epithelial cell where it acts as a chloride Cl^- ion channel, as well as having regulatory influence over many other proteins engaged in the vectorial transport of salts and water. Mutations in the CF gene give rise to aberrant forms of CFTR where impaired opening, conductance or trafficking affect performance (Welsh & Smith, 1993), a major consequence of which is reduced mucociliary clearance in the lungs, following which bacterial infection supervenes with the inevitable destruction of lung tissue (Pilewski & Frizzell, 1999).

Early examples of agents that activate CFTR are genistein (Illek & Fischer, 1998), MPB compounds (Becq *et al*., 1999) and the benzimidazolone, NS004 (Gribkoff *et al*., 1994), but more recently novel benzoflavone and benzimidazolone compounds, with submicromolar potencies have been found using high-throughput screening of chemical libraries (Caci *et al*., 2003). Some of the benzimidazolone compounds (e.g. UCCF-853) are thought to have additional minor effects on basolateral K-channels. The activation of basolateral K-channels in the epithelial cells leads to cellular hyperpolarisation, increasing the electrochemical gradient for Cl^-

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efflux through CFTR. More recently, further screening has revealed some tetrahydrobenzothiophenes that are capable of activating ΔF508 CFTR in the presence of cyclic adenosine monophosphate (cAMP), where the protein has been trafficked by exposure of the cells to low temperature (Yang *et al*., 2003). This is an important development, as the most common mutation in cystic fibrosis is ΔF508, with some 75% of patients having at least one ΔF508 allele. However, the mutant protein is not normally trafficked to the membrane, but can show Cl^- channel activity if coaxed to the membrane by, for example, by lowering the temperature (Denning *et al*., 1992).

As explained above, agents activating basolateral K^+-channels in epithelia might be useful in situations where CFTR function is minimal as they boost the efflux of Cl^- ions through CFTR *via* cellular hyperpolarisation. The classical examples of this type of agent are 1-ethyl-2-benzimidazolone (EBIO), zoxazolamine, chlorzoxazone, together with the more recent and potent dichloro-EBIO (Singh *et al*., 2001). EBIO itself was also shown to have some stimulating activity on CFTR (Cuthbert *et al*., 1999; Devor *et al*., 1999).

In a series of studies on both natural epithelia and cultured epithelial monolayers, the activities of a series of phenanthrolines and benzoquinolines have been investigated. Both 7,8-benzoquinoline and 5,6-benzoquinoline were powerful activators of chloride secretion in murine colonic epithelia and monolayers of cultured Calu-3 cells, derived from the serous cells of submucosal glands of human airways (Cuthbert, 2003; Cuthbert & MacVinish, 2003). Evidence indicated that both these benzoquinolines had dual actions on both apical CFTR and basolateral K^+-channels.

Here, we report on the activity of only one new compound, namely, 4-chloro-benzo[*F*]isoquinoline (CBIQ). It is shown to be a potent secretagogue of anion transport in intact epithelia. We demonstrate that CBIQ affects both CFTR Cl-channels and the intermediate-conductance calcium-sensitive K^+-channel, KCNN4, in Calu-3 cells.

Methods

Calu-3 cell culture

Calu-3 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and were grown on 75 cm^2 culture flasks containing Eagle's minimum essential medium (Vitacell, ATCC, VA, U.S.A.) or Dulbecco's modified Eagle's medium with 10% foetal calf serum (Gibco BRL), kanamycin, 100 μM ml^{-1} and fungizone, 1.25 mg ml^{-1} or alternatively with gentamycin sulphate, 5 μg ml^{-1}, penicillin G, 6 μg ml^{-1} and streptomycin, 10 μg ml^{-1}. Cells were incubated at 37°C in humidified air containing 5% CO_2. Cells from confluent monolayers were collected by trypsinisation and subcultured either on Snapwell polycarbonate membrane inserts (1 cm^2, 0.4 μM pore size) (Costar U.K., Ltd, Buckinghamshire, U.K.) or placed upon 15 mm coverslips (Fisherbrand, Pittsburg, PA, U.S.A.). Cultures on membrane inserts were refed every 3–4 days and used between 17 and 29 days after subculture for the inserts or after at least 12 h for the cells on coverslips.

Short-circuit current (SCC) recording

The detachable rings of the Snapwell inserts, bearing the cultured monolayers, were inserted into CHM5 Ussing chambers with associated electrodes (WPI, Herts, U.K.) and voltage clamped at zero potential using a WPI Dual Voltage Clamp-1000 (WPI, Herts, U.K.). Both sides of the epithelium were bathed in 5 or 6 ml of Krebs Henseleit solution (KHS) that was continually circulated through the half chambers, maintained at 37°C and continuously bubbled with 95% O_2/5% CO_2. SCCs were recorded continuously using an ADInstruments PowerLab/8SP (NSW 2154, Australia) and displayed on a computer screen.

KHS had the following composition (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11.1 glucose (pH 7.4). Drugs applied to both sides of the epithelium are indicated by bs, while drugs applied to the apical or basolateral surfaces only are designated by ap or bl, respectively. In general, drugs were allowed to act for 10 min, unless it was clear that steady state had been reached before then.

Modifications of the standard SCC procedure

To examine the effects on the basolateral membranes of Calu-3 epithelia, the apical membrane was treated with nystatin (200–250 μg ml^{-1}) until the SCC increased to a new steady state. In these experiments, the apical bathing solution was changed to potassium gluconate Krebs (PGK) and the basolateral solution to sodium gluconate Krebs (SGK), thus imposing a K^+ gradient in the apical to basolateral direction. For examining the effects of agents on the apical membranes, the method described by Cuthbert (2001) was used. A high potassium containing solution (PGK) is used to depolarise the basolateral membrane, while the apical membrane remained bathed in KHS, thus imposing an apical to basolateral Cl^- gradient. Alternatively, the basolateral membrane was treated with nystatin with an imposed apical to basolateral chloride gradient (i.e. apical bathing solution, KHS; basolateral bathing solution, SGK). Using these methods, a negative SCC (−SCC) was established after clamping, showing a slow, near linear increase. Extrapolation of this allowed an estimate of the effects of agents increasing apical chloride conductance as the vertical distance (in μA) between the trace and the extrapolated prediction. The modified solutions PGK (SGK) contained (mM): 120 K gluconate (Na gluconate), 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 4 $CaCl_2$ and 10 glucose.

Measurement of $^{36}Cl^-$ fluxes in Calu-3 monolayers

Calu-3 monolayers were used in matched pairs, each bathed in 6 ml KHS on each side. After the SCC had stabilised, a few μCi of $^{36}Cl^-$ was added to the basolateral side of one monolayer and to the apical side of the other. For the radioactive fluxes to achieve equilibrium, 20 min were allowed, after which samples were taken from the side to which the radioisotope had not been added. The sample size was 2 ml and was immediately replaced with 2 ml of fresh KHS. Second samples were taken 20 min later, with the same protocol, and CBIQ (20 μM) added to both sides of the epithelia. A third set of samples was taken

after a further 20 min. The SCC current was recorded throughout the experiment and small samples (200 μl) were taken from the 'hot' sides periodically. Unidirectional chloride fluxes were calculated from the specific activity and making allowance for the dilution caused by removing samples. The areas under the SCC traces were obtained using the integrator facility on the ADI PowerLab/8SP. The paired experiment was repeated nine times.

Patch clamping methods

Fire polished pipette electrodes were prepared with resistances of 4–8 MΩ for whole-cell recordings and 5–20 MΩ for single channel recordings. Cells on coverslips were washed with bath solution and mounted in an open bath chamber (Warner Instruments Inc., Hamden, CT, U.S.A.), maintained at 37°C and fixed to the stage of an Olympus Inverted Research Microscope (Lake Success, NY, U.S.A.). After the pipette was immersed in the bath solution, offset potentials were compensated before forming a GΩ seal. After seal formation, the whole-cell configuration was obtained by applying suction and the amplifier used to compensate for the series resistance and capacitance. Currents were recorded using an Axopatch 200A amplifier and Clampex 8.0 software (Axon Instruments, Foster City, CA, U.S.A.). Currents were reported with reference to zero in the bath and the data were analysed using pClamp 8.0 (Axon Instruments) and Microcal Origin 6.0 (Northampton, MA, U.S.A.) software.

Whole-cell recordings

Whole-cell recordings were made with a bath solution containing (mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10.5 HEPES and 10 glucose (pH 7.4). The pipette solution contained (mM): 5 NaCl, 135 KCl, 0.2 $CaCl_2$, 1.8 $MgCl_2$, 10 HEPES, 0.5 EGTA and 10 glucose (pH 7.4). In addition, MgATP, 1 mM and LiGTP, 0.2 mM were included in the pipette solution in some experiments. After the whole-cell configuration was achieved, the cell was clamped at –40 mV. Current records were obtained at 1 min intervals in response to voltage steps, each lasting 200 ms, from –80 to 80 mV in 20 mV increments. Traces were normalised to 1 pF, in order to remove variability due to differences in cell size, and the current–voltage relationship was obtained from the mean current during the central 140 ms of each recording.

Single channel recordings

Excised inside-out patch-clamp experiments were performed in a bath solution containing (mM): 145 K gluconate, 5 KCl, 0.78 $CaCl_2$, 1 $MgCl_2$, 1 EGTA and 10 HEPES (pH 7.2). The pipette solution contained (mM): 140 K gluconate, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (pH 7.2). Continuous recordings of the channel activity, sampled at 10 kHz, were made. Voltage was briefly stepped from –80 to 80 mV in 20 mV increments to obtain a current–voltage relationship. All kinetic data (NP_O measurements) were obtained at –60 mV. Recordings were filtered at 100 Hz, using an 8-Pole Bessel Filter, histograms were made and fitted with Gaussian functions in order to determine the unitary current amplitude (i). The channel activity was measured by NP_O (i.e. number of channels multiplied by the individual channel open probability), calculated as the mean current (I) divided by the unitary current amplitude ($NP_O = I/i$), from recordings that were at least 60 s long. Cell-attached patch-clamp experiments were performed in a similar way, with a few modifications. The bath and pipette solutions contained (mM): 135 Na gluconate, 5 K gluconate, 1 Mg $(gluconate)_2$, 2 Ca $(gluconate)_2$, 10 glucose and 10.5 HEPES (pH 7.4). The applied voltage is quoted as the negative of the pipette potential ($-V_{PIP}$) since the intracellular potential is uncertain, and all the kinetic data (NP_O) were obtained at $-V_{PIP} = 60$ mV.

Data analysis

All the data are presented as mean values ± s.e., where n refers to the number of experiments. For the SSC data, the unpaired Student's t-test was used, unless otherwise stated. For the single channel and whole-cell current measurements, the paired Student's t-test was used to compare the means of two groups. Statistically significant differences among the means of multiple groups were determined by one-way analysis of variance (ANOVA) with the Tukey–Kramer post-test using Graphpad Inst 3.05 software (San Diego, CA, U.S.A.). Values of $P < 0.05$ were considered statistically significant.

Source of materials

The following were obtained from Sigma-Aldrich Co. Ltd, Poole, Dorset, U.K.: 7,8-benzoquinoline, charybdotoxin (ChTX), clotrimazole, collagenase Type 1A, gentamycin, glibenclamide, nystatin and isobutylmethylxanthine (IBMX). Diphenylamine carboxylate (DPC) was from Lancaster Synthesis, Lancashire, U.K. and chromanol 293B was from Hoechst AG, Frankfurt, Germany. CBIQ was obtained by custom synthesis from Ubichem plc, Eastleigh, U.K.

Results

Effects of CBIQ on SSC in Calu-3 monolayers

Concentration–response curves for CBIQ were measured as changes in SCC of Calu-3 monolayers when added to both sides of the epithelia. EC_{50} values were obtained for each curve by interpolation and the mean value ± s.e. for all curves is given in Figure 1. For comparison, similar experiments were made with 7,8-benzoquinoline and 5,6 benzoquinoline, compounds resulting from an earlier search for CFTR activators (Cuthbert & MacVinish, 2003). All three compounds produced a maximal increase in current of ca. 150 μA cm^{-2}, with inhibition appearing at high concentrations with both 7,8-benzoquinoline and 5,6-benzoquinoline. The EC_{50} value for CBIQ (4.0 ± 1.3 μM) was lower than those for either 7,8- or 5,6-benzoquinoline. The addition of IBMX, 100 μM, to the bathing solution potentiated the responses to all three agents, although alone it produced only a minor effect on SCC (20.7 ± 4.2 μA cm^{-2}, $n = 4$). IBMX affected the responses in several ways, the maximal responses were increased, the concentration–response curves became steeper and the EC_{50} values were reduced, although this was

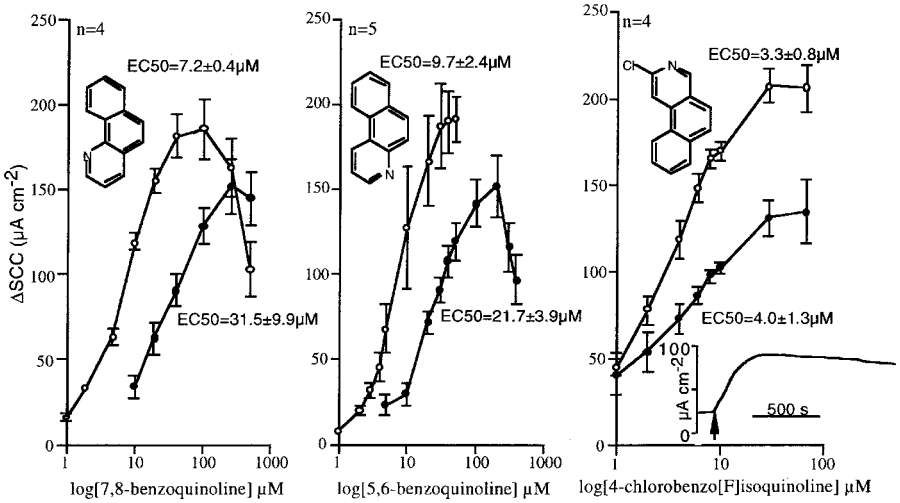

Figure 1 Shows the responses (mean values ± s.e.) of Calu-3 monolayers to CBIQ, 7,8-benzoquinoline and 5,6-benzoquinoline in the absence (closed circles) and in the presence (open circles) of IBMX (100 μM, bs). Each individual curve, indicated by *n* values, was analysed to give an EC_{50}value and the mean values ± s.e. for these are shown adjacent to each curve. The structures of the three compounds are shown. An inset shows a maintained response to CBIQ, 20 μM given at the arrow.

Table 1 Simultaneous measurement of $^{36}Cl^-$ fluxes and SCC in Calu-3 monolayers

	Control period	*Plus CBIQ*	*Difference*	P-*value*	n
$^{36}Cl^-$ flux (ap–bl) (μEq cm^{-2} h^{-1})	1.13 ± 0.19	1.51 ± 0.23	0.38 ± 0.09		9
				< 0.001	
$^{36}Cl^-$ flux (bl–ap) (μEq cm^{-2} h^{-1})	2.39 ± 0.79	4.35 ± 0.69	1.96 ± 0.14		9
$^{36}Cl^-$ net flux (bl–ap) (μEq cm^{-2} h^{-1})	1.26 ± 0.81	2.84 ± 0.73	1.58 ± 0.17		9
				NS	
SCC (μEq cm^{-2} h^{-1})	0.93 ± 0.07	2.24 ± 0.16	1.31 ± 0.12		18

Nine pairs of Calu-3 monolayers between 13 and 29 days were used to measure fluxes and SCCs simultaneously. Mean values and s.e. of the mean are shown throughout. The concentration of CBIQ used was 20 μM. SCC, short-circuit current; NS, not significant; ap, apical; bl, basolateral.

marginal with CBIQ. Responses to CBIQ were well maintained (see inset in Figure 1), were reversible and could be repeated.

The stimulation of current by CBIQ was largely removed by the addition of the NaK2CL cotransport inhibitor, bumetanide (see later), suggesting that the current increase to CBIQ was mediated by electrogenic chloride secretion, as already shown for 1,10-phenanthroline (Duszyk *et al*., 2001) and for 7,8-benzoquinoline (Cuthbert *et al*., 2003). However, to confirm that this was so bidirectional Cl^- flux studies were carried out (see below).

Effects of CBIQ on chloride fluxes in Calu-3 monolayers

Matched pairs of Calu-3 monolayers were used to measure $^{36}Cl^-$ fluxes simultaneously with SCCs. Pairs varied in age from 13–29 days from seeding. The data from nine paired experiments are given in Table 1. The essential finding is that CBIQ increases the net flux of Cl^- to an extent that is not different to that predicted from SCC measurements, while the basolateral to apical Cl^- flux due to CBIQ is significantly greater than the corresponding apical to basolateral flux ($P < 0.0001$). The net flux of Cl^- due to CBIQ measured with $^{36}Cl^-$ was 1.58 ± 0.17 μEq cm^{-2} h^{-1} ($n = 9$), whereas SCC records predicted 1.31 ± 0.12 μEq cm^{-2} h^{-1} ($n = 18$); these values are not significantly different. Nevertheless, the SCC values are 0.27 μEq cm^{-2} h^{-1} less than those from flux measurements. However, this amount is virtually the same as overestimated by flux measurements during the control period (0.33 μEq cm^{-2} h^{-1}). Assuming whatever ion movements underlie the basal SCC continue during the effects of CBIQ the measurements of flux would be expected to overestimate the SCC values by the actual amount found. Therefore, the major ion transport process underlying the effect of CBIQ on SCC must be the electrogenic secretion of chloride ions.

Bicarbonate-dependent chloride secretion due to CBIQ in Calu-3 monolayers

Flux analysis, given above, suggests that CBIQ activates only chloride secretion in Calu-3 monolayers. Previous work with this cell line has shown that basolateral chloride entry depends

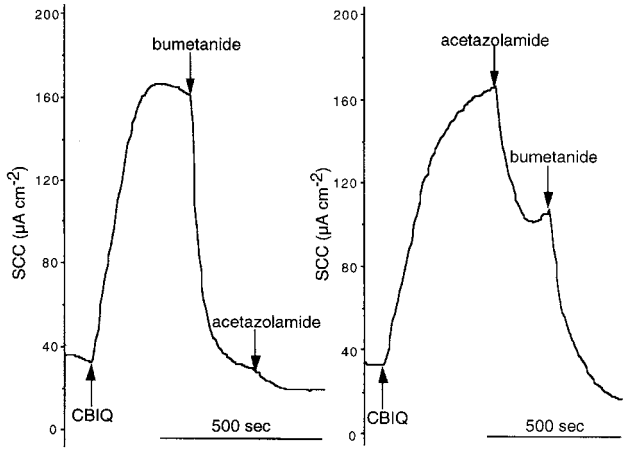

Figure 2 Shows the effect of acetazolamide (100 μM, bs) and bumetanide (20 μM, bl) on the responses to CBIQ (50 μM, bs) in two Calu-3 monolayers. Note that the extent of the inhibition to either inhibitor is dependent on the order in which they are given.

on two separate mechanisms when activated by 7,8-benzoquinoline, one of which is bicarbonate dependent. To examine if CBIQ activated bicarbonate-dependent chloride secretion, the ratio of the inhibition of transport by bumetanide and acetazolamide was measured. Bicarbonate-dependent chloride secretion is indicated if the bumetanide/acetazolamide ratio depends on the order in which the inhibitors are applied (Cuthbert *et al*., 2003).

Figure 2 illustrates one of five paired experiments in which the inhibition of the SCC stimulated by CBIQ was inhibited with acetazolamide and bumetanide, but in which the order of addition was reversed in the two sets of measurements. The ratio of the current removed by bumetanide to that removed by acetazolamide (bumet/acet ratio) was calculated for both sets of experiments. When acetazolamide was added first the bumet/acet ratio was 1.9 ± 0.3, whereas if bumetanide was given first the ratio was 12.4 ± 1.2 ($P < 0.001$, $n = 5$). The significant difference of the ratios cannot be related to differences in stimulation caused by CBIQ, as these were not different in the two sets of experiments, being $100.9 \pm 8.8\,\mu A\,cm^{-2}$, when acetazolamide was given first and $91.1 \pm 12.2\,\mu A\,cm^{-2}$ when bumetanide was given first (NS, $n = 5$). When acetazolamide was given first the reduction in SCC was $40.2 \pm 6.6\,\mu A\,cm^{-2}$ ($n = 5$), whereas it was only $7.5 \pm 0.6\,\mu A\,cm^{-2}$ ($n = 5$) when given after bumetanide ($P < 0.002$). The figure shows that after bumetanide and acetazolamide, independent of the order given, the final SCC falls below the original baseline value. The mean value of the initial baseline current was $27.5 \pm 1.9\,\mu A\,cm^{-2}$ ($n = 10$), but was reduced by $9.4 \pm 1.2\,\mu A\,cm^{-2}$ at the end of the experiments, indicating that part of the basal current is sensitive to either bumetanide or acetazolamide. It can be concluded that CBIQ activates bicarbonate-dependent chloride secretion in Calu-3 monolayers.

Effect of CBIQ on basolateral K^+-channels in Calu-3 monolayers

Other agents in the CBIQ series have been shown to activate basolateral K^+-channels in Calu-3 epithelia. To examine if this was so for CBIQ, Calu-3 monolayers were exposed to an apical to basolateral potassium ion gradient in the absence of permeant anions as described in the Methods section. The epithelia were exposed to nystatin (200–250 $\mu g\,ml^{-1}$) on the apical side that permeabilises the membrane to small cations to produce an increase in SCC. Thereafter the experiments took one of two forms as illustrated in Figure 3a, where essentially CBIQ was given before or after the inhibitors ChTX and 293B. The former blocks calcium-dependent intermediate-conductance K^+-channels, KCNN4, while the latter blocks cAMP-dependent K^+-channels, KCNQ1/KCNE3. Each experiment was repeated a total of six times and the cumulative data are shown in Figure 3b.

Several observations and conclusions can be drawn from the experiments as follows:

1. The response to CBIQ is greater in the absence of ChTX and 293B than in the presence of the inhibitors (180.8 ± 21.2 *versus* $31.7 \pm 6.6\,\mu A\,cm^{-2}$, $P < 0.001$, $n = 6$).
2. The effect of ChTX is greater after CBIQ than after nystatin (-161.2 ± 24.8 *versus* $-50.7 \pm 7.7\,\mu A\,cm^{-2}$, $P < 0.002$, $n = 6$).
3. The effect of 293B is greater after CBIQ than after nystatin (28.2 ± 2.7 *versus* $8.8 \pm 1.7\,\mu A\,cm^{-2}$, $P < 0.001$, $n = 6$).
4. It can be concluded that CBIQ causes a major activation of K^+-channels that are inhibited by ChTX, with a minor effect on K^+-channels inhibited by 293B.
5. Permeabilisation of the apical membrane by nystatin reveals that some fraction of K^+-channels sensitive to ChTX or 293B are constitutively active in the absence of CBIQ.

The importance of the activation of basolateral K^+-channels in generating a response under short circuit conditions is emphasised by the data given in Figure 4. Paired Calu-3 monolayers were exposed to a maximally effective concentration of CBIQ either in the presence or absence of basolateral K^+-channel blockers. In the presence of the latter responses were almost obliterated.

Evidence for the activation of apical CFTR Cl-channels by CBIQ in Calu-3 cells

To investigate the effect of CBIQ on apical CFTR Cl-channels two approaches were used. In the first, the basolateral membranes of the cells were depolarised with PGK in the presence of an apical to basolateral Cl^- gradient (Cuthbert, 2001), while in the second, nystatin was used to make the basolateral membranes permeable to small ions, again in the presence of a Cl^- gradient, directed apically to basolaterally.

Using the first method CBIQ, 10 μM, caused an increase in $-$SCC amounting to $-17.9 \pm 1.0\,\mu A\,cm^{-2}$ ($n = 4$), the effect being reversed by DPC, applied apically. Similar results were obtained using nystatin treatment of the basolateral membranes, when again an increase in $-$SCC was recorded in response to CBIQ, 10 μM ($-26.8 \pm 5.0\,\mu A\,cm^{-2}$ ($n = 4$)). Examples of responses using the two methods are given in Figure 5.

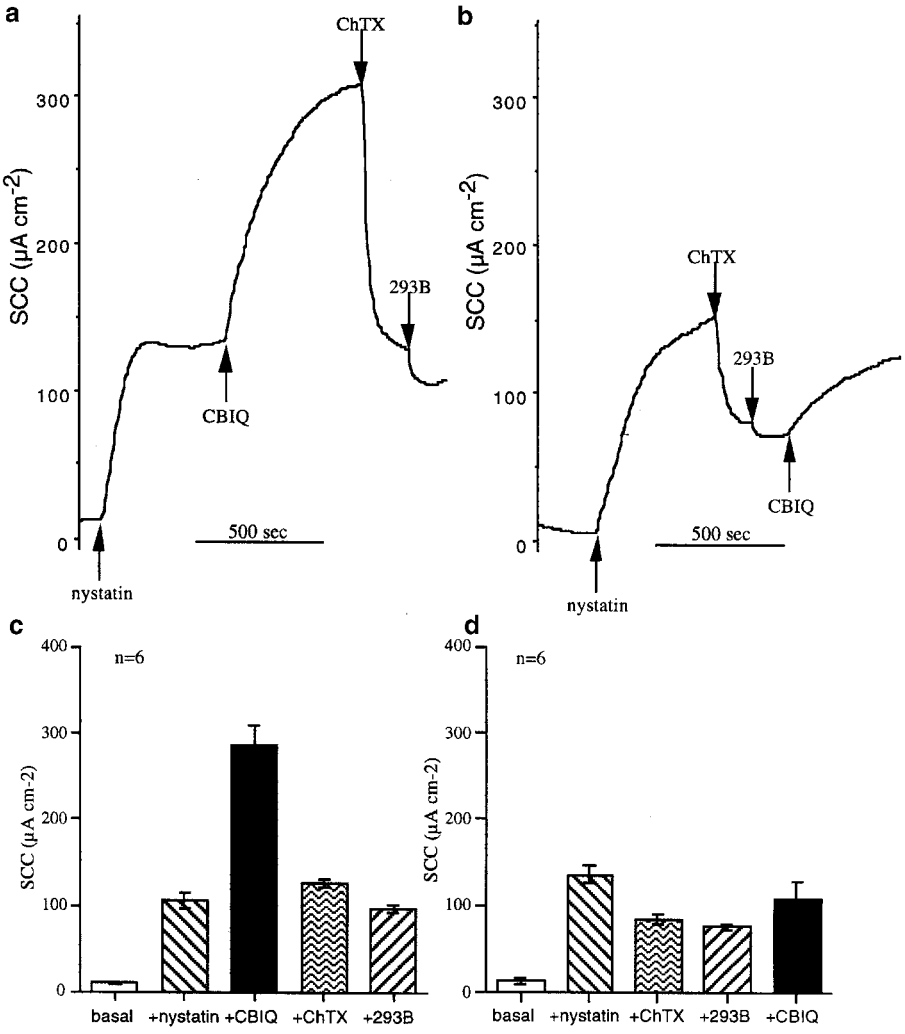

Figure 3 (a) and (b) illustrate a paired experiment with Calu-3 monolayers. Both monolayers were initially treated on the apical surface with nystatin, 200 μg ml^{-1}, in the presence of an apical to basolateral potassium gradient and in the absence of permeant anions. In (a) after nystatin, CBIQ (10 μM, bs) was added, causing a current increase that was reversed by the addition of ChTX (50 nM, bl), with a smaller extra inhibition by 293B (20 μM, bl). In the control experiment (b), the inhibitors were added after nystatin. When CBIQ (10 μM, bs) was added subsequently, a small response was produced compared to that seen in (a). In (c) and (d), the cumulative data for six paired experiments are given. The response to CBIQ, 10 μM, shown in (d) was significantly smaller than the value given in (c). ($P<0.001$, $n=6$).

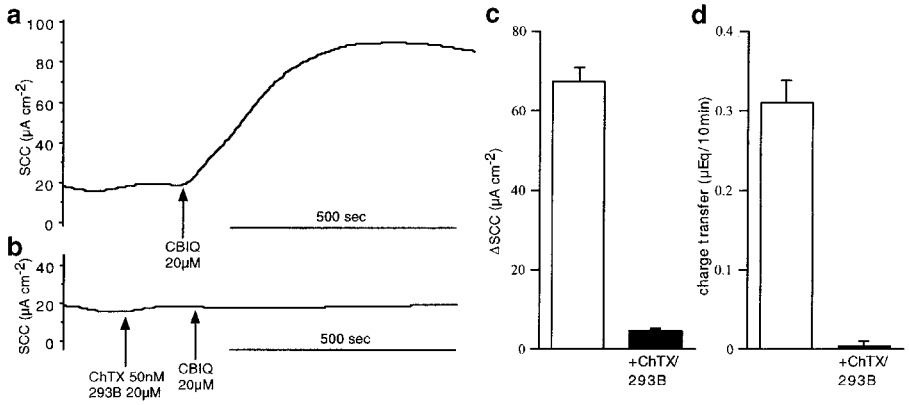

Figure 4 (a) and (b) illustrate a paired experiment with Calu-3 monolayers, one of which was pre-exposed to ChTX, 50 nM and 293B, 20 μM (both added basolaterally). Each monolayer was then treated with CBIQ, 20 μM (bs). (c) and (d) show, respectively, the maximal SCC increases and the charge transfers during 10 min for three paired experiments without (open columns) and with blocking agents (closed columns). The increases in SCC and charge transfer were significantly greater in the absence of the blocking agents ($P<0.001$ and <0.002, respectively).

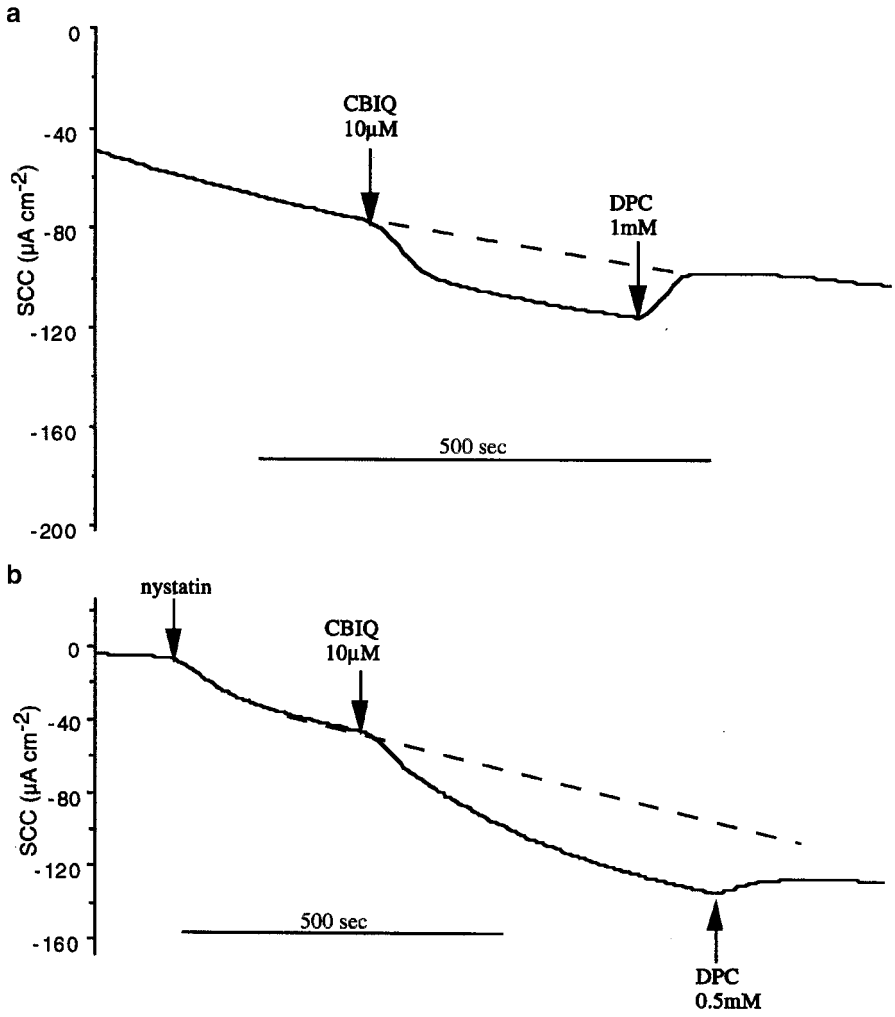

Figure 5 Shown are representative examples of responses of Calu-3 monolayers subjected to an apical to basolateral chloride gradient. In (a), the basolateral membrane was depolarised with PGK solution, while in (b), the basolateral surface was permeabilised by treatment with nystatin, as described in the Methods section. CBIQ, 10 μM (bs) and DPC (bl) were added as indicated.

Whole-cell recording in Calu-3 cells

The nature of the response to CBIQ in the whole-cell recording mode was dependent on the presence or absence of ATP and GTP in the recording pipette. Figure 6 shows representative traces demonstrating the effect of CBIQ, 10 μM, on whole-cell current traces in response to a series of voltage commands ranging from –80 to +80 mV in the absence of ATP and GTP. Clotrimazole, 30 μM, was able to block the current increases due to CBIQ. Also shown are the I/V relationships corresponding to the current traces. After CBIQ, the I/V curve showed inward rectification, characteristic of KCNN4 channels, together with a leftward (hyperpolarizing) change in reversal potential (Table 2), again consistent with the activation of K^+-channels.

When the pipette contained ATP and GTP there were appreciable currents before CBIQ was added, suggesting that the some channels requiring ATP/GTP were already activated. Addition of CBIQ, 10 μM, activated an inwardly rectifying current, but without any overall change in reversal potential (Table 2). The addition of glibenclamide, 100 μM, a CFTR channel blocker, resulted in the inhibition of the majority of the whole-cell current and a leftward (hyperpolarizing) shift in the reversal potential. The residual whole-cell current was reminiscent of the inwardly rectifying current stimulated by CBIQ when ATP/GTP were excluded from the pipette solution.

Single channel recording in excised inside-out patches

Application of CBIQ, 10 μM, to excised inside-out patches from Calu-3 cells caused the appearance of single channel events when clamped at –60 mV (Figure 7). Initially, no channel activity is seen until CBIQ is added, after which the activity is continuous until clotrimazole, 30 μM, is added, which quickly reduces all the activity. In separate experiments, the value of NP_O rose from zero to 1.1 ± 0.44 ($n = 3$). When patches were clamped from –80 to +80 mV in 20 mV steps, the data revealed an inwardly rectifying I/V

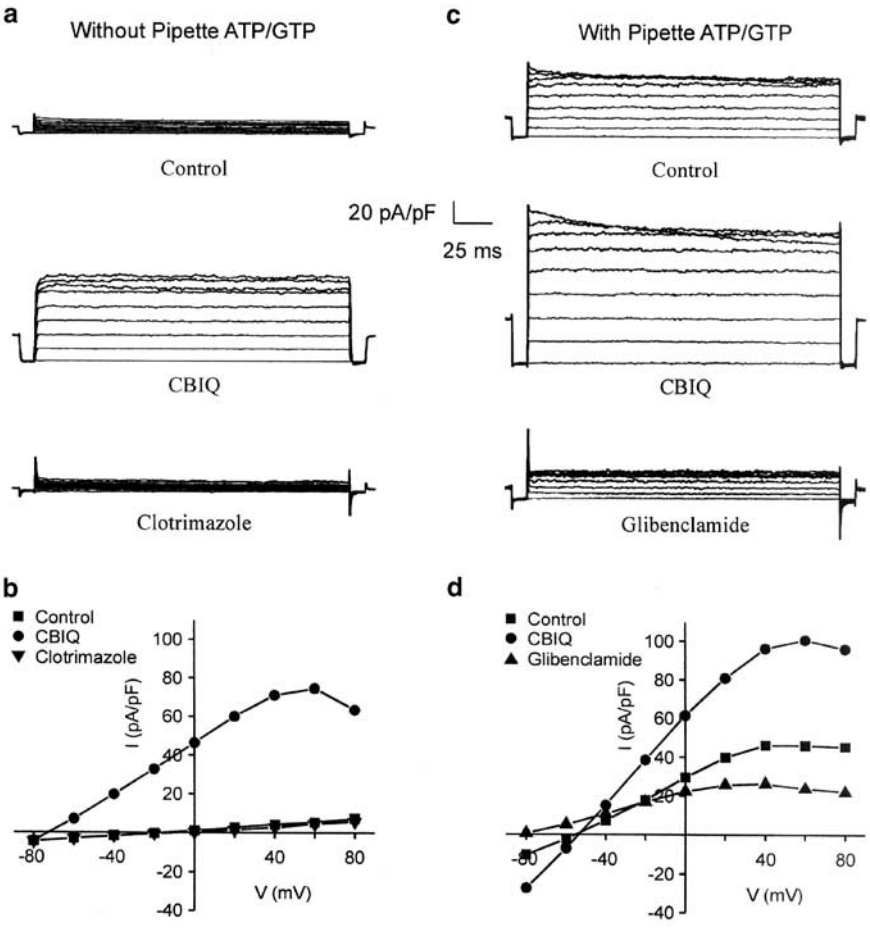

Figure 6 Whole-cell recording in Calu-3 cells. (a) Shows a representative whole-cell recording in the absence of ATP and GTP and demonstrating the effect of CBIQ, 10 μM, followed by clotrimazole, 30 μM. (b) Shows the I/V relationship corresponding to the traces depicted in (a). Note the hyperpolarizing shift in the reversal potential. Cumulative data from six experiments are given in Table 2. (c) Is a representative whole-cell recording showing the effects of CBIQ, 10 μM, with subsequent addition of glibenclamide, 100 μM, in the presence of ATP and GTP. (d) Shows the I/V relationship corresponding to the traces depicted in (c). Cumulative data from several experiments are given in Table 2.

Table 2 Conductance (G) and reversal potential (RP) measurements in Calu-3 cells

	No ATP/GTP						*With ATP/GDP*					
	G (mS pF^{-1})	P-*value*	n	*RP* (mV)	P-*value*	n	G (mS pF^{-1})	P-*value*	n	*RP* (mV)	P-*value*	n
Control	158±64	<0.01	6	−34±6	<0.02	6	317±135	<0.01	5	−38±9	NS	5
+CBIQ	687±152	—	6	−60±7	—	6	1145±206	—	4	−38±16	—	4
+Clotrimazole	109±28	<0.04	3	−19±8	<0.01	3	—	—	—	—	—	—
+Glibenclamide	—	—	—	—	—	—	184±44	<0.02	3	−72±7	NS	3

Calu-3 cells were treated with CBIQ, 10 μM, after control observations were made. When ATP/GTP were not included in the pipette solution, CBIQ was followed by clotrimazole, 30 μM. When ATP/GTP were present in the pipette CBIQ was followed by glibenclamide, 100 μM. Tests for significance were used to compare the values in the presence of CBIQ to control values or those in the presence of inhibitors. n values refer to the number of cells used. Conductance values were calculated from the slope conductance between 0 and −80 mV. NS = not significant.

plot where the unitary conductance was 12.0±0.4 pS at positive potentials (measured from the linear fit of the points from 0 to 80mV) and 23.0±0.9 pS at negative potentials (measured from the linear fit of the points from 0 to −80 mV) (Figure 8).

Cell-attached patch recording from Calu-3 cells

Cell-attached patches were used to record from single channels with the characteristics of CFTR. In the absence of stimulation, the channel activity is low ($NP_O = 0.52 \pm 0.51$, $n = 3$), but

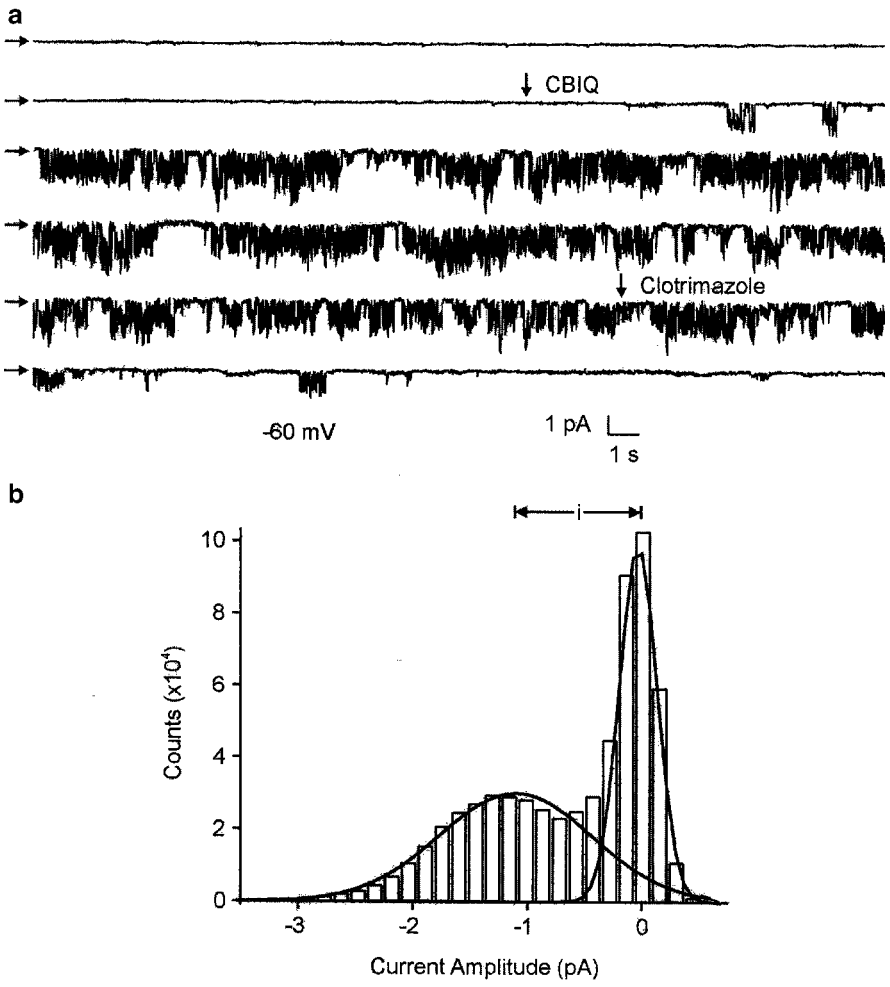

Figure 7 Representative recording from an excised inside-out patch (260 s) of a Calu-3 cell. (a). No channel activity is recorded under control conditions at −60 mV until CBIQ, 10 μM, is added, indicated by the arrow. Subsequent addition of clotrimazole, 30 μM, as indicated, completely eliminated the channel activity. Arrows, to the left of the traces, designate the closed channel state. (b). The all points histogram was derived from 60 s of the recording shown in (a), starting at a point 20 s after the addition of CBIQ. The unitary current amplitude (*i*) was calculated as the difference between the mean peak currents of a two peak Gaussian fit.

after the addition of CBIQ, 10 μM, the channel activity was stimulated ($NP_O = 2.63 \pm 0.38$, $n = 3$, $P < 0.03$) (Figure 9). The addition of glibenclamide, 100 μM, to the bathing fluid slowly abolished the channel activity during 10 min. However, we could not distinguish blockade from rundown with confidence. Clamping presumed CFTR channel containing patches at potentials between –80 and 80 mV gave linear I/V plots, characteristic of CFTR with a unitary conductance value of 6.7 ± 0.5 pS ($n = 3$) (Figure 10).

Discussion

CBIQ is the most potent of the benzoquinolines so far discovered. This study shows that its actions are not dissimilar to other compounds in this series and reported previously (Cuthbert, 2003; Cuthbert & MacVinish, 2003). Here, we have shown that CBIQ causes a sustained, reversible and repeatable increase in SCC in Calu-3 monolayers, and that biophysical measurements correspond to the net movement of Cl^- measured from the net flux of $^{36}Cl^-$. 1,10-Phenanthroline and 7,8-benzoquinoline were earlier shown, by Cl^- flux and SCC measurements, to activate the net movement of Cl^- across the epithelia (Duszyk *et al*., 2001; Cuthbert & MacVinish, 2003). Further, it is shown that the chloride secretion is potentiated by IBMX, as it is with two other benzoquinolines, namely, 5,6- and 7,8-benzoquinoline. The EC_{50} for 7,8-benzoquinoline reported in this study (31.5 ± 9.9 μM) is smaller than a value reported earlier of 123.2 ± 14.0 μA (Cuthbert & MacVinish, 2003). From recent work (Caci *et al*., 2003; Cuthbert *et al*., 2003; Derand *et al*., 2003), it is becoming apparent that the maximal response to and the EC_{50} of CFTR activators are dependent on the state of phosphorylation of CFTR, which is crucially involved in the chloride secretory process in Calu-3 cells. IBMX, 100 μM, causes a 50-fold increase in basal cAMP levels in Calu-3 cells (Cuthbert & MacVinish, 2003) and presumably affects the state of phosphorylation of CFTR, with a consequent reduction in EC_{50} and increased maximal responses.

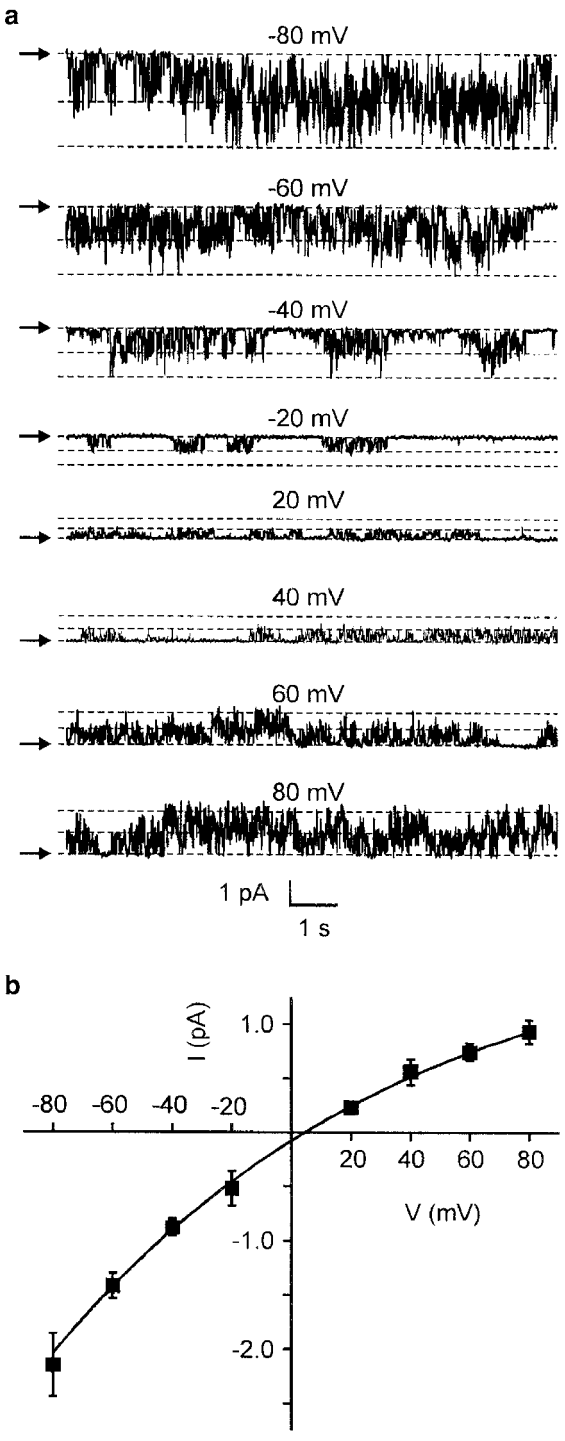

Figure 8 Single channel properties of the CBIQ activated K^+-channels in excised inside-out patches of Calu-3 cells. In the presence of CBIQ, 10 μM, the patch was clamped from –80 to 80 mV in 20 mV steps, each lasting 10 s. Representative recordings at each step are shown. Arrows to the left of the traces designate the closed state. (b) Shows the I/V relationship with inward rectification ($n=3$).

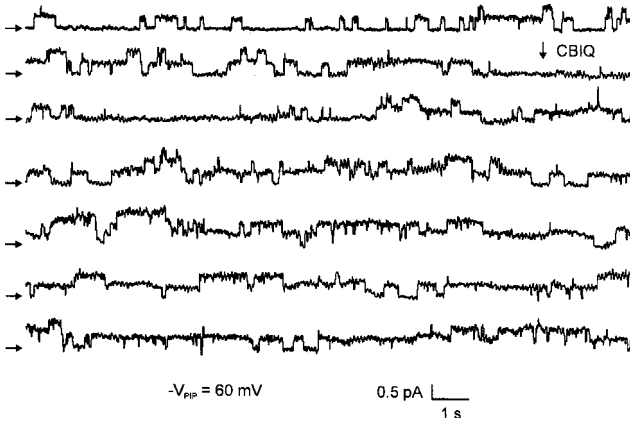

Figure 9 Recording from a cell-attached patch of a Calu-3 cell. Shown is a representative recording (110 s) demonstrating a relatively low channel activity under control conditions when clamped at a negative pipette potential ($-V_{PIP}$) of 60 mV. The addition of CBIQ, 10 μM (at the arrow), significantly stimulated channel activity. NP_O values were 0.52 ± 0.51 ($n=3$) in control conditions, increasing to 2.63 ± 0.38 ($n=3$) ($P<0.03$) after CBIQ. Arrows to the left of the traces indicate the closed state.

Sensitivity to acetazolamide in the secretory epithelia is often taken to indicate that they are bicarbonate secreting. However, this is not necessarily the case, and as we have shown (Cuthbert *et al*., 2003) chloride secretion can be partly bicarbonate dependent when chloride enters the basolateral side of the cells using a parallel arrangement of a chloride–bicarbonate exchanger together with a sodium–proton exchanger, the rest of the chloride entering on the NaK2Cl cotransporter. The crucial test for bicarbonate-dependent chloride secretion is the bumetanide/acetazolamide ratio, which varies dependent on the order in which they are added. Here, this ratio varied significantly with the order when CBIQ was used to stimulate chloride secretion, showing that both methods of chloride entry were operating, as with 7,8-benzoquinoline.

By permeabilisation of either the apical or basolateral membranes and the application of ion gradients, it was demonstrated that CBIQ had actions on both faces of the cell. Use of blocking agents suggested that CBIQ could activate CFTR Cl-channels in the apical membrane as well as intermediate-conductance calcium-sensitive K-channels, KCNN4, on the basolateral aspect of the cells. Both these channels are known to be present in Calu-3 cells (Leidtke *et al*., 2001; Cowley & Linsdell, 2002). There was also evidence that CBIQ could also activate the cAMP-sensitive K^+-channel (KCNQ1/KCNE3), sensitive to 293B, but the effect was minor and was not pursued. It is noted that in the presence of ChTX and 293B, CBIQ was still able to elicit a small increase in SCC. There are several possible reasons for this. First, 293B inhibits both KCNQ1/KCNE3 channels and homopolymeric KCNQ1 channels, the latter with lower affinity (Busch *et al*., 1997). At 20 μM 293B, the complexed channels will have an occupancy of 75% ($K_d=6.7\,\mu$M) and the homopolymeric channels even less. Thus, the residual effect of CBIQ in the presence of ChTX and 293B may result from the activation of both unoccupied complexed and homopolymeric channels, plus possibly other unidentified basolateral K^--channels.

Under SCC conditions in the presence of basolateral K^+-channel inhibitors, the effects of CBIQ on transport were almost obliterated, although as noted in Figure 3d, CBIQ was able to produce some change in K^+ conductance under these

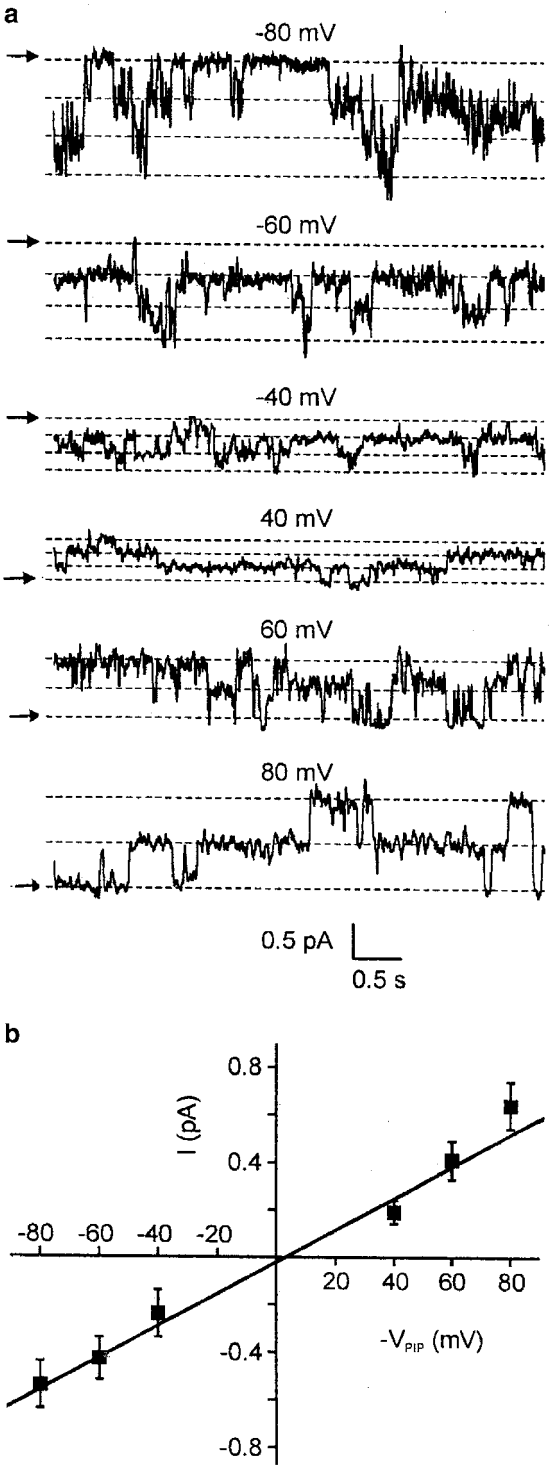

Figure 10 Single channel properties of the CBIQ activated channels in attached patches of Calu-3 cells. (a). In the presence of CBIQ, the membrane was clamped at negative pipette potentials ($-V_{PIP}$) from −80 to 80 mV in 20 mV increments, each lasting 5 s. Representative recordings from each step are shown. Arrows to the left of the traces indicate the closed state. (b) The I/V relationship of the channel collected from three experiments as in (a) is shown in (b). Mean values and s.e. are shown. From a linear fit of all points, the channel is shown to have a unitary conductance of 6.7 ± 0.5 pS.

conditions. Presumably, CFTR channels were still activated as with the CFTR channel opener NS004. This latter has no K^+-channel activity but failed to stimulate chloride secretion across T84 monolayers (Devor *et al*., 1999). From these results and those from an earlier study (Cuthbert *et al*., 1999), the importance of a favourable electrochemical gradient for chloride exit is emphasised.

Thus far, apart from increased potency, the actions of CBIQ are the same as those of other benzoquinolines and phenanthrolines (Duszyk *et al*., 2001; Cuthbert & MacVinish, 2003; Cuthbert *et al*., 2003). However, it was important to obtain more direct evidence that the targets for CBIQ had been correctly identified.

CFTR requires both phosphorylation of the R-domain and ATP binding and hydrolysis at the nucleotide binding folds to effect channel gating (Ikuma & Welsh, 2000). Using whole-cell recording in Calu-3 cells, but without ATP or GTP in the pipette, it is unlikely therefore that CFTR will contribute to the whole-cell currents. Under these conditions, whole-cell currents activated by CBIQ displayed an inwardly rectifying I/V relationship, a change in reversal potential towards E_K, both characteristic of KCNN4 channels (Devor & Frizzell, 1993) and blockade by the specific KCNN4 antagonist clotrimazole. With ATP and GTP present in the pipette, it might be expected that both CFTR and KCNN4 would be activated by CBIQ. In this situation, the currents were partially inhibited by glibenclamide, a CFTR blocking drug, leaving a residual current, presumably due to KCNN4. Inwardly rectifying currents were replicated in inside-out membrane patches activated by CBIQ with single channel properties characteristic of KCNN4 channels (Devor & Frizzell, 1993) and indicate that the benzoquinoline increases open channel probability without affecting channel conductance.

Single channels with a conductance of around 7 pS were detected in cell-attached patches and had a linear I/V relation, characteristic of CFTR (Tabcharani *et al*., 1991). CBIQ increased the open channel frequency without any effect on conductance. As the membrane patches were not accessible to CBIQ applied extracellularly, activation must be dependent on penetration through the cell membrane and activation from the intracellular aspect. This is hardly surprising as only small connecting loops of the transmembrane segments of the CFTR protein are extracellular.

It seems possible that CBIQ acts directly on the KCNN4 and CFTR channel proteins themselves, but absolute proof will require a demonstration of the activation with these proteins incorporated into lipid bilayers. Nevertheless, here we have provided more direct evidence for the involvement of KCNN4 and CFTR in the actions of CBIQ.

The search for activators of CFTR is driven by the possibility that they may act as chaperones for incorrectly trafficked mutant forms, as activators of CFTR mutants or as adjuncts in gene therapy. To do this highly potent agents will be needed, but we have not determined the actual EC_{50} value for CBIQ on CFTR, simply the EC_{50} value for the total electrogenic chloride transporting process. We have shown that blockade of basolateral K^+-channels severely inhibits the response to CBIQ, thus agents with no K^+-channel activity may not appear potent, yet have powerful actions on CFTR. Others searching for CFTR channel activators (Galietta *et al*., 2001; Ma *et al*., 2002; Caci *et al*., 2003) have used an alternate test system consisting of

monolayers of Fischer rat thyroid cells (FRT). FRT monolayers, expressing CFTR, were permeabilised on the basolateral side with amphotericin and subjected to basolateral to apical chloride gradient to assay potential activators. In this way, CFTR activators without K^+-channel activity are detected. Similar alternate methodology is needed to discover the K_d for CBIQ on CFTR.

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