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4-Chlorobenzo[F]isoquinoline (CBIQ), a novel activator of CFTR and ΔF508 CFTR

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Abstract

4-Chlorobenzo[F]isoquinoline (CBIQ) is a novel compound, here shown to activate both CFTR (cystic fibrosis transmembrane conductance regulator) Cl^- ion channels and KCNN4, intermediate conductance, calcium-sensitive K^+-channels, present in transporting epithelia by the use of heterologous expression systems. Earlier studies with other benzoquinolines, namely 7,8- and 5,6 benzoquinoline, showed they too could activate CFTR and KCNN4, but the evidence was only indirect. However this study also shows that CBIQ can also activate ΔF508 CFTR, the most common mutant form of CFTR present in approximately 75% of patients with cystic fibrosis. This property is not shared with the other benzoquinolines. As activation of CFTR and KCNN4 work in unison to promote epithelial chloride secretion, CBIQ is a new chemical scaffold for developing agents that may be useful in cystic fibrosis.

Keywords: CFTR (Cystic fibrosis transmembrane conductance regulator); Chloride channel; Chloride secretion; KCNN4, Intermediate conductance calcium-sensitive potassium channel; Cystic fibrosis

1. Introduction

Recently we showed that 4-chlorobenzo[F]isoquinoline (CBIQ) was a potent stimulant of chloride secretion measured in epithelial monolayers of human Calu-3 cells, with an apparent K_d of 4 μM (Szkotak et al., 2004). Thus CBIQ is more potent than other benzoquinolines and phenanthrolines described in earlier studies designed to discover novel openers of the cystic fibrosis transmembrane conductance regulator (CFTR) (Duszyk et al., 2001; Cuthbert, 2003; Cuthbert and MacVinish, 2003). Indirect evidence suggested that CBIQ activated both CFTR, located on apical epithelial membranes, and KCNN4, an intermediate conductance Ca^{2+}-sensitive K^+-channel, located basolaterally in Calu-3 cells (Szkotak et al., 2004). Activation of basolateral KCNN4 channels hyperpolarizes cells, thus increasing the electrochemical gradient for anion efflux, of both Cl^- and HCO_3^-, across the apical membrane. This propitious combination of activities increases the potential for transepithelial anion flux, a desirable end point in the pharmacotherapy of cystic fibrosis. While cellular hyperpolarisation also promotes sodium absorption, an undesirable feature in cystic fibrosis, this depends on the presence of epithelial sodium channels that are not present in Calu-3 cells. These cells are derived from human, airway submucosal gland serous cells that contain highest concentration of CFTR in human airways, and are therefore an important target for drugs that might be used to treat cystic fibrosis (Engelhardt et al., 1992).In all previous studies with benzoquinolines and phenanthrolines the conclusions that they affected both CFTR and KCNN4 were a result of indirect measurements, and were made with epithelia in which either the apical or basolateral membranes had been functionally removed by permeabilisation. The major aim of this study was to obtain direct evidence for their

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activity on CFTR and KCNN4 by expressing these two channels separately in cells not normally showing these activities. This has been done using the *Xenopus* oocyte system, and in addition CFTR has been expressed in Fischer rat thyroid cells. A secondary aim was to examine if CBIQ could also activate ΔF508 CFTR, the most common mutant form of CFTR carried by three-quarters of cystic fibrosis patients.

2. Materials and methods

Full-length clones of human CFTR, ΔF508 CFTR and KCNN4 were kindly supplied by Dr. D. Korbmacher (Physiology Department, University of Oxford), Dr. W. Fuller (King's College, London) and Dr. D. Klaerke (Panum Institute, University of Copenhagen). In vitro transcription was achieved using capped RNA made from *Eco*RV-linearised cDNA using the T7 polymerase for CFTR and for ΔF508 CFTR, or *Pvu*I-linearised cDNA using SP6 polymerase for KCNN4 using mMESSAGE mMACHINE Kits (Ambion) according to the manufacturer's instructions.

Xenopus laevis frogs were anaesthetised with MS222 and bunches of oocytes were removed, after which the animals were sacrificed. The protocol complies with the European Community guidelines for the use of experimental animals. The oocytes were exposed to collagenase (0.1%) in calcium-free ND96 for 45 min, de-folliculated by dissection and microinjected with CFTR cRNA (50 ng/oocyte), ΔF508 CFTR cRNA (75 ng/oocyte) or KCNN4 cRNA (20 ng/oocyte), using a volume of 50 or 100 nl. 100 nl was given as two 50 nl injections with a short interval to allow the oocyte to accommodate. Water injected oocytes were used as controls. Oocytes were incubated in ND96 containing sodium pyruvate, 2 mM and gentamicin, 0.1 mg ml^{-1} at 18 °C for several days before use. ND96 had the following composition, mM: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1 and HEPES 5.

Two-electrode voltage clamp was carried out using glass microelectrodes, filled with 3 M KCl (<2 MΩ). External voltage and current electrodes consisted of fine chloride coated silver wires. Oocytes were continuously perfused with ND96 (2 ml/min) and the flow could be switched to ND96 containing drugs. Oocytes were voltage clamped at −60 mV with continuous current recording.

Fischer rat thyroid cells expressing CFTR or ΔF508 CFTR plus YFP-H148Q, a yellow fluorescent protein sensitive to halide ions, were produced as described in detail elsewhere (Galietta et al., 2001; Ma et al., 2002). These cells were grown in 96-well plates. In mammalian epithelial cells expressing ΔF508 CFTR the protein is not normally expressed in the apical membrane because of a trafficking defect, the mutant protein being destroyed in the proteasome. However cooling cells allow membrane expression to take place (Denning et al., 1992). Consequently ΔF508 CFTR expressing cells were kept at 27 °C for 24 h before assay to allow trafficking of the mutant protein to the membrane. The microplates were processed using a fluorescence microplate reader equipped with filters to measure yellow fluorescent protein. Cells were pre-incubated with forskolin alone or with other compounds added to the cells. Subsequent replacement of the Cl^- containing bathing solution with one containing I^- allows ion exchange, and loss of fluorescence, at a rate dependent on the efficiency of the compounds as channel openers (Ma et al., 2002). The rate of change of fluorescence with time is given in arbitrary units (AU).

3. Results

3.1. Oocytes expressing KCNN4

Oocytes expressing KCNN4 bathed in ND96 demonstrated outward currents in response to 7,8-benzoquinoline, 5,6-benzoquinoline and CBIQ when examined by two-electrode voltage clamp at −60 mV, whereas water injected oocytes failed to respond. Responses were reversible by washing and were repeatable and could be rapidly curtailed by addition of the specific blocking agents, charybdotoxin or clotrimazole, added to the perfusion solution in the presence of the benzoquinolines. At high concentrations responses to all three agents were less than at lower concentrations, so giving bell-shaped concentration–response curves. Concentrations of all three agents giving half-maximal responses were obtained by interpolation to give K_d values for stimulatory activity. Concentration–response curves were obtained in 4–5 different oocytes for all three benzoquinolines and the mean K_d±S.E.M. values obtained. The order of potency as activators of KCNN4 is CBIQ>7,8-benzoquinoline>5,6-benzoquinoline (potency ratios 1:0.2:0.07). Representative examples of concentration–response relations for all three agents are shown in Fig. 1, together with the statistical data. Previous studies with the compounds showed they did not raise intracellular Ca^{2+} excluding this as possible mechanism of action on KCNN4 (Szkotak et al., 2004; Duszyk et al., 2001; Cuthbert and MacVinish, 2003).

3.2. Oocytes expressing CFTR

Activation of CFTR in oocytes with the benzoquinolines produced inward currents due to the movement of chloride ions out of the cells, while water injected oocytes failed to respond, when examined by two-electrode voltage clamp and bathed in ND96 and clamped at −60 mV. CBIQ was added cumulatively until a maximal response was achieved. In common with other studies of CFTR channel openers it was necessary to prime CFTR expressing oocytes with a cAMP generating cocktail (Berger et al., 2005; Caci et al., 2003; Dearand et al., 2003; Galietta et al., 2001; Ma et al., 2002.) to produce a minimal level of phosphorylation of the multiple sites in the R-domain of CFTR necessary for activation. For oocytes we used a cocktail consisting of forskolin, 1 μM with isobutyl methyl xanthine (IBMX), 100 μM, the effectiveness of which is dependent on the endogenous adenylate cyclase system. Oocytes were exposed to the cocktail for sufficient time to allow a steady state to be achieved. The need for priming is illustrated in Fig. 2A and B. Note the increase in current that occurred, due to CBIQ, when the cocktail was changed from IBMX, 100 μM to one with IBMX, 100 μM with forskolin, 1 μM. The responses of five oocytes exposed to the complete cocktail are shown in Fig. 2B, together with a representative current trace, shown in Fig. 2A. The trace indicates that the maximal effect was achieved at ~1 μM CBIQ and from the histogram that the K_d was ~100 nM. In

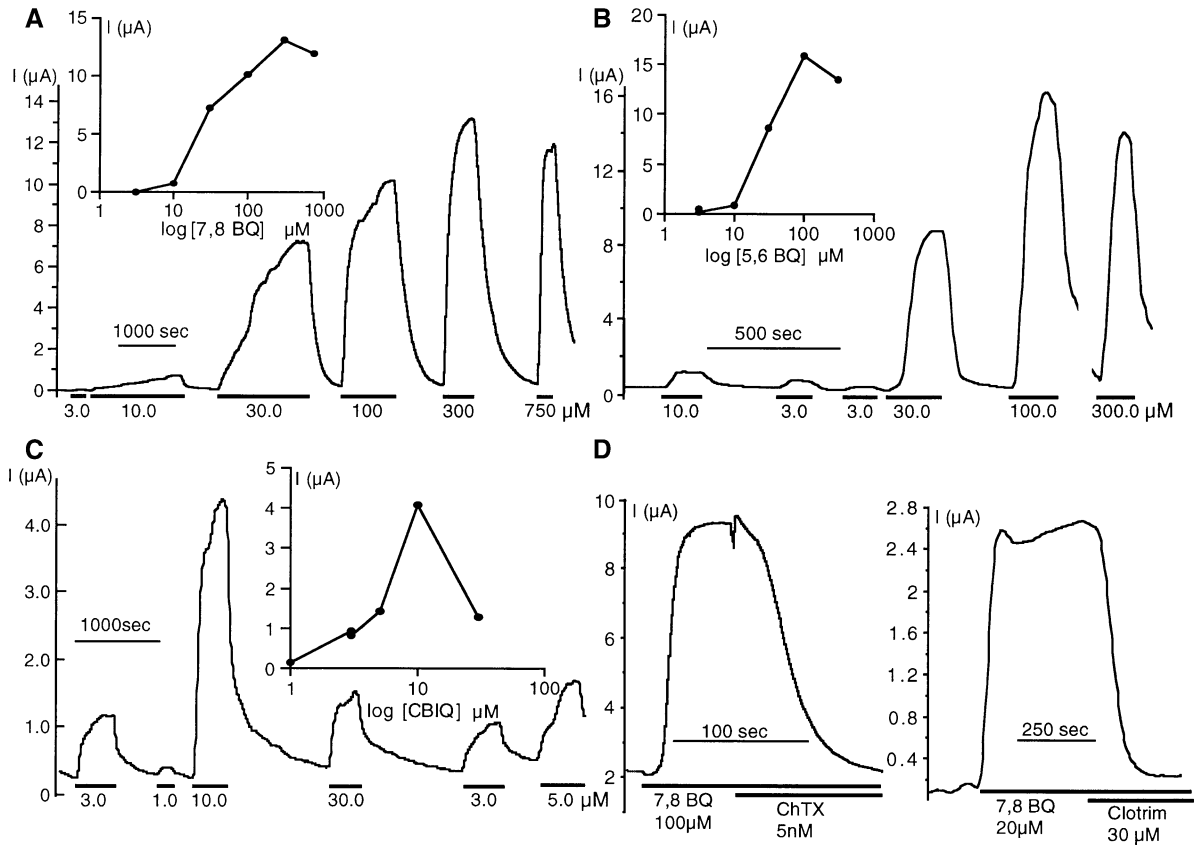

Fig. 1. Current responses, in oocytes expressing KCNN4 when exposed to 7,8-benzoquinoline (A), 5,6-benzoquinoline (B) or CBIQ(C) and clamped at −60 mV. Concentrations of the agents in μM are indicated. Insets show the concentration–response relationships from which the half-maximal stimulatory concentration (K_d) was measured. Each of the experiments in A–C was repeated 4–5 times. The mean±S.E.M. K_d values were 3.9±1.6 μM (n=4) for CBIQ, 19.4±5.7 μM (n=5) for 7,8-benzoquinoline and 53.3±10.1 μM (n=5) for 5,6-benzoquinoline. The K_d for CBIQ was significantly smaller than for 7,8-benzoquinoline (P<0.03, single tail t-test) or 5,6-benzoquinoline (P<0.004, t-test) The effects of charybdotoxin (ChTX) and clotrimazole (Clotrim) on KCNN4 dependent currents are shown in D.

this set of experiments the cocktail alone had no measurable effect on the basal current (+0.1±0.06 μA, n=5). In a second set of experiments (Fig. 2C,D) the cocktail significantly affected the basal current, causing a change of −1.2±0.2 μA (n=5). Here the responses of oocytes to CBIQ peaked at ~100 nM, in the presence of the complete cocktail, with an apparent K_d of >10 nM. These two sets of results indicate that forskolin with IBMX is better than IBMX alone in priming oocytes and that primed oocytes expressing CFTR are sensitive to CBIQ in the nanomolar range. Furthermore it can be argued that the level of priming, dependent also on the endogenous adenylate cyclase system, determines the sensitivity to CFTR channel openers, as others have found (Caci et al., 2003). When higher concentrations of CBIQ were added to oocytes after the maximal response had been achieved a decline, but not abolition, of current was seen, producing bell-shaped curves.

The phenanthrolines and benzoquinolines investigated in earlier studies (Duszyk et al., 2001; Cuthbert, 2003; Cuthbert and MacVinish, 2003) were investigated on intact epithelia. To examine if CBIQ was unique in activating CFTR expressed in oocytes some of the earlier compounds were examined by this system. All those examined activated CFTR expressing oocytes, including the least potent, 1,10-phenanthroline (Fig. 3A) and 7,8-benzoquinoline (Fig. 2E,F) but none showed sensitivity in the nanomolar range as did CBIQ. Responses of primed oocytes expressing CFTR to 5,6-benzoquinoline were similarly insensitive, as with 7,8-benzoquinoline (data not shown). Responses to all the agents that activated CFTR were inhibited by addition of the CFTR channel blocker diphenyl carboxylate (DPC).

3.3. Oocytes expressing ΔF508 CFTR

Mammalian cells expressing ΔF508 CFTR fail to traffic the mutant protein to the membrane, unless the cells are incubated at low temperature (Denning et al., 1992). However since *Xenopus* oocytes are normally incubated at 18 °C, ΔF508 CFTR is trafficked to the cell membrane. ΔF508 CFTR expressing oocytes in ND96 and primed with the standard cocktail and clamped at −60 mV also responded to CBIQ but at much higher concentrations than required for CFTR. Oocytes showed no responses at 1 μM but did so at 10 μM (−0.59±0.14 μA, n=4), comparable to the responses to genistein, 50 μM under the same conditions (−0.28±0.06 μA, n=4). Concentrations of CBIQ greater than 10 μM caused a decline in the total current (Fig. 3B), reminiscent of the responses of CFTR expressing oocytes.

3.4. Fischer rat thyroid cells expressing CFTR or ΔF508 CFTR

Fischer rat thyroid cells expressing either CFTR or ΔF508 CFTR were used to examine the effects of CBIQ, 7,8-benzoquino-

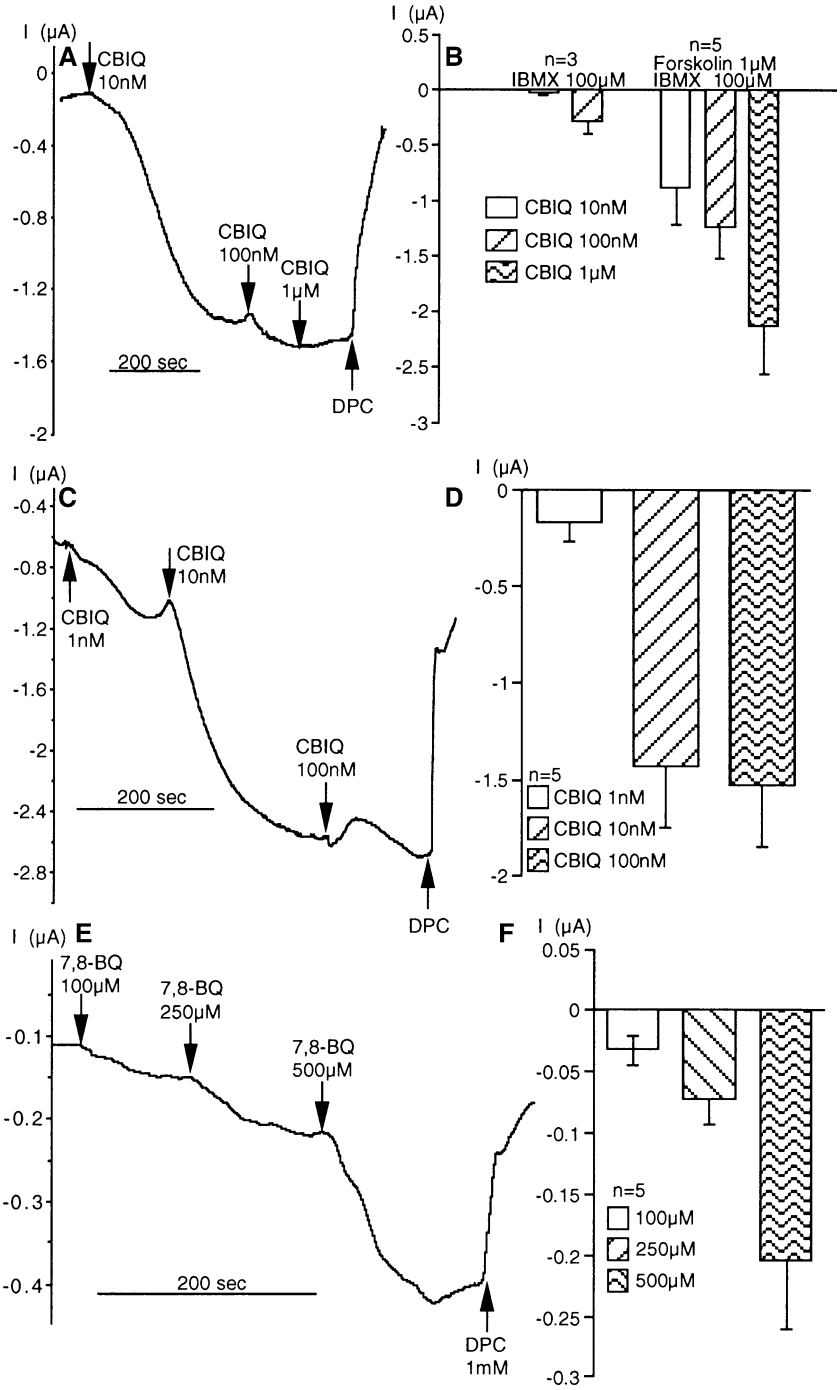

Fig. 2. Current responses in oocytes expressing CFTR when exposed to either CBIQ (A–D) or 7,8-benzoquinoline (E/F). B shows the effects of varying the priming protocol from IBMX, 100 μM to forskolin, 1 μM plus IBMX, 100 μM. The current trace shown in A was in the presence forskolin, 1 μM plus IBMX, 100 μM. The data from 5 similarly treated oocytes is shown in B. All 8 oocytes represented in A/B were from a single batch. The current record shown in C and the histogram in D are data from 5 oocytes from a single batch, expressing CFTR and primed with forskolin, 1 μM and IBMX, 100 μM. Current responses to 7,8-benzoquinoline and the total data from a batch of 5 primed CFTR expressing oocytes are shown in E/F. Throughout DPC indicates the addition of diphenyl carboxylate, 1 mM. Histograms show mean values±S.E.M.

line and 5,6-benzoquinoline. Wild-type FRT/CFTR cells were primed with forskolin, 0.5 μM and exposed to CBIQ (Fig. 4A). Curve fitting gave a K_d value of 12.1 μM for CBIQ and a Hill slope of 1.0. The maximal responses to CBIQ, 7,8- and 5,6-benzoquinoline were compared in the same experiment with the standard opener, genistein. Only 7,8-benzoquinoline was significantly less active ($P<0.05$) than genistein under the experimental conditions used (Fig. 4B). ΔF508 CFTR expressing FRT cells were incubated at 27 °C for 24 h to rescue the protein from the endoplasmic reticulum. Forskolin, 20 μM, was used to prime the cells, after which CBIQ gave the concentration–response curve shown in Fig. 4C, giving a K_d of 3.6 μM and a Hill slope of 1.3. No responses to 7,8- or 5,6-benzoquinoline were seen at 100 μM, a major difference from CBIQ. In these experiments the maximal response

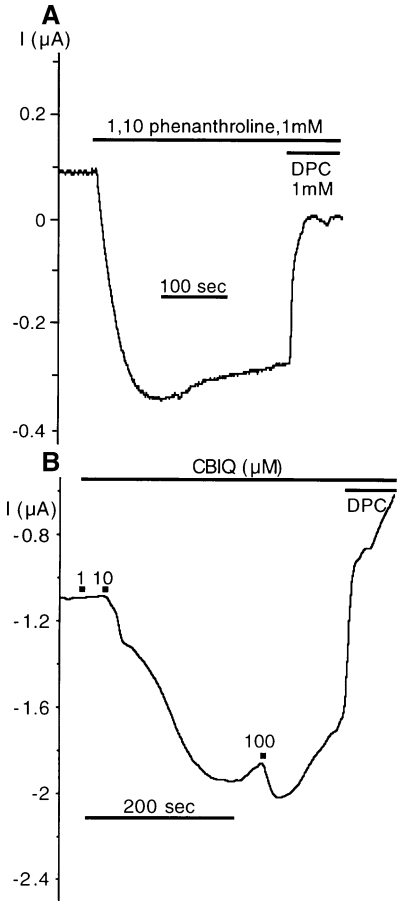

Fig. 3. Current trace of the response of a primed CFTR expressing oocyte to 1,10 phenanthroline, 1 mM and its inhibition by diphenyl carboxylate (DPC) is shown in A. Representative current trace from a oocyte expressing ΔF508 CFTR and exposed to CBIQ in the presence of a priming cocktail, consisting of forskolin, 1 μM and IBMX, 100 μM. DPC, 1 mM was added as indicated (B).

to genistein, 50 μM was 52.5, greater than the maximal response to CBIQ (29.1). Exactly the same experiment with CBIQ, as shown in Fig. 4C, was repeated using different concentrations of forskolin to prime the cells giving K_d values that were dependent on the level of priming. Without priming the K_d for CBIQ was 43.5 μM, while with 20 μM forskolin the K_d fell to 4.3 μM (Fig. 4D), comparable to the previous value (3.6 μM) at this concentration (Fig. 4C). It is to be noted that at 0.5 μM forskolin CBIQ had a K_d of 12.1 μM versus CFTR (Fig. 4A) and a K_d of 18.7 μM versus ΔF508 CFTR (Fig. 4D).

4. Discussion

Direct evidence that CBIQ can activate KCNN4, CFTR and ΔF508 CFTR is shown here for the first time, using heterologous expression systems namely the *Xenopus* oocyte and Fischer rat thyroid cell systems expressing these channels. It is still not known if the actions are directly upon the channel proteins or involves other cellular components. It is unlikely that CBIQ acts as an inhibitor of phosphodiesterase or an activator of adenylate cyclase since one or both activities are present in cocktails used to prime the cells. Furthermore, CBIQ is effective on ΔF508 CFTR expressing Fischer rat thyroid cells in the presence of forskolin concentrations sufficient to maximally activate adenylate cyclase.

It is shown that CBIQ is more active than either 7,8- or 5,6-benzoquinoline as an activator of KCNN4. Others have reported that some CFTR channel openers have minor activity on epithelial K^+-channels, for example, the benzimidazolone derivative, UCCF-853 (Caci et al., 2003). Since KCNN4 channels are calcium-sensitive can the action of CBIQ be due to an increase in intracellular Ca^{2+}? Other works with benzoquinolines showed that they caused no increase in intracellular Ca^{2+} (Duszyk et al., 2001; Cuthbert and MacVinish, 2003). Furthermore with inside-out membrane patches from Calu-3 cells, where the Ca^{2+} concentration was controlled at intracellular levels, CBIQ caused rapid bursting of KCNN4 channels (Szkotak et al., 2004). In this respect CBIQ is like 1-ethyl-2-benzimidazolone (EBIO) that directly activates KCNN4 channels without affecting Ca^{2+} concentration (Devor et al., 1996). An analysis by Pedersen et al. (1999) showed the presence of Ca^{2+} was necessary for the activation of KCNN4 by EBIO that acted directly on the channel. It is to be noted that on Calu-3 cell monolayers, the K_d for CBIQ for the activation of chloride secretion is 4.0±1.3 μM, similar to the value obtained when KCNN4 is expressed in oocytes (3.9±1.6 μM). This may indicate that the degree of hyperpolarisation acts as a rate limiting step for transepithelial chloride ion secretion in Calu-3 monolayers.

The high sensitivity of CFTR to CBIQ shown in oocytes was not replicated in Fischer rat thyroid cells. We have no explanation for this, but others (Cuppoletti et al., 2001) have discussed how channel properties may depend on the expression system, the degree of phosphorylation and differences in assembly with other proteins, to which can be added the presence of IBMX in the *Xenopus* oocyte cocktail. However both in the *Xenopus* oocyte and the Fischer rat thyroid cell system we have provided evidence that the degree of phosphorylation has a profound effect on the apparent K_d for CBIQ. It was unfortunate that the effects of different forskolin concentrations could not be studied in Fischer rat thyroid cells expressing wild-type CFTR. However, concentrations of forskolin greater than 0.5 μM produced such steep changes in the rate of fluorescence decline that it was not possible to obtain quantitative data with CBIQ under these conditions. As with the majority of CFTR channel openers, when these are applied to intact tissues, the effects are dependent on a functioning endogenous adenylate cyclase system. This is well illustrated in epithelial monolayers of bronchial epithelial cells and Calu-3 cells where the apparent K_d of CFTR channel openers depends on the basal transporting activity, a marker of adenylate cyclase activity (Caci et al., 2003; Cuthbert et al., 2003).

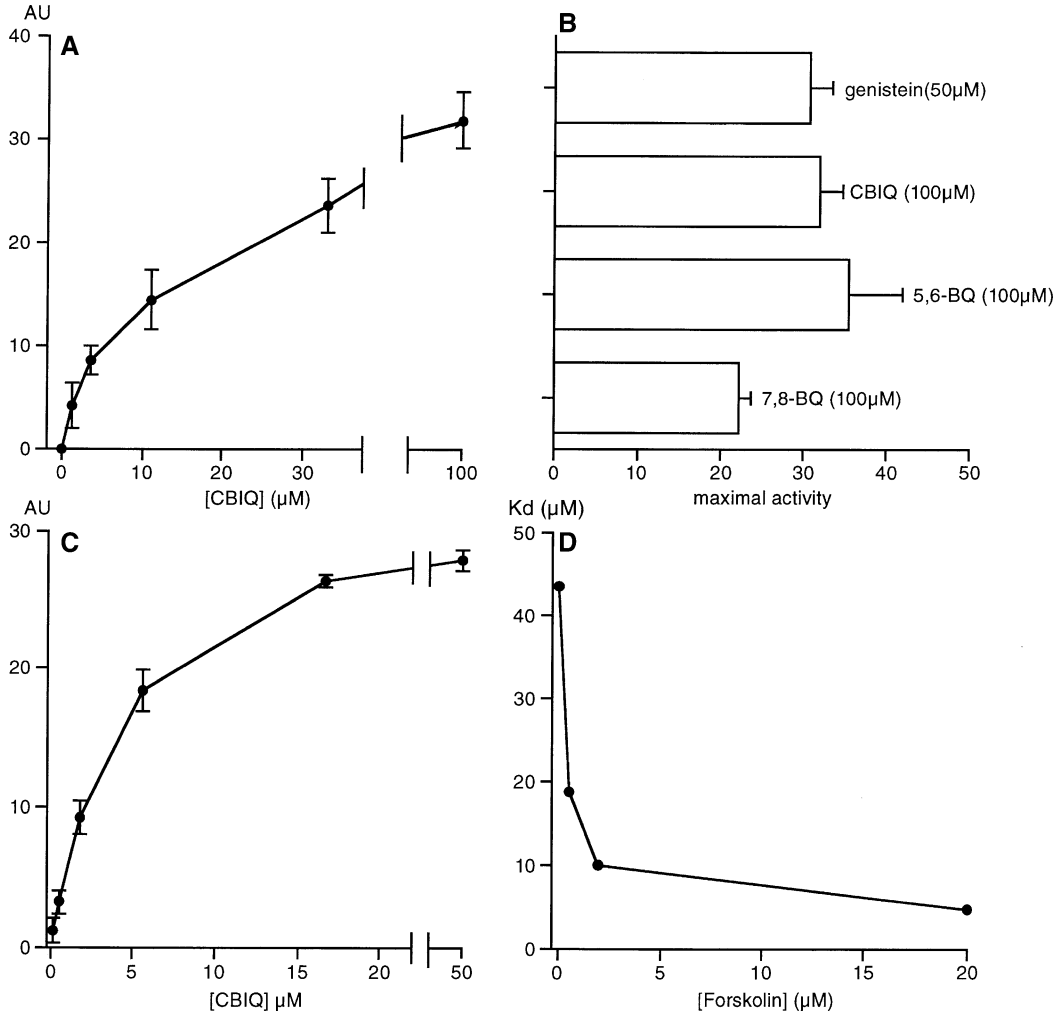

Fig. 4. Shows results from plate reader assays using Fischer rat thyroid cells expressing either CFTR (A–B) or ΔF508 CFTR (C–D). The concentration–response relationship for activation of CFTR in Fischer rat thyroid cells, primed with 0.5 μM forskolin, by CBIQ is given in A (K_d=12.1 μM). B shows the maximal effects of CBIQ, 7,8-benzoquinoline and 5,6-benzoquinoline (all at 100 μM) under the same conditions, compared to genistein, 50 μM. Fischer rat thyroid cells expressing ΔF508 were incubated at 27 °C to traffic the protein to the membrane. Cells were exposed to forskolin, 20 μM plus various concentrations of CBIQ to obtain the concentration–response curve shown in C (K_d=3.6 μM). This experiment was repeated using different concentrations of forskolin and the K_d values obtained are plotted versus the forskolin concentration in D. In these experiments the increased rate of fluorescence loss (dI/dt) is given in arbitrary units (AU). Values shown are means±S.E.M. of 4–11 observations.

We show here for the first time that CBIQ also activates ΔF508 CFTR when expressed in either oocytes or Fischer rat thyroid cells, a property not shared with either 7,8- or 5,6-benzoquinoline. We have noted earlier that when compared under exactly the same conditions (0.5 μM forskolin) the K_d for CBIQ was similar in wild-type (12.2 μM) and ΔF508 CFTR (18.7 μM).

The close chemical similarity between the different benzoquinolines means that CBIQ provides an important clue towards the design of ΔF508 CFTR activators, since 7,8- and 5,6-benzoquinoline were unable to activate ΔF508 CFTR. Introduction of an halogen atom at position 4 enhanced efficacy, as was also found for the benzoquinoliziniums (Marivingt-Mounir et al., 2004).

The search for CFTR activators is a relatively recent activity. Becq et al. (1999) produced a novel group of benzo[*c*]quinoliziniums and subsequently Ma et al. (2002) developed high throughput methods. Based upon genistein and the benzoquinoliziniums, 223 compounds were assembled of which the benzoflavone, UCCF-029, turned out to be the most potent with a K_d of ~5 μM (Galietta et al., 2001). Using UCCF-029 as a structural guide a further 77 analogues were examined, the most potent being UCCF-339, with a K_d of 1.7 μM (Springsteel et al., 2003). Ma et al. (2002) examined a further 60,000 miscellaneous compounds. Fifty-seven strong activators were found with activities greater than apigenin, some with K_d values as low as 200 nM. We have used a structure activity approach starting with 1,10 phenanthroline (Duszyk et al., 2001), graduating to benzoquinolines (Cuthbert, 2003) and then to CBIQ. We have shown that the latter has unique properties and, as far as we are aware, CBIQ is chemically different to other agents that can activate CFTR. Thus CBIQ is a further low molecular weight scaffold upon which to build

molecules that may be useful in cystic fibrosis, be it as chaperones to correct trafficking defects, activators of ΔF508 CFTR or adjuncts to gene therapy.

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