J Membrane Biol (2007) 220:1–9
DOI 10.1007/s00232-007-9066-4

Characterization of a 7,8-Benzoflavone Double Effect on CFTR Cl^- Channel Activity

Loretta Ferrera · Chiara Pincin · Oscar Moran

Received: 12 January 2007 / Accepted: 21 July 2007 / Published online: 18 September 2007

Abstract The human cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) transporter ATPases. This protein forms a Cl^- channel with a complex regulation; gene mutations cause cystic fibrosis disease. We investigated the interaction between the protein and the flavone UCCF-029 using the patch-clamp technique in the excised inside-out configuration in order to study the molecular mechanism of action for this potentiator on completely phosphorylated channel (25 U/ml protein kinase A) and a relatively low level of ATP (0.3 mM). Low concentrations of UCCF-029 (<50 nM) increase the open probability (p_o), favoring the channel transition to an activated state, while high UCCF-029 (>50 nM) levels determine inhibition of the CFTR by a reduction of the total open time. Our data suggest that this drug can potentiate CFTR by binding to a specific site on the nucleotide binding domain, promoting dimer formation. The response of CFTR to variable concentrations of ATP is not modified by application of the potentiator UCCF-029 at either low, activatory, concentration or high, inhibitory, levels. Hence, we conclude that the potentiator may not interfere with binding of ATP but probably acts at an independent site in the protein, interacting directly with CFTR to modulate channel activity.

Keywords Cystic fibrosis transmembrane conductance regulator · 7,8-Benzoflavone · Cl^- channel

L. Ferrera · C. Pincin · O. Moran (✉)
Istituto di Biofisica, CNR, Via De Marini 6, 16149 Genoa, Italy
e-mail: moran@ge.ibf.cnr.it

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family that forms a Cl^- channel with complex regulation (Riordan et al., 1989). It is mainly expressed in the apical membrane of epithelia, where it forms a pathway for anion movement and controls the rate of fluid flowthrough. Defective function of CFTR is responsible for cystic fibrosis (CF), the most common lethal genetic disease (Welsh & Smith, 1993; Tsui & Durie, 1997). Other diseases, such as autosomal dominant polycystic kidney disease (ADPKD) and secretory diarrhea, likely involve increased activity of the CFTR Cl^- channel (Welsh & Smith, 1993; Sullivan, Wallace & Grantham, 1998). The prevalence of these diseases suggests that modulators of the CFTR Cl^- channel have significant therapeutic potential. Although the exact mechanism by which decreased CFTR Cl^- permeability produces lung and pancreatic disease in CF remains unclear, it is generally believed that restoration of CFTR Cl^- permeability will be clinically beneficial.

Several small organic compounds are able to increase CFTR Cl^- conductance in vitro, such as the flavonoids and xanthines (Al-Nakkash et al., 2001; Illek, Fischer & Machen, 1998; Illek et al., 2000; Zegarra-Moran et al., 2002; Guay-Broder et al., 1995; Haws et al., 1996; Smit et al., 1993). Flavones/isoflavones (e.g., apigenin and genistein) are thought to interact directly with the nucleotide binding domain regions of CFTR and not by the inhibition of tyrosine kinases (French et al., 1997; Wang et al., 1998; Randak et al., 1999), although the exact mechanism has not been determined (Schultz et al., 1999).

Galietta and collaborators (2001) used a load-based combinatorial synthesis approach and high-throughput

screening to identify a novel class of potent CFTR potentiators, the 7,8-benzoflavones. An interesting observation was that the activating potency of these compounds depended on basal CFTR function/phosphorylation state (Caci et al., 2003; Moran, Galietta & Zegarra-Moran, 2005; Moran & Zegarra-Moran, 2005). Compounds of the 7,8-benzoflavone class, which are structurally intermediate between flavones and benzo[*c*]quinoliziums, were effective CFTR potentiators (Becq et al., 1999). The benzoflavone UCCF-029 (Fig. 1) (we kept the name "UCCF-029" as originally described by Galietta et al., 2001) is a strong potentiator of CFTR-dependent Cl^- secretion in the intact human airway epithelium (Caci et al., 2003).

Like the flavonoids apigenin and genistein, UCCF-029 potentiated and inhibited channel activity as a function of its concentration. In particular in human bronchial epithelial cells low UCCF-029 concentrations (μ1 M) determined a current increase, while high quantities of this compound decreased Cl^- flow (Caci et al., 2003; Moran et al., 2005; Zegarra-Moran et al., 2007). Here, we studied the CFTR single-channel Cl^- currents in excised inside-out membrane patches from cells expressing human CFTR to understand better the mechanism of UCCF-029 action. Since the molecular mechanisms with which the potentiators change the channel activity remain unclear, we investigated the relationships between the potentiator and the gating channel kinetics in a fully phosphorylated channel. Moreover, we analyzed the interaction between UCCF-029 and the modulation of CFTR gating by ATP, concluding that, most probably, while the potentiator favors the open state of the channel, it does not compete with the binding of the nucleotide.

Methods

Cell Culture and Electrophysiology

Experiments were done on mouse fibroblast (NIH3T3) cells stably expressing wild-type CFTR channels (Taddei et al., 2004). Cells were grown in standard conditions in Dulbecco's modified Eagle medium (DMEM-F12, 1:1) supplemented with 10% fetal calf serum, 2 mM glutamine and 0.05 mg/100 ml gentamicin. Currents were measured using the patch-clamp technique in the excised inside-out configuration (Hamill et al., 1981) using a standard patch-clamp amplifier (Axopatch-200; Axon Instruments, Union City, CA). Borosilicate glass micropipettes (Hilgemberg, Mansfield, Germany) were fire-polished to a tip diameter yielding a resistance $\geq$15 MΩ in the working solutions. Pipettes were filled with (in mM) *N*-methyl-D-glucamine (NMDG)-Cl 140, $CaCl_2$ 5, $MgCl_2$ 2, NMDG-4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 (pH 7.4). The bath solution had the following composition (in mM): NMDG-Cl 150, $MgCl_2$ 2, ethyleneglycoltetraacetic acid (EGTA) 10, NMDG-HEPES 10 (pH 7.4). The pH of solutions was adjusted after addition of ATP. Except when indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). After excision of inside-out membrane patches, CFTR Cl^- channels were activated by the addition to the bath solution of 2.5 mM ATP and of 50 U/ml protein kinase A (PKA) to phosphorylate completely the CFTR protein for at least 5 min. Bath working solutions contained variable ATP concentrations (0.3–1.5 mM ATP) and a standard PKA concentration of 25 U/ml to keep constant the protein phosphorylation. When potentiator (UCCF-029) and inhibitor ($CFTR_{inh}$-172) were used, aliquots of freshly thawed small volumes of stock solutions (100 μM in dimethyl sulfoxide [DMSO]) of both compounds (Asinex, Moscow, Russia) were mixed with the bath working solution to obtain the desired concentration before each measurement.

CFTR channel currents were recorded at room temperature (20 $\pm$ 1°C), filtered at 100 Hz with a low–pass four-pole Bessel filter (Krohn-Hite, Brockton, MA) and sampled at 1 kHz. The membrane patch was kept at a holding potential of -100 m*V*. Potential control and data acquisition used 16-bit D/A-A/D converters (ITC–16; Instrutech, Port Washington, NY) controlled with Pulse software (Heka Electronik, Lambrecht, Germany).

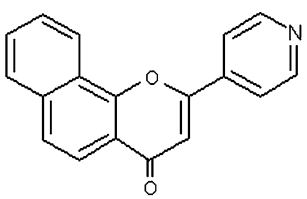

Fig. 1 Chemical structure of the CFTR potentiator (2-pyridin-7,6,benzoflavone) UCCF-029

Data Analysis

The amplitude of single-channel currents was estimated from the fit of the gaussian distributions of the current amplitude histograms. In multichannel patches, the open probability was estimated as a proportion of the time average of the current record. Statistics of the burst duration were taken from the idealized traces that presented one bona fide channel (in records where there were no double events). The minimal duration of an interburst was arbitrarily defined as 5 ms. In this condition, the dwell time histograms for interburst and burst events were consistent with single populations, indicating that we were not including a significant number of events occurring during

the burst. Data were analyzed using custom software developed in the Igor environment (Wavemetrics, Lake Oswego, OR). Data are expressed as means ± standard error of the mean (SEM, number of experiments). Statistical comparisons were done with Student's *t*-test, and statistical significance was defined at $p < 0.05$.

Results

Gating of CFTR Cl^- Channel by ATP

The CFTR Cl^- channel requires both phosphorylation by the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) and the presence of intracellular ATP to open. We observed that, in our experiments, treatment of membrane patches with 50 U/ml of the catalytic subunit of PKA and 2.5 mM ATP for 5–7 min leads to channel activation, which remains roughly constant for at least 20 min after membrane excision, even after removal of PKA (*data not shown*). However, to prevent the spontaneous dephosphorylation, we kept the PKA concentration (25 U/ml) constant during the whole experiment. In this condition, we evaluated the channel gating by adding different ATP concentrations, ranging 0.3–1.5 mM. Single-channel amplitude measured at -100 m*V* was 0.872 ± 0.014 pA, which corresponds to a conductance of 8.7 pS (*see* Fig. 2a) and is consistent with the single-channel conductance of CFTR (Lansdell et al., 1998). To further control that single-channel events were mediated by CFTR Cl^- channels, we tested the blockage of the activity by the CFTR-specific blocker $CFTR_{inh}$-172 (Taddei et al., 2004) (*data not shown*). CFTR currents were measured at a membrane potential of –100 m*V* for at least 240 s. Figure 2a shows traces recorded at three different ATP concentrations; we observed an increase of channel activity as intracellular (bath) ATP increased. To evaluate the response to ATP, we measured the mean current in the patch (with one or more channels) at different nucleotide concentrations and fitted the data of each experiment with

$$\frac{I}{I_{max}} = \frac{[ATP]}{K_d + [ATP]} \quad (1)$$

where I is the current measured for each ATP concentration, I_{max} is the maximum current estimated in a given experiment, [ATP] is the nucleotide concentration and K_d is the dissociation constant of ATP for the CFTR channel. The mean value of I/I_{max} calculated for four different patches is presented in Figure 2b, where the continuous line is the fit of data with equation 1. The fit yielded a K_d of 0.48 ± 0.04 mM, which is similar to data reported elsewhere (Lansdell et al., 2000).

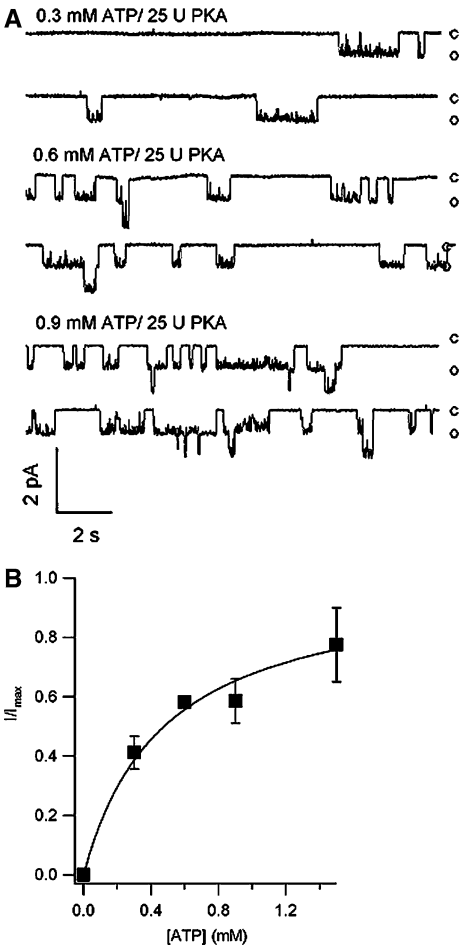

Fig. 2 Gating of CFTR Cl^- channel by ATP. (**a**) Records were obtained from a multichannel inside-out patch recorded at −100 m*V*. Channels were previously phosphorylated, and records were acquired in the presence of 25 units/ml of PKA and variable concentrations of ATP of 0.3, 0.6 and 0.9 mM. (**b**) Normalized average current as a function of the intracellular (bath) concentration of ATP. Data are the mean of at least four experiments; bars represent the standard deviation of the mean. *Continuous line* is the best fit of data with equation 1, yielding a K_d = 0.48 ± 0.04 mM

Modification of CFTR Activity by the UCCF-029 Potentiator

Most of the successive measurements to analyze the effect of UCCF-029 on the single-channel properties were done at a constant ATP concentration of 0.3 mM, which led to CFTR activity of about 38% of its maximum. This level of submaximal activity allowed us to resolve the action of UCCF-029 as it was still possible to observe an increase of CFTR channel activity by an effect of the potentiator. We defined the effect of the potentiator as the time average current increase over the current elicited by 25 U/ml PKA and 0.3 mM ATP in the absence of this compound, $I(0)$. The addition of DMSO (<0.1%), used as potentiator solvent, did not affect CFTR channel activity (*data not shown*).

Application of UCCF-029, up to 50 nM, significantly increased CFTR channel activity (Fig. 3a, center panel); but a further increase of the potentiator resulted in strong inhibition of channel activity, below the control conditions (Fig. 3a, bottom panel). The effect of UCCF-029 on CFTR activity was depicted with a sequential model of activation and inhibition, as described by Moran & Zegarra-Moran (2005):

$$\frac{I}{I(0)} = \frac{(f_a[c]+K_a)K_i}{[c]^2+[c]K_i+K_aK_i} \quad (2)$$

where I is the current measured in the presence of UCCF-029, normalized by the current measured without the potentiator, $I(0)$; f_a is the maximum increase of channel open probability (or normalized time average current); $[c]$ is the potentiator concentration; K_a is the apparent dissociation constant for CFTR activation; and K_i is the apparent dissociation constant for the inhibitory effect. The dose-response curve shown in Figure 3b yielded a K_a of 24.3 ± 2.4 nM, K_i of 187 ± 25 nM and f_a of 2.3 ± 0.2.

To investigate the mechanism of action of UCCF-029 on the single-channel kinetics, we analyzed membrane patches that contained one bona fide single active channel. When membrane patches were perfused with a solution containing 12.5 nM UCCF-029, we observed a 56.4 ± 7.2% increase of the single channel open probability, p_o, in three different experiments, from 0.14 ± 0.004 to 0.22 ± 0.009 (Fig. 4a, b). The increase in p_o was due to a significant reduction of the interburst interval, from 2.8 ± 0.2 s in control conditions to 1.3 ± 0.2 s in the presence of 12.5 nM UCCF-029 (Fig. 4d), while the mean burst duration was not significantly modified, yielding 127 ± 21 ms and 98 ± 2 ms for control and with the potentiator, respectively (Fig. 4c).

The CFTR Cl^- channel remains closed for long periods, separating bursts of channel activity, the active state of the channel. In this state, the openings are interrupted by brief closures. ATP regulates the transition between the long closed periods and the active state. The major effect of the potentiator UCCF-029 (12.5 nM) on the channel activity was a time reduction of long closed periods.

As mentioned before, UCCF-029 produces an inhibition of the CFTR-mediated current when applied at high concentration. We observed a decrease of 54% in p_o by perfusion of 100 nM of UCCF-029 on membrane patches (Fig. 5a, b). Analysis of data obtained on single-channel patches in the presence of 100 nM UCCF-029 showed a significant decrease of the mean burst duration and an increase of the interburst time (Fig. 5c, d). Use of higher concentrations of the drug was restricted by the solubility of UCCF-029 as it forms aggregates (probably micelles) at concentrations higher than 100 nM, as observed by the changes on the absorption spectra of the solutions. It is noteworthy that application of UCCF-029, even at high concentrations (100 nM), did not change the single-channel conductance. It follows that the inhibition of the current is due to a modification of the gating, without any significant contribution of pore blockage of the channel by the potentiator.

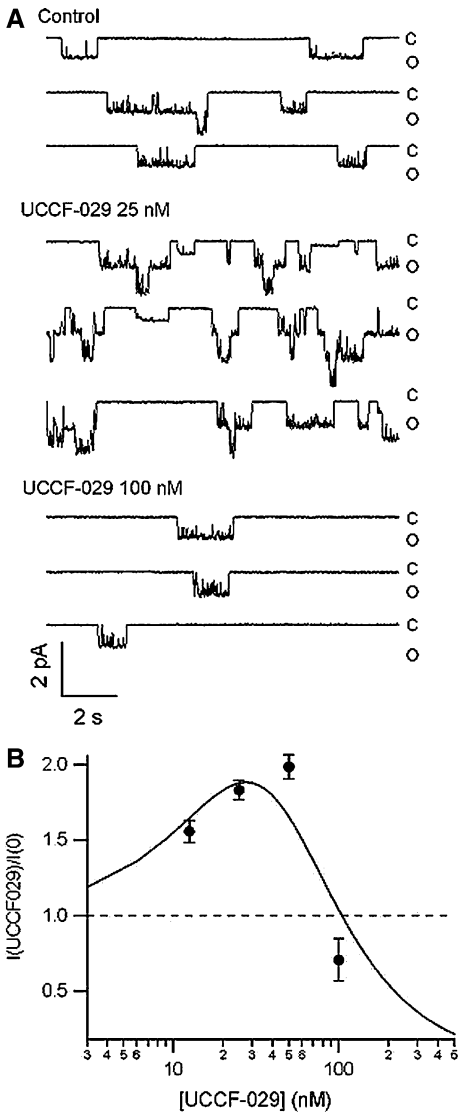

Fig. 3 Modulation of CFTR Cl^- channel activity by the potentiator UCCF-029. (**a**) Unitary events recorded in a membrane patch containing at least two channels in the presence of 0.3 mM ATP and 25 U/ml PKA. Addition of 25 nM UCCF-029 increased channel activity almost twofold, and a further increase of UCCF-029 to 100 nM reduced the channel open probability. (**b**) The time averages of current, normalized to the current in the absence of the potentiator, are plotted against the concentration of UCCF-029. Symbols represent the mean ± SEM of at least three experiments. *Continuous line* is the best fit of data with equation 2

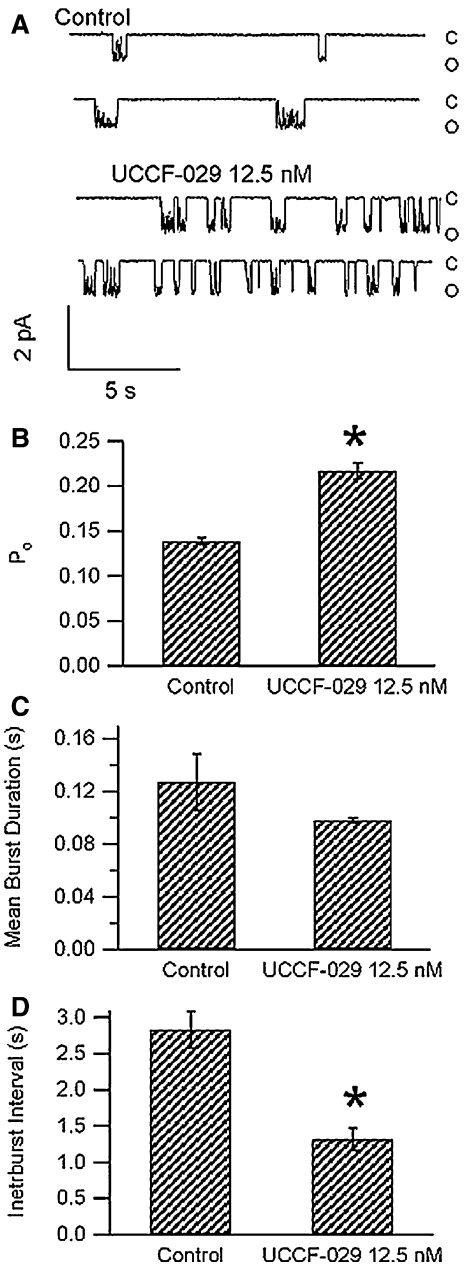

Fig. 4 Changes of CFTR single-channel activity by application of 12.5 nM UCCF-029. (**a**) Single-channel currents recorded from an excised patch containing one channel. The potential was kept at –100 m*V*. The intracellular (bath) solution contained 0.3 mM ATP and 25 U/ml PKA. Observe the significant increase of channel activity upon application of 12.5 nM UCCF-029. Open channel probability, p_o (**b**); mean burst duration (**c**); and interburst interval (**d**) measured in control conditions and after perfusion with 12.5 nM. Data represent the mean of at least three different patches, and bars are SEM. *Asterisks* indicate values that are significantly different from the control value ($p < 0.05$)

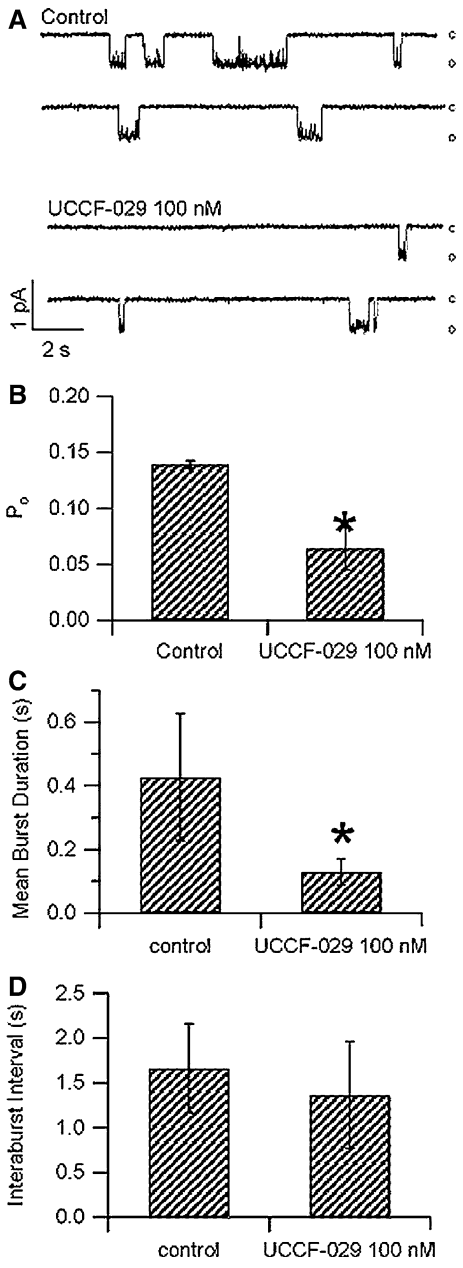

Fig. 5 Inhibition of CFTR channel activity by 100 nM UCCF-029. (**a**) Single-channel currents recorded from an excised patch containing one channel. The potential was kept at –100 m*V*. The intracellular (bath) solution contained 0.3 mM ATP and 25 U/ml PKA. Observe the significant reduction of channel activity upon application of 100 nM UCCF-029. Open channel probability, p_o (**b**); mean burst duration (**c**); and interburst interval (**d**) measured in control conditions and after perfusion with 100 nM UCCF-029. Data represent the mean of at least three different patches, and bars are SEM. *Asterisks* indicate values that are significantly different from the control value ($p < 0.05$)

Effect of ATP on UCCF-029 Modulation of CFTR

It has been suggested that CFTR potentiators exert their action by interfering with the binding of ATP or even by substituting the nucleotide in its binding site (French et al., 1997; Wang et al., 1998; Randak et al., 1999). Conversely, based on simulated docking of several ligands in a molecular model of the nucleotide binding domains

(NBDs), it has been proposed that potentiators would bind the NBD1-NBD2 interface in a putative site independent from the ATP binding site (Moran et al., 2005; Zegarra-Moran et al., 2007).

To test whether there is an interaction between the potentiator and the bound ATP during channel activity, we investigated the CFTR response to different concentrations of ATP in the presence of the potentiator. In the absence of ATP on the intracellular side of the membrane, there was virtually no channel activity; and addition of the potentiator at concentrations of 25 or 100 nM did not induce any activity at the CFTR. We measured the open channel probability in the presence of 0.1 to 1.5 mM ATP in control conditions and in the presence of 25 or 100 nM UCCF-029 (*see* Fig. 6a). These data were used to estimate the dissociation constant for ATP, K_d, at different concentrations of the potentiator (Fig. 6b). Observe the superposition of the curves when probability data were normalized. Indeed, in the inset of Figure 6b we compare the values of K_d measured in control conditions (0.48 ± 0.04 mM) after potentiation of CFTR by application of 25 nM UCCF-029 (0.49 ± 0.07 mM) and when the channel was inhibited by perfusion with 100 nM UCCF-029 (0.38 ± 0.11 mM). These dissociation constants for ATP are not statistically different, showing that affinity to ATP is not modified by application of UCCF-029, either at concentrations that increase the channel activity or at concentrations that inhibit CFTR. This observation suggests that the activity of the potentiator is independent of gating of CFTR by ATP binding.

Discussion

Modulation of CFTR Cl^- channel activity by potentiators occurs in a bimodal manner: low concentrations ($\leq$50 nM) of UCCF-029 potentiate CFTR protein, while higher concentrations ($\geq$100 nM) reduce channel activity, as previously demonstrated by measuring the apical membrane current in cell monolayers (Caci et al., 2003; Moran et al., 2005). We have confirmed the double effect of potentiator at the single-channel level, and this observation in an excised patch suggests that UCCF-029 interacts directly with the CFTR protein, plausibly excluding an action mediated by intracellular signaling pathways, as previously reported (Galietta et al., 2001). Phosphorylation of CFTR is necessary to activate the channel, rendering the NBDs available for an ATP-dependent dimerization that determines the channel gating (Mense et al., 2006). It has been demonstrated that the phosphorylation level (in terms of concentration of cAMP) may determine the apparent equilibrium constants for activation and inhibition of CFTR by potentiators (Moran & Zegarra-Moran, 2005). However, the level of phosphorylation occurring in the regulatory domain of the CFTR remains difficult to precisely define because of the elevated number of putative phosphorylation sites in this domain (Vais, Zhang & Reenstra, 2004). We have overcome this problem by inducing maximum phosphorylation of the channel by application of saturating concentrations of PKA and ATP at the beginning of the experiment. Still, because the very high channel activity obtained in these conditions could hide the effect of the potentiator, we made all our records in a submaximal concentration of ATP, to obtain a relatively low open channel probability that could be clearly increased by application of the potentiator. It is interesting to note that, after the channel was phosphorylated, the activity was maintained roughly constant for more than 20 min upon removal of PKA and then slowly decayed. This would indicate that this particular cell line may have a very low, probably almost absent, membrane-bound phosphatase activity. Indeed, compared with two other cell lines which we have independently tested, FRT cells stably transfected and CHO cells transiently transfected, CFTR activity in inside-out patches decays in a few minutes after removal of PKA. In any case, we kept PKA on the intracellular side of the channel during the whole experiment to be sure that changes in CFTR activity were not due to variations in the phosphorylation level of the polypeptide.

When phosphorylated, CFTR gating occurs upon dimerization of the NBDs induced by the binding of two ATP molecules, and the activity period of the channel would be determined by the separations of the NBDs after the hydrolysis of one ATP in the complex (Mense et al., 2006; Vergani, Nairn & Gadsby, 2003; Vergani et al., 2005). In this scheme, the burst of activity of the channel would represent the dimeric state of the NBDs, while the long interburst closure corresponds to the conformation with separated NBDs. According to this activation cycle, the increase of the Cl^- current by low concentration of potentiator was due to a reduction of the interburst interval observed here (Fig. 4d) and should be related to a decrease of the occupancy of the nondimerized state; thus, UCCF-029 would favor dimerization of the NBDs and increasing the open channel probability by an increase of the burst frequency. This is consistent with the increase of the permanency in the active state observed in the single-channel analysis, representing a more frequent transition to the active state favored by 12.5 nM UCCF-029. Our results, obtained in single-channel experiments on UCCF-029 activation, can be compared with the activation mediated by genistein, phloxine B and capsaicin. Genistein at 50 μM induces a prolonged open state with a direct bind to the CFTR Cl^- channel (Wang et al., 1998). Phloxine B increases the channel activity as UCCF-029: at low concentrations it prolongs the duration of channel opening by

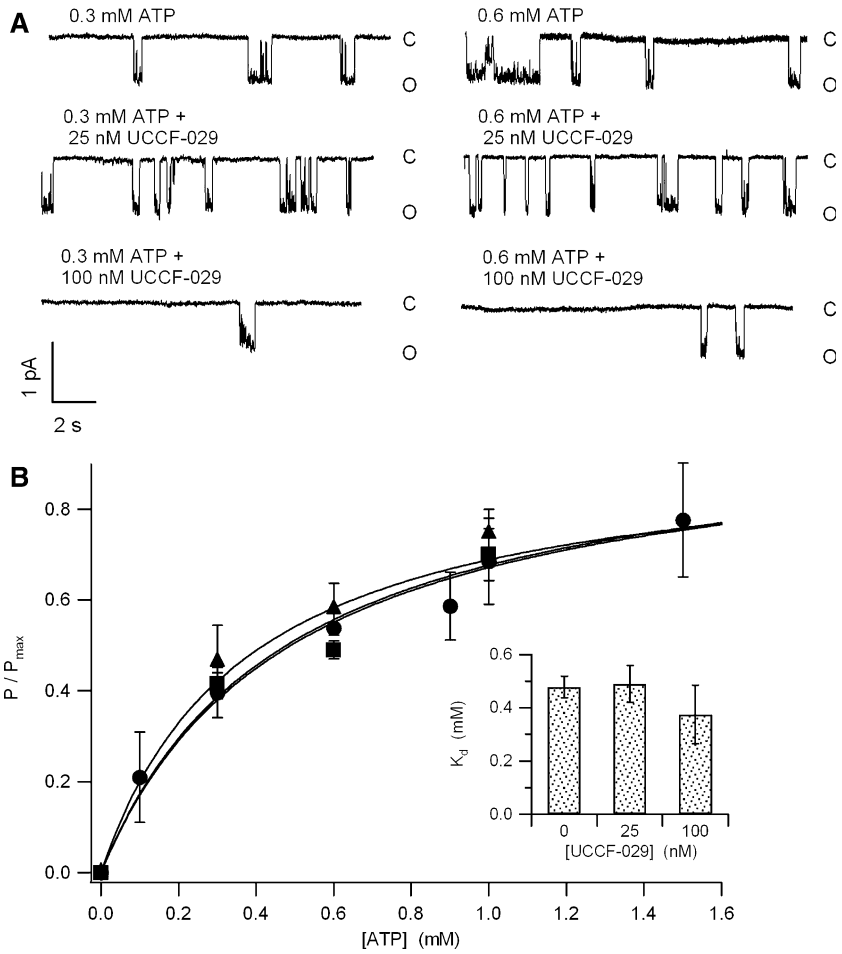

Fig. 6 ATP effect on UCCF-029 modulation of CFTR. (**a**) Currents recorded from excised patch kept at –100 m*V* at different ATP and UCCF-029 concentrations as indicated. (**b**) Normalized open probability, measured in control conditions (*circles*) and in the presence of 25 nM UCCF-029 (*squares*) or 100 nM UCCF-029 (*triangles*), is plotted against the concentration of ATP. Data represent the mean of al least three experiments, and bars are SEM. *Continuous line* is the best fit with equation 1, yielding a maximum open probability of 0.35 ± 0.02, 0.53 ± 0.12 and 0.17 ± 0.02 for control, 25 nM UCCF-029 and 100 nM UCCF-029, respectively. These values were used to normalize the curves for comparison. Dissociation constants of ATP, K_d, evaluated in these three experimental conditions are shown in the *inset*. Values represent mean ± SEM from at least three experiments at a given UCCF-029 concentration. Values of K_d (0.48 ± 0.08, 0.49 ± 0.27 and 0.38 ± 0.11 nM for control, 25 nM UCCF-029 and 100 nM UCCF-029, respectively) are not statistically different from each other

direct binding with the channel (Cai & Sheppard, 2002). Capsaicin potentiates CFTR protein by increasing the opening rate and decreasing the closing rate of the channel (Ai et al., 2004).

Increase of the UCCF-029 potentiator concentration causes inhibition of CFTR activity (*see* Fig. 5a). In this condition we observed that the mean burst duration is strongly reduced and the interburst duration increases with respect to the control conditions. This behavior is in contrast with the hypothesis that CFTR potentiators exert their activatory and inhibitory actions through two different binding sites. An independence of the binding sites would imply that when the inhibitory site is occupied, the effect of the activatory site, i.e. the reduction of the interburst interval, would remain reduced. However, a high concentration of UCCF-029, which induces a reduction of the open channel probability by shortening the burst duration, also removes the hastening of the dimerization process induced by the activatory action of the potentiator. This interference of the inhibition on the activatory effect of the potentiator strongly suggests that this double effect may occur by the action of the drug on a single receptor or on two tightly correlated receptors, as suggested by Zegarra-Moran and collaborators (2007) studying the effect of three potentiators on several mutants of their putative binding site in the NBDs. This hypothesis cannot exclude, however, the possibility that UCCF-029 would also produce a mechanical blockage of the channel, as described for genistein and phloxine B (Lansdell et al., 2000; Bachmann et al., 2000; Cai & Sheppard, 2002). However, a fast-block mechanism, similar to that described for genistein and phloxine B, has to be excluded for UCCF-029 as the apparent single-channel conductance is not varied by potentiator concentrations that produce reduction of the current.

An important aspect that has been pointed out by our experiments is the disjunction between the binding of ATP to the NBDs and the action of the CFTR potentiator, as already suggested for the potentiation of CFTR expressed in frog oocytes by genistein (Weinreich et al., 1997). The independence of the dissociation constant of ATP measured in the presence of UCCF-029 reveals that the activatory and inhibitory effects of the drug are not due to a competitive binding of the potentiator at an ATP site. This is consistent with the activatory effect of flavones on CFTR, even at high intracellular ATP concentration, that

occurs in integral cell preparations (Caci et al., 2003; Moran et al., 2005).

In conclusion, UCCF-029 can modify the gating of CFTR, probably by altering the equilibrium of the ATP-dependent dimerization of the NBDs via a noncompetitive mechanism. The activatory and inhibitory effects of the potentiator may be strongly correlated, probably acting at a common binding site. These observations are in agreement with the molecular model of the potentiator binding site, located in the interface of the NBD1-NBD2 dimer, in a position that is independent of the sites occupied by ATP (Moran et al., 2005; Zegarra-Moran et al., 2007) and possibly interacts with the interdomain interactions.

Acknowledgments This work was partially supported by the Italian Cystic Fibrosis Research Foundation (grant FFC#4/2006), Gruppo Progetto Madri per la Ricerca and Delegazione FFC di Bologna.

References

Ai T, Bompadre SG, Wang X, Hu S, Li M, Hwang TC (2004) Capsaicin potentiates wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride-channel currents. Mol Pharmacol 65:1415–1426

Al-Nakkash L, Hu S, Li M, Hwang TC (2001) A common mechanism for cystic fibrosis transmembrane conductance regulator protein activation by genistein and benzimidazolone analogs. J Pharmacol Exp Ther 296:464–472

Bachmann A, Russ U, Waldegger S, Quast U (2000) Potent stimulation and inhibition of the CFTR Cl^- current by phloxine B. Br J Pharmacol 131:433–440

Becq F, Mettey Y, Gray MA, Galietta LJ, Dormer LR, Merten M, Metaye T, Chappe V, Marvingt-Mounir C, Zegarra-Moran O, Tarran R, Bulteau L, Derand R, Pereira MM, McPherson MA, Rogier C, Joffre M, Argent BE, Sarrohuilhe D, Kammouni W, Figarella C, Verrier B, Gola M, Vierfond JM (1999) Development of substituted benzo[*c*]quiniolizinium compounds as novel activators of the cystic fibrosis chloride channel. J Biol Chem 274:27415–27425

Caci E, Folli C, Zegarra-Moran O, Ma T, Springsteel MF, Sammelson RE, Nantz MH, Kurth MJ, Verkman AS, Galietta LJ (2003) CFTR activation in human bronchial epithelial cells by novel benzoflavone and benzimidazolone compounds. Am J Physiol 285:L180–L188

Cai Z, Sheppard DN (2002) Phloxine B interacts with the cystic fibrosis transmembrane conductance regulator at multiple sites to modulate channel activity. J Biol Chem 277:19546–19553

French P, Bijman J, Bot A, Boomaars W, Scholte B, De Jonge H (1997) Genistein activates CFTR Cl^- channels via a tyrosine kinase- and protein phosphatase-independent mechanism. Am J Physiol 273:C747–C753

Galietta L, Springsteel M, Eda M, Niedzinski E, By K, Haddadin M, Kurth M, Nantz M, Verkman A (2001) Novel CFTR chloride channel activators identified by screening of combinatorial libraries based on flavone and benzoquinolizinium lead compounds. J Biol Chem 276:19723–19728

Guay-Broder C, Jacobson KA, Barnoy S, Cabantchik ZI, Guggino WB, Zeitlin PL, Turner RJ, Vergara L, Eidelman O, Pollard HB (1995) A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine selectively activates chloride efflux from human epithelial and mouse fibroblast cell lines expressing the cystic fibrosis transmembrane regulator delta F508 mutation. Biochemistry 34:9079–9087

Hamill O, Marty A, Neher E, Sakmann B, Sigworth F (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391:85–100

Haws CM, Nepomuceno IB, Krouse ME, Wakelee H, Law T, Xia Y, Nguyen H, Wine JJ (1996) Delta F508-CFTR channels: kinetics, activation by forskolin, and potentiation by xanthines. Am J Physiol 270:C1544–C1555

Illek B, Fischer H, Machen TE (1998) Genetic disorders of membrane transport. II. Regulation of CFTR by small molecules including HCO_3^-. Am J Physiol 275:G1221–G1226

Illek B, Lizarzaburu M, Lee V, Nantz M, Kurth M, Fischer H (2000) Structural determinants for the activation and block of CFTR-mediated chloride currents by apigenin. Am J Physiol 279:C1838–C1846

Lansdell KA, Delaney SJ, Lunn DP, Thomson SA, Sheppard DN, Wainwright BJ (1998) Comparison of the gating behaviour of human and murine cystic fibrosis transmembrane conductance regulator Cl^- channels expressed in mammalian cells. J Physiol 508(Pt 2):379–392

Lansdell KA, Cai Z, Kidd JF, Sheppard DN (2000) Two mechanism of genistein inhibition of cystic fibrosis transmembrane conductance regulator Cl^- channels expressed in murine cell line. J Physiol 524:317–330

Mense M, Vergani P, White DM, Altberg G, Nairn AC, Gadsby DC (2006) In vivo phosphorylation of CFTR promotes formation of a nucleotide-binding domain heterodimer. EMBO J 25:4728–4739

Moran O, Galietta L, Zegarra-Moran O (2005) Binding site of activators of the cystic fibrosis transmembrane conductance regulator in the nucleotide binding domains. Cell Mol Life Sci 62:446–460

Moran O, Zegarra-Moran O (2005) A quantitative description of the activation and inhibition of CFTR by potentiators: genistein. FEBS Lett 579:3979–3983

Randak C, Auerswald EA, Assfalg-Machleidt I, Reenstra WW, Machleidt W (1999) Inhibition of ATPase, GTPase and adenylate kinase activities of the second nucleotide-binding fold of the cystic fibrosis transmembrane conductance regulator by genistein. J Biochem 340:227–235

Riordan JR, Rommens JM, Kerem B, et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245:1066–1073

Schultz BD, Singh AK, Devor DC, Bridges RJ (1999) Pharmacology of CFTR chloride channel activity. Physiol Rev 79:S109–S144

Smit LS, Wilkinson DJ, Mansoura MK, Collins FS, Dawson DC (1993) Functional roles of the nucleotide-binding folds in the activation of the cystic fibrosis transmembrane conductance regulator. Proc Natl Acad Sci USA 90:9963–9967

Sullivan LP, Wallace DP, Grantham JJ (1998) Epithelial transport in polycystic kidney disease. Physiol Rev 78:1165–1191

Taddei A, Folli C, Zegarra-Moran O, Fanen P, Verkman AS, Galietta LJ (2004) Altered channel gating mechanism for CFTR inhibition by a high-affinity thiazolidinone blocker. FEBS Lett 558:52–56

Tsui LC, Durie P (1997) Genotype and phenotype in cystic fibrosis. Hosp Pract (Minneapolis) 32:15–18, 123–129, 134, passim

Vais H, Zhang R, Reenstra WW (2004) Dibasic phosphorylation sites in the R domain of CFTR have stimulatory and inhibitory effects on channel activation. Am J Physiol 287:C737–C745

Vergani P, Lockless SW, Nairn AC, Gadsby DC (2005) CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. Nature 433:876–880

Vergani P, Nairn A, Gadsby D (2003) On the mechanism of MgATP-dependent gating of CFTR Cl^- channels. J Gen Physiol 121:17–36

Wang F, Zeltwanger S, Yang I, Nairn A, Hwang T (1998) Actions of genistein on cystic fibrosis transmembrane conductance regulator channel gating. Evidence for two binding sites with opposite effects. J Gen Physiol 111:477–490

Weinreich F, Wood P, Riordan J, Nagel G (1997) Direct action of genistein on CFTR. Pfluegers Arch 434:484–491

Welsh MJ, Smith AE (1993) Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 73:1251–1254

Zegarra-Moran O, Romio L, Folli C, Caci E, Becq F, Vierfond JM, Mettey Y, Cabrini G, Fanen P, Galietta LJ (2002) Correction of G551D-CFTR transport defect in epithelial monolayers by genistein but not by CPX or MPB-07. Br J Pharmacol 137:504–512

Zegarra-Moran O, Monteverde M, Galietta LJV, Moran O (2007) Functional analysis of mutations in the putative binding site for cystic fibrosis transmembrane conductance regulatot potentiators. Interaction between activation and inhibition. J Biol Chem 282:9098–9104