Pflügers Arch – Eur J Physiol (1998) 436:175–181 © Springer-Verlag 1998

ORIGINAL ARTICLE

Horst Fischer · Beate Illek
Terry E. Machen

Regulation of CFTR by protein phosphatase 2B and protein kinase C

Received: 29 October 1997 / Received after revision: 13 February 1998 / Accepted: 2 March 1998

Abstract The activity of the CFTR Cl^- channel is dependent on its phosphorylation status set by kinases and phosphatases. We report here that protein phosphatase 2B (PP2B) and protein kinase C (PKC) are potential regulators of the cystic fibrosis conductance regulator (CFTR). Treating CFTR-expressing 3T3 cells with either of the two specific PP2B blockers cyclosporin A (CsA, 1 μM) or deltamethrin (DM, 30 nM) caused rapid activation of CFTR in cell-attached patches. As determined by noise analysis of multi channel patches, DM- or CsA-activated CFTR displayed gating kinetics comparable to those of forskolin-activated CFTR. After activation of CFTR by blocking PP2B, CFTR still inactivated. CFTR-mediated currents were, on average, 6.1 times larger when cells were stimulated by forskolin during PP2B block compared to stimulation by forskolin alone. This suggests that, in CFTR-expressing 3T3 cells, a phosphorylation site of CFTR is regulated by cellular PKA, PP2B and another phosphatase. However, in the epithelial cell lines Calu-3 and HT-29/B6, CsA and DM had no effect on CFTR activity in both cell-attached patch-clamp and transepithelial experiments. In contrast, when exogenous PP2B was added to patches excised from 3T3 or Calu-3 cells, PKA-activated CFTR currents were quickly inactivated. This indicates that free exogenous PP2B can inactivate CFTR in patches from both cell types. We propose that in order to regulate CFTR in an intact cell, PP2B may require a selective subcellular localization to become active. When excised patches were PKC-phosphorylated, the gating kinetics of CFTR were significantly different from those of PKA-phosphorylated CFTR. Addition of PP2B also inactivated PKC-activated CFTR showing the indiscriminate dephosphorylation of different phosphorylation sites by PP2B.

Key words CFTR gating · Chloride transport · Cyclosporin A · Deltamethrin · Noise analysis · Patch clamp

H. Fischer (✉) · B. Illek · T.E. Machen
University of California, Department of Molecular and Cell Biology, Division of Cell and Developmental Biology, 231 Life Science Addition, Berkeley, CA 94720, USA

Present address:
H. Fischer · B. Illek
Children's Hospital Oakland, Research Institute, 747 Fifty Second Street, Oakland, CA 94609, USA,

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is the major epithelial apical membrane Cl^- channel regulated by phosphorylation of its regulatory domain (R domain). In vivo phosphorylation studies have shown that at least five different serines are phosphorylated by protein kinase A (PKA) and two others by protein kinase C (PKC; [9]). There is recent evidence for an ordered phosphorylation of R domain serines during stimulation [4, 5]. CFTR dephosphorylation and inactivation by phosphatases is probably similarly organized, but neither biochemical nor functional data are available that distinguish between effects of phosphatases on specific sites of CFTR.

Serine/threonine-specific phosphatases are present in all cell types [18], including epithelial cells [23, 24]. Phosphatases have been classified into two groups according to their dependence on intracellular factors. Type 1 protein phosphatase (PP1) is inhibited by two intracellular proteins, termed inhibitors 1 and 2, while type 2 phosphatases are not. The latter were further subdivided by their dependence on divalent cations. PP2A is active in the absence of divalents, while PP2B and PP2C are active only in the presence of Ca^{2+} and Mg^{2+}, respectively [18]. In in vitro assays phosphatases show a very broad and overlapping substrate specificity, though targeted localization of phosphatases to certain cell domains might result in spatial specificity.

Whether CFTR is regulated by a certain phosphatase might therefore depend on the cellular localization and environment. CFTR is expressed in many different cell types, which makes cell-type-specific regulation of CFTR

possible owing to the differential cellular expression of regulatory complexes including kinases, phosphatases and anchoring proteins in micro domains near the membrane.

PP2B is a Ca^{2+}/calmodulin-dependent phosphatase [15, 22] and is a target of very specific phosphatase blockers. The immunosuppressant cyclosporin A (CsA) specifically binds to an intracellular immunophilin, termed cyclophilin, and the CsA-cyclophilin complex binds and inactivates PP2B [10, 21]. This sequence makes CsA a very selective probe. The chemically unrelated insecticide deltamethrin (DM) specifically inactivates PP2B by direct binding to PP2B at nanomolar concentrations [6].

In this report we show that PP2B can inactivate PKA- or PKC-phosphorylated CFTR in excised patches. By using PP2B blockers on intact cells we show that PP2B controls an important phosphorylation site during activation of CFTR when expressed in mouse fibroblasts, but not in the epithelial cell lines HT-29 or Calu-3, even though PP2B was found in all cell types investigated [15]. This apparent contradiction may be explained by different subcellular localizations of PP2B between cell types. In contrast to data presented here, a previous report showed that PP2B does not dephosphorylate immunoprecipitated CFTR and does not inactivate CFTR in patches excised from 3T3 cells [3], although some effects of PP2B on the phosphorylation of the isolated R domain were noted [5]. Possible reasons for the difference will be discussed.

Materials and methods

Cells

Mouse NIH 3T3 fibroblasts stably transfected with the wild-type CFTR and its mock-transfected control line were grown in H-21 Dulbecco's minimal essential medium (DMEM) supplemented with 10% newborn calf serum as described [7]. Calu-3 cells, a human cell line of pulmonary adenocarcinoma origin [11], were kindly provided by Dr. J.H. Widdicombe (Children's Hospital Oakland). HT-29/B6, a subclone derived from the human adenocarcinoma cell line HT-29, was given by Dr. K.M. Kreusel, FU Berlin [16]. These Cl^- secretory epithelial cell lines were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine (all from University of California at San Francisco Cell Culture Facility). For patch-clamping cells were seeded in low density on glass coverslips and used after 1–2 days. For transepithelial experiments the epithelial cell lines were seeded on porous membrane filters (0.45 µm pore size, 12 mm diameter, Falcon, Becton Dickinson, Franklin Lakes, N.J., USA). Cells grown as monolayers were used between 5 and 8 days.

Patch-clamp recordings

Measurements were performed with the cells placed on the stage of a microscope in an open, perfusable chamber (≅0.5 ml) at 37°C as described in detail before [7, 8]. All recordings in this report contained multiple CFTRs per patch. Current noise from multichannel recordings was analysed as described elsewhere [7]. Spectral densities (S) were fitted with the sum of two Lorentzians resulting in fit-estimates of the corner frequencies (f_{cl}, f_{ch}) and the amplitudes (S_{0l}, S_{0h}) of the low-frequency (l) and the high-frequency (h) Lorentzians [7, 8]. Current noise spectra were used to characterize the gating properties of CFTR and to identify CFTR in multi-channel recordings. The applied potential is reported as the negative pipette potential ($-V_p$) in the cell-attached mode, and as the membrane potential (V_m) in the excised mode.

The composition of the standard bath solution was (in mM): 141 NaCl, 4 KCl, 1 KH_2PO_4, 1 $MgCl_2$, 1.7 $CaCl_2$, 10 4-(2-hydroxyethyl)1-piperazineethanesulphonic acid (HEPES), 25 glucose, pH=7.4. Pipette filling solution for all cell-attached or excised recordings was (in mM): 147 *N*-methyl-D-glucamine chloride (NMDG-Cl), 1.7 $CaCl_2$, 10 HEPES, 25 glucose, pH=7.4. Bath solution for excised patches was (mM): 147 NMDG-Cl, 1.7 $MgCl_2$, 0.1 ethylenebis(oxonitrilo)tetraacetate (EGTA), 1 MgATP, 25 glucose, pH=7.4.

Transepithelial recordings

Monolayers grown on filters were cut out and mounted in a modified Ussing chamber as described [13]. Monolayers were voltage-clamped to 0 mV and pulsed every 20 s to 2 mV. The resulting short-circuit current (I_{sc}) was recorded by a computer. Negative currents were defined as anion movement from mucosa to serosa. Both chamber compartments were separately and continuously perfused at 37°C. The exposed epithelial area was 0.5 cm^2. The basolateral membranes of monolayers were permeabilized by using the pore-forming agent *Staphylococcus aureus* α-toxin (250 U/ml, Calbiochem, La Jolla, Calif., USA), which irreversibly permeabilizes cell membranes for molecules <4000 Da. α-Toxin permeabilization yields a dialysed preparation with its intracellular milieu given by the extracellular solution, which allows us to clamp cytosolic regulators of CFTR, i.e. cAMP and ATP levels [13]. A mucosal-to-serosal Cl^- gradient was imposed. The mucosal solution contained (in mM): 135 NaCl, 1.2 $MgSO_4$, 1.2 $CaSO_4$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 20 HEPES and 10 glucose. The serosal solution contained (in mM): 135 Na-gluconate, 0.1 $CaCl_2$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 20 HEPES, 10 glucose and 5 MgATP (Sigma). cAMP, Na salt (100 mM, Sigma) was added to the serosal solution to stimulate CFTR. All solutions were adjusted to pH 7.4 with NMDG.

Drugs and enzymes

Forskolin (Calbiochem), the adenylyl cyclase activator, was made as a 100 mM stock in dimethyl sulphoxide (DMSO) and used at 1–10 µM. cAMP, Na salt (Sigma) was directly dissolved in Ringer solution. DM (Alamone, Israel) was made as 100 mM stock in DMSO and used at 30 nM; CsA (gift from Sandoz Pharmaceutical, East Hanover, N.J., USA) was made as a 1 mM stock in ethanol and used at 1 µM. The catalytic subunit of protein kinase A (Promega, Madison, Wis., USA) was used at a final activity of ≅100 U/ml. PP2B (Promega) was added together with 1 mM $NiCl_2$, 1 mg/ml bovine serum albumin (BSA, molecular biology grade, New England Biolabs, Beverly, Mass., USA) and 10 µg/ml calmodulin (CaM, Boehringer Mannheim) at a final activity of ≅2 U/ml. Addition of cofactors alone had no effect on CFTR activity in eight cases. PKC (containing PKCα and PKCβ, Promega) was added together with 20 µg/ml phosphatidyl serine (PS) and 5 mM $MgCl_2$. Experiments using PKC or PP2B were done using solutions with 2 mM $CaCl_2$ added to satisfy the Ca^{2+} requirements of these enzymes. None of the cofactors mimicked the effects of the respective enzymes.

Results

Effects of CsA and DM on CFTR activity

In a resting cell, in which kinase and phosphatase activities are in equilibrium, blocking a phosphatase is expect-

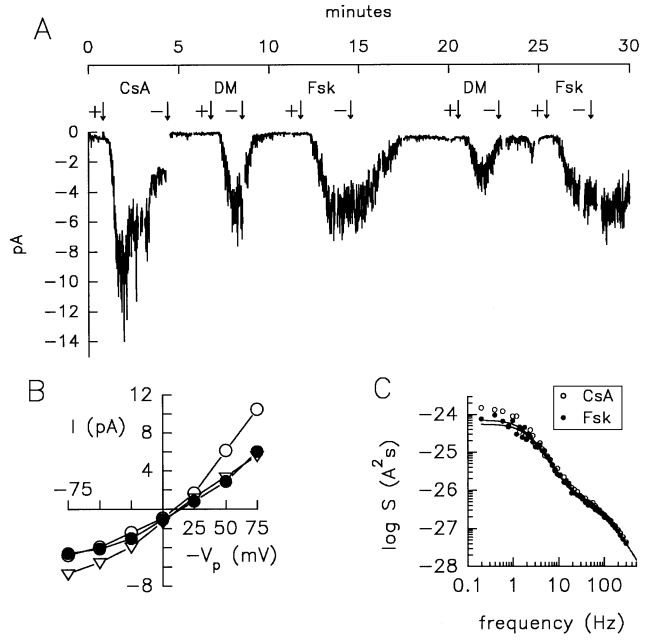


Fig. 1A–C Activation of cystic fibrosis conductance regulator-(CFTR-) mediated currents by cyclosporin A (*CsA*) and deltamethrin (*DM*) in 3T3 cell. **A** Cell-attached recording from a CFTR-expressing 3T3 cell. CsA (1 μM), DM (30 nM), or forskolin (1 μM, *Fsk*) was perfused in (+) and washed out (–) where indicated by *arrows*. Repeated treatment with the phosphatase blockers CsA and DM or with forskolin led to repeated stimulation of CFTR-mediated currents. Bath contained NaCl Ringer solution, pipette *N*-methyl-D-glucamine chloride (*NMDGCl*) Ringer solution. Current trace was sampled at 10 Hz. Breaks in current trace are where current/voltage (*I*/*V*) relations were recorded. Potential was $-V_p$=–75 mV; current activation is downward. **B** Total-current/voltage relations, CsA-stimulated (*open circles*), DM-stimulated (*filled circles*), and forskolin-stimulated (*triangles*) for the first three stimulations of the experiment shown in **A**. Reversal potentials (in the cell-attached mode) were on average 8.0±4.1 mV and not different for the three conditions. **C** Current noise spectra were recorded during each of the five consecutive stimulations in **A**. The corner frequencies for the first stimulation (CsA, *open circles*) were: f_c=2.04 and 97.8 Hz, and for the third stimulation (Fsk, *filled circles*): f_c=2.0 and 97.2 Hz. Spectra recorded during the other stimulations in **A** had the following corner frequencies: 2nd stimulation (*DM*): f_c=2.24 and 95 Hz; 4th stimulation (*DM*): f_c=2.03 and 93.2 Hz; and 5th stimulation (*Fsk*): f_c=2.3 and 87.2 Hz (not shown)

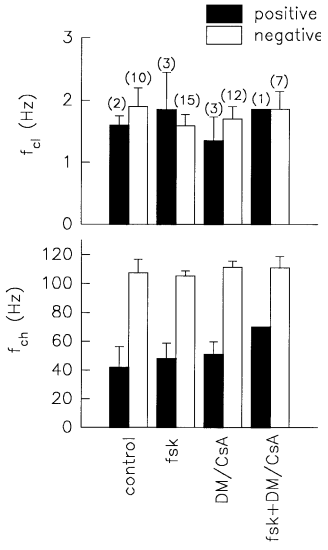


Fig. 2A, B Average corner frequencies of spectra recorded in cell-attached patch-clamp mode. Average corner frequency of slow Lorentzian (f_{cl}, **A**) and of fast Lorentzian (f_{ch}, **B**) are shown. Four treatment groups were: control, forskolin-stimulated (*fsk*), deltamethrin or cyclosporin A-treated (*DM/CsA*), and forskolin stimulation during deltamethrin or cyclosporin A treatment (*fsk+DM/CsA*). Treatment groups were split into positive (65–85 mV) and negative (–60 mV to –90 mV) holding potentials. f_{cl} was not affected by treatment or holding potential; f_{ch} was similar for all treatments and significantly dependent on potential (P<0.0001, ANOVA). Number of spectra for each group are given in **A**. Data are mean ± SEM

ed to have three effects: (1) CFTR activity should be stimulated through basal kinase activity, (2) CFTR should not inactivate, and (3) effects of kinase activation and phosphatase block should be synergistic. Figure 1 shows the effects of the two PP2B inhibitors, CsA and DM, on a CFTR-expressing 3T3 cell in a cell-attached patch recording. Perfusion of the cell with 1 μM CsA or 30 nM DM readily activated current to levels similar to those activated by 1 μM forskolin. Under the recording conditions negative currents were indicative for Cl^- currents. Total current/voltage (*I*/*V*) relations resulted in the CFTR-typical outward rectification in the cell-attached mode (Fig. 1B; [7, 8]); reversal potentials were slightly positive, which was expected for Cl^- currents recorded from a secreting cell. Current noise spectra recorded during the different stimulations were very similar (Fig. 1C). See Fig. 2 for average corner frequencies of spectra recorded under the different conditions. Gating kinetics, as judged by noise analysis, were identical for all treatments (i.e. forskolin, CsA, DM). The low-frequency Lorentzian (f_{cl}) averaged 1.69±0.1 Hz (n=53) and was unaffected by potential, and the high-frequency Lorentzian was strongly voltage dependent [f_{ch}=49.4±5.02 Hz (n=9) for positive potentials; f_{ch}=108.4±2.95 (n=41), for negative potentials]. These gating characteristics were independent of the treatment. In mock-transfected 3T3 control cells CsA, DM, or forskolin had no effects on currents (not shown). This suggests that both block of PP2B (by CsA or DM) and activation of PKA (by forskolin) activates CFTR through a PKA-dependent phosphorylation, supporting the notion that PP2B tonically inhibits CFTR by controlling a phosphorylation site of CFTR, which, if PP2B is blocked, is relieved and CFTR activated. Because the gating properties were very similar during PP2B block or PKA activation (by forskolin), it appears likely that CFTR is in a similar phosphorylation state during each treatment (for comparison, see Fig. 8 for distinct gating properties of PKC-phosphorylated CFTR).

Note that currents inactivated despite the continued presence of PP2B-blocker. In none of 11 stimulations did block of PP2B inhibit inactivation of CFTR. This indicates that another inactivation step, different from PP2B, is also present, suggesting that another phosphatase is also active.

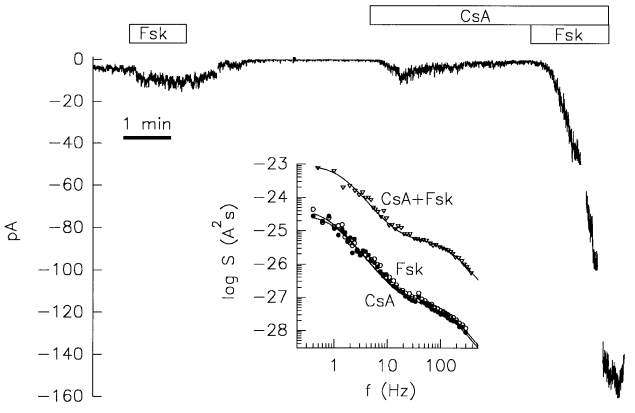


Fig. 3 Synergy between CsA-induced and forskolin-induced activation. Cell-attached recording from a CFTR-expressing 3T3 cell. $-V_p$=–70 mV. CsA (1 μM) and forskolin (1 μM) were perfused into the bath as indicated by *boxes*. *Breaks* in current trace were caused by repeated saturation and re-setting of patch amplifier. *Inset* shows current noise spectra during CsA treatment (f_{cl}=0.88 Hz, f_{ch}=112 Hz), forskolin (f_{cl}=0.74 Hz, f_{ch}=108 Hz) and after full activation by forskolin (f_{cl}=1.0 Hz, f_{ch}=125 Hz). The typical double-Lorentzian was used as an indicator for CFTR whose gating behaviour appeared unaltered by different treatments

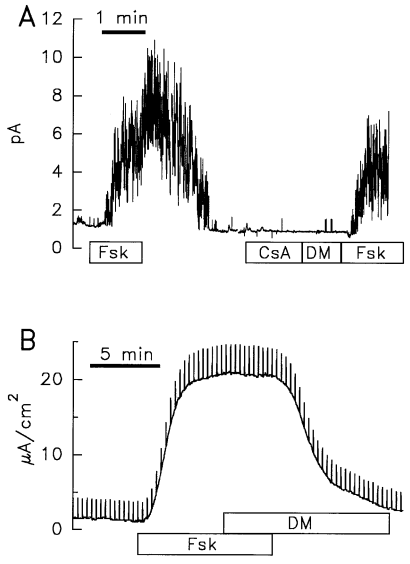


Fig. 4A, B Lack of activation of CFTR by CsA or DM in Calu-3 and HT-29/B6 cells. **A** Cell-attached recording, $-V_p$=78 mV, 1 mM forskolin (*Fsk*), 1 mM CsA, or 30 nM DM was present where indicated by *boxes*. This recording was performed at a positive potential because CFTR-mediated currents in Calu-3 showed strong outward rectification in the cell-attached mode, such that at negative potentials currents were very small. **B** Transepithelial recording from an HT-29/B6 monolayer. DM (300 nM, mucosal) neither increased currents nor inhibited inactivation of current after forskolin removal

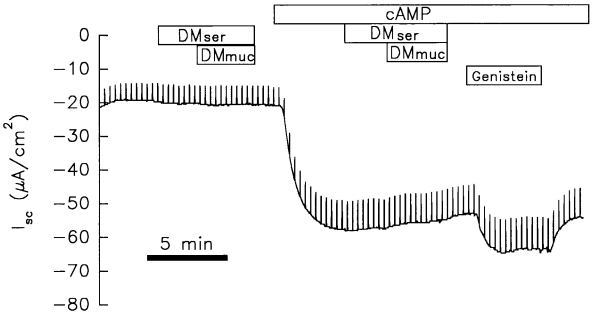


Fig. 5 Lack of effect of DM in HT-29/B6 epithelium. Transepithelial current recording from an HT-29/B6 tissue basolaterally permeabilized with α-toxin. Current activation is downward. DM (100 nM) added to the serosal (*ser*) or mucosal (*muc*) side had no effects on Cl^- currents, either in the absence or the presence of cAMP (100 μM). In the presence of cAMP, current was further activated by addition of genistein (50 μM) to the mucosal solution

CsA- and DM-induced stimulations of CFTR could have also been due to an increase in cAMP concentrations [2]. This possibility was addressed in the next experiment. Figure 3 shows a cell-attached patch experiment which compares current activations by forskolin or CsA alone and in combination. Forskolin's stimulatory effect was much larger when in the presence of CsA compared to its effect alone. On average, forskolin-stimulated currents were 6.1±3.3 fold (n=5) larger in the presence of CsA or DM compared to those elicited by forskolin alone. This suggests that during stimulation by forskolin currents are partially inhibited by PP2B, and that when PKA is activated and PP2B is blocked, the currents are fully activated. This synergism indicates that, in 3T3 cells, PKA activates and PP2B inactivates a common step. This synergism also rules out the hypothesis that CsA or DM activates CFTR by increasing cAMP levels, because during forskolin treatment PKA can be expected to be saturated with cAMP [1].

Corner frequencies of Lorentzian fits to current noise spectra recorded during PP2B block, forskolin stimulation, or combined treatment with PP2B blocker and forskolin were not changed by the treatment despite significant changes in current (Figs. 2, 3). This indicated that CFTR's gating in that frequency domain (0.2–400 Hz) is not regulated by these interventions, but that current activation is caused by slower events, e.g. increasing the apparent number of active channels.

The role of PP2B in controlling CFTR was also tested in experiments on Calu-3 airway epithelial cells and in HT-29/B6 colonic crypt-like epithelial cells. Figure 4 A shows a cell-attached recording on a Calu-3 cell. Neither CsA nor DM elicited currents, while forskolin readily activated currents (n=6). In addition, DM had no effect either on transepithelial recordings from intact Calu-3 monolayers (n=4, not shown) or intact HT-29/B6 monolayers (n=3, Fig. 4B). These results suggest that in these two epithelial cell lines PP2B does not play the same role in CFTR regulation as it does in 3T3 cells. These data indicate that a stricter polarization of cells does not influence the effects of PP2B, because PP2B block had no effect on CFTR in either polarized monolayers or in single cells used for patch clamping. However, another explanation may be that basal PKA activity in these cells was very low so that block of PP2B did not activate CFTR. This possibility was tested in the next set of experiments.

Figure 5 shows a typical (n=4) transepithelial current recording from an HT-29/B6 monolayer with its basolat-

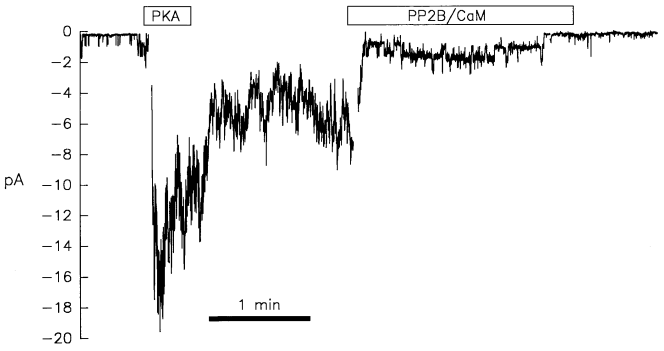


Fig. 6 Inactivation of CFTR-mediated current by protein phosphatase 2B (*PP2B*) in a patch excised from a CFTR-expressing 3T3 cell. V_m=–71 mV; pipette, NMDG-Cl Ringer; bath, NMDG-Cl Ringer plus 2 mM $CaCl_2$. After inactivation of current by PP2B, subsequent addition of PKC reactivated current in this patch (not shown)

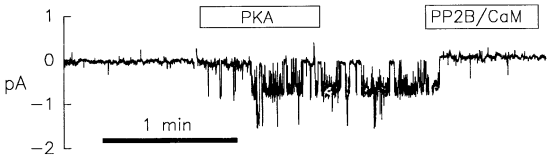


Fig. 7 Inactivation of protein-kinase-A- (*PKA*-) phosphorylated CFTR by PP2B in a patch excised from a Calu-3 cell. V_m=–70 mV; pipette, NMDG-Cl Ringer; bath, NMDG-Cl Ringer plus 2 mM $CaCl_2$. Addition of PKA to the bath activated two CFTRs in this patch. PP2B/calmodulin fully inactivated CFTRs

eral membrane permeabilized by α-toxin. Under unstimulated conditions DM, added to the serosal and mucosal sides, had no effect on current. Addition of cAMP (100 μM) to the serosal bath activated Cl^- current, but addition of DM to either side of the tissue still had no effect. However, addition of genistein, a compound that has all the typical effects of a phosphatase blocker [13], further increased currents. These data suggest that the lack of response of intact cells to DM is not due to low PKA activity but instead is due to the fact that PP2B has no significant role in regulating CFTR in HT-29 or Calu-3 cells.

Effects of PP2B after PKA phosphorylation of CFTR

We tested the effects of PP2B on excised membrane patches to clarify whether direct application of PP2B to phosphorylated, active CFTR dephosphorylates and inactivates CFTR from different cells. Figure 6 shows a current recording from a patch excised from a CFTR-expressing 3T3 cell. The patch was excised from an unstimulated cell. Initially the patch showed little channel activity. Addition of PKA stimulated many CFTR channels. Addition of PP2B (together with its cofactor CaM) inactivated most of the current immediately, and the three remaining CFTRs were inactivated after ≅2 min of incubation with PP2B. This partial and delayed inactivation was noticed in two of six experiments and may have

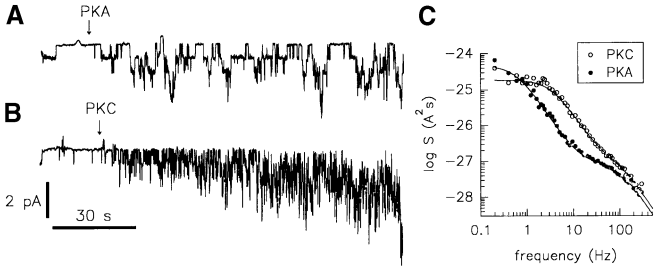


Fig. 8A–C CFTR activated by PKA or PKC shows different gating behaviour. V_m=–70 mV; pipette, NMDG-Cl Ringer; bath, NMDG-Cl Ringer plus 2 mM $CaCl_2$ and 5 mM $MgCl_2$; current traces were sampled at 20 Hz. Addition of PKA (**A**) or PKC (**B**) led to clearly distinct gating behaviour of CFTR. Both traces are from a continuous recording. After PKA activation CFTR was inactivated with λ-phosphatase (not shown [8]). Current noise spectra (**C**) quantify gating. Fitted values were: for PKA, f_{cl}=0.58 Hz, f_{ch}=97 Hz, for PKC, f_{cl}=3.3 Hz, f_{ch}=103 Hz

reflected channels in the patch that were not readily accessible to the enzyme added to the bath. This experiment shows that exogenous PP2B inactivates PKA-phosphorylated CFTR in patches isolated from 3T3 cells. Similar experiments were performed using a membrane patch excised from a Calu-3 cell. Figure 7 shows a typical experiment (*n*=4). Addition of PKA activated two CFTR channels in this patch, which were quickly inactivated after addition of PP2B. Thus, independent of the cell type used, exogenous PP2B readily inactivated CFTR. This indicates that PP2B is an inactivator of CFTR in cells where PP2B can access CFTR.

Effects of PP2B after PKC phosphorylation of CFTR

We used PKC-phosphorylated CFTR to test whether PP2B is specific for the site it dephosphorylates. PKC has previously been shown to phosphorylate two R domain serines that are not recognized by PKA, and one site common to PKA and PKC [9]. Figure 8 shows an excised patch (of *n*=6 experiments) that was first phosphorylated by PKA (A), then inactivated by treating with λ-phosphatase (not shown), and finally activated with PKC (Fig. 8B). PKA and PKC induced very different CFTR activity: PKA activated CFTR maximally after ≅30 s and induced slow gating, while PKC activated CFTR much more slowly so that currents were maximal only after ≅2 min. In addition, PKC-phosphorylation induced much faster gating behaviour of CFTR. Steady-state gating was quantified in current noise spectra (Fig. 8C). PKC phosphorylation shifted the low-frequency Lorentizan significantly to the right to, on average, f_{cl}=2.76±0.15 Hz. In all six experiments, PKC treatment increased f_{cl} compared to PKA treatment of the same patch. The sequence of phosphorylation of CFTR by PKC or PKA had no effect on this gating pattern. This difference in behaviour of CFTR after PKA or PKC phosphorylation suggests that the two kinases phosphorylated different site(s) of CFTR.

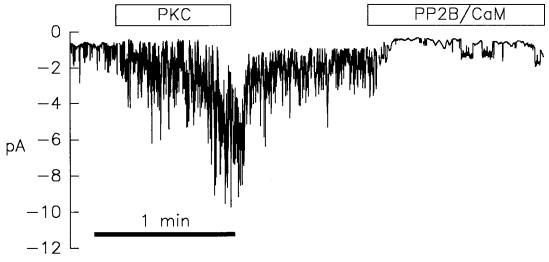
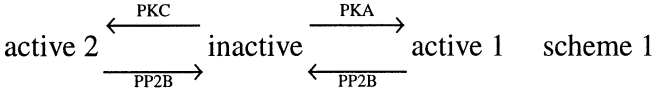


Fig. 9 Inactivation of PKC-phosphorylated CFTR by PP2B. V_m=–72 mV; pipette, NMDG-Cl Ringer; bath NMDG-Cl Ringer plus 2 mM $CaCl_2$ and 5 mM $MgCl_2$. Patch showed basal channel activity after excision. Addition of PKC activated many CFTR channels which were readily inactivated by PP2B/CaM

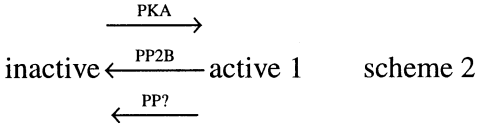
Figure 9 shows a typical recording (n=3) from an excised patch that was first activated by PKC. Addition of PP2B quickly inactivated PKC-phosphorylated CFTR. Since PP2B inactivated both PKA- and PKC-activated CFTR, PP2B appears not to be selective for specific phosphorylation sites of CFTR (e.g. PKA sites over PKC sites) when freely diffusible in solution.

Discussion

CFTR in the plasma membrane is activated by phosphorylation and inactivated by dephosphorylation. We have shown in this report that in excised patches CFTR can be phosphorylated and activated by both PKA and PKC and inactivated by PP2B. Our data further indicate that PKA and PKC cause different patterns of phosphorylation of CFTR [19] which can be indiscriminately dephosphorylated and inactivated by PP2B. These results may be described by a simple scheme:



where PKA- and PKC-dependent phosphorylation results in two different states of activity (distinguished by their gating characteristics), and PP2B dephosphorylates and inactivates both states. In intact 3T3 cells blocking of PP2B leads to a rapid activation of CFTR consistent with Scheme 1. If under resting conditions basal PKA or PKC activity is lower than basal PP2B activity, CFTR is inactive. The large synergizing effect of CsA on forskolin-induced activation of CFTR (Fig. 3) suggests (Scheme 1) that PP2B had a high activity during forskolin-induced stimulation and that CFTR can be maximally stimulated only when PP2B is blocked. We also observed that CFTR inactivated even when PP2B was blocked, suggesting an inactivation step that is independent of PP2B. This necessitates a second inactivation step in Scheme 1 by introducing a phosphatase that is not blocked by CsA or DM:



where PP? is an unknown phosphatase or another inactivation step. Under resting conditions PP? must have a lower activity than PP2B, otherwise block of PP2B would not activate CFTR. Note that it was not tested whether PP? also inactivates PKC-phosphorylated CFTR, i.e. state active 2 in Scheme 1. Although PP2B appears to play a dominant role in inactivating CFTR in 3T3 cells, the PP(s) that regulate(s) CFTR in other cell types remain(s) unknown. The PP2B inhibitors did not affect CFTR in the epithelial cell lines Calu-3 and HT-29, indicating that another, unidentified, phosphatase substitutes for PP2B's effects in these cells. When used at low concentrations specific for phosphatases, neither of the PP1/2A blockers okadaic acid or calyculin A affected CFTR-mediated currents in either HT-29 or Calu-3 cells (not shown). These results indicated that neither PP1, 2A nor 2B inactivated CFTR in intact epithelial cells. Previously Berger et al. [3][1] showed that PP2A inactivates CFTR-mediated currents by 67% in patches excised from 3T3 cells and, recently, Travis et al. [24] showed that PP2C fully inactivates and dephosphorylates CFTR excised from HeLa cells. These data, combined with those presented here, indicate that although CFTR may be dephosphorylated and inactivated by multiple phosphatases in excised patches, it is necessary to examine the activity of CFTR in intact cells to determine whether any of these phosphatases actually regulates CFTR in a specific cell type.

Travis et al. [23] have detected mRNA coding for PP1, 2A, 2B and 2C in the epithelial chloride secretory cell line T84. However, blocking either PP2A or 2B with okadaic acid or FK506, respectively, had no effect on CFTR-mediated currents in intact T84 cells [24], and PP2B appears also not to be involved in regulating CFTR in intact Calu-3 and HT-29 cells (present data). In sweat gland duct cells CFTR was shown to be inactivated by a phosphatase that is exquisitely sensitive to okadaic acid [20], indicating that PP2A is likely to be the only phosphatase responsible for inactivating CFTR in these cells. In heart muscle cells, CFTR is serially inactivated by an okadaic-acid-sensitive phosphatase (likely PP2A) and another, unidentified phosphatase (possibly PP2C; [5, 12]).

Taken together, these data indicate that it is likely that most cells express multiple phosphatases, and, though all are capable of dephosphorylating and inactivating CFTR, only one or two specific PP appear to operate in a given cell. In the present work we have shown that PP2B totally inactivates both PKA- and PKC-activated CFTR in excised patches from both fibroblasts and epithelial

[1] Berger et al. [3] were the first to add PP2B to excised patches, and they found no effects. Because we have found no obvious differences in methodology, we can only speculate that the PP2B preparations that have only become commercially available during the last few years are more active than the self-made preparation used by Berger et al. [3]. Our data showed that PP2B blockers and PP2B enzyme have complementary effects. Consistent with our data, Nairn et al. (personal communication and [5]) recently used PP2B (from the same source that we use) in phosphorylation assays of CFTR's R domain. Their data suggest that PP2B dephosphorylates specific site(s) of CFTR, while PP2A and PP2C dephosphorylate virtually all sites (with different kinetics).

cells, and previous work has shown that PP2B is present in epithelial cells [15, 23]. Experiments on intact cells have shown that PP2B regulates CFTR only in fibroblasts. We propose that this cell-type-specific activity of PP2B is caused by selective localization of the phosphatase to a specific subcellular domain near the CFTR. Thus, the physiological regulation of CFTR in intact cells will be dominated by this cell-specific localization of phosphatases.

Although cell-specific targeting of PP2B has not been shown, recent reports about localization and expression of AKAP79, a scaffold protein that binds PP2B, PKA and PKC [14], support the notion of a selectively targeted and tethered PP2B. The observed cell-type-specific effects of inhibiting CFTR by PP2B in intact cells (i.e. in cell-attached patches and in transepithelial recordings) are consistent with a cell-type-dependent selective localization of PP2B. Similarly, PKC is ubiquitously expressed in all cells and is a very potent stimulator of CFTR in excised patches. Several tethering proteins have been reported for PKC, including AKAP79 [14] and RACK (receptor for activated C kinase; [17]). We therefore propose that cell-type-specific regulation of CFTR by PP2B and PKC may involve selective localization of these enzymes.

Acknowledgements This study was supported by the Cystic Fibrosis Foundation (to H.F.), by Cystic Fibrosis Research, Inc., California (to H.F. and T.E.M.), by the Deutsche Forschungsgemeinschaft (to B.I.), and the National Institutes of Health (to T.E.M.).

References

1. Adams SR, Harootunian AT, Buechler YT, Taylor SS, Tsien RY (1991) Fluorescence ratio imaging of cyclic AMP in single cells. Nature 349:694–697
2. Antoni FA, Barnard RJO, Shipston MJ, Smith SM, Simpson J, Paterson JM (1995) Calcineurin feedback inhibition of agonist-evoked cAMP formation. J Biol Chem 270:28055–28061
3. Berger HA, Travis SM, Welsh MJ (1993) Regulation of the CFTR by specific protein kinases and protein phosphatases. J Biol Chem 268:2037–2047
4. Borchardt R, Kole J, Cohn J (1996) Phosphorylation of CFTR Ser-737 by PKA (abstract). Pediatr Pulmonol Suppl 13:212
5. Chan KW, Qin J, Chait BT, Gadsby DC, Nairn AC (1997) Dephosphorylation of CFTR by protein phosphatase 2A and 2C (abstract). Pediatr Pulmonol Suppl 14:213
6. Enan E, Matsumuru F (1992) Specific inhibition of calcineurin by type II synthetic pyrethroid insecticides. Biochem Pharmacol 43:1777–1784
7. Fischer H, Machen TE (1994) CFTR displays voltage-dependence and two gating modes during stimulation. J Gen Physiol 104:541–566
8. Fischer H, Machen TE (1996) The tyrosine kinase $p60^{c\text{-}src}$ regulates the fast gate of the CFTR Cl channel. Biophys J 71:3073–3082
9. Gadsby DC, Nairn AN (1994) Regulation of CFTR Cl channel gating. Trends Biochem Sci 19:513–518
10. Galat A (1993) Peptidylproline cis-trans-isomerases: immunophilins. Eur J Biochem 216:689–707
11. Haws C, Finkbeiner WE, Widdicombe JH, Wine JJ (1994) CFTR in human airway cells: channel properties and role in cAMP-activated Cl conductance. Am J Physiol 266:L502–L512
12. Hwang TC, Horie M, Gadsby DC (1993) Functionally distinct phospho-forms underlie incremental activation of protein kinase-regulated Cl^- conductance in mammalian heart. J Gen Physiol 101:629–650
13. Illek B, Fischer H, Machen TE (1996) Alternate stimulation of apical CFTR by genistein in epithelia. Am J Physiol 270: C265–C275
14. Klauk TM, Faux MC, Labudda K, Langeberg LK, Jaken S, Scott JD (1996) Coordination of three signalling enzymes by AKAP79, a mammalian scaffold protein. Science 271:1589–1592
15. Klee CB, Draetta, GF, Hubbard MJ (1988) Calcineurin. Adv Enzymol 61:149–200
16. Kreusel KM, Fromm M, Schultzke JD, Hegel U (1991) Cl secretion in epithelial monolayers of mucus-forming human colon cells (HT-29/B6). Am J Physiol 261:C574–C582
17. Mochly-Rosen D (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 268: 247–251
18. Mumby MC, Walter G (1993) Protein serine/threonine phosphatases: structure, regulation, and function in cell growth. Physiol Rev 73:673–699
19. Picciotto M, Cohn JA, Bertuzzi G, Greengard P, Nairn AC (1992) Phosphorylation of the cystic fibrosis transmembrane conductance regulator. J Biol Chem 267:12742–12752
20. Reddy MM, Quinton PM (1996) Deactivation of CFTR-Cl conductance by endogenous phosphatases in the native sweat duct. Am J Physiol 270:C474–C480
21. Schreiber Sl, Crabtree GR (1992) The mechanism of action of cyclosporin A and FK506. Immunol Today 13:136–142
22. Steward AA, Ingebritsen TS, Manalan A, Klee CB, Cohen P (1982) Discovery of a Ca^{2+}- and calmodulin-dependent protein phosphatase: probable identity with calcineurin (CaM-BP80). FEBS Lett 137:80–84
23. Travis SM, Berger HA, Welsh MJ (1995) Identification of CFTR phosphatases from T84 epithelial cells (abstract). Pediatr Pulmonol Suppl 12:190
24. Travis SM, Berger HA, Welsh MJ (1997) Protein phosphatase 2 C dephosphorylates and inactivates CFTR. Proc Natl Acad Sci USA 94:11055–11060