Differential regulation of single CFTR channels by PP2C, PP2A, and other phosphatases

JIEXIN LUO, MARY D. PATO, JOHN R. RIORDAN, AND JOHN W. HANRAHAN

1Department of Physiology, McGill University, Montreal, Quebec H3G 1Y6; 2Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0; and 3S. C. Johnson Medical Research Center, Mayo Clinic Scottsdale, Scottsdale, Arizona 85259

Luo, Jieixin, Mary D. Pato, John R. Riordan, and John W. Hanrahan. Differential regulation of single CFTR channels by PP2C, PP2A, and other phosphatases. Am. J. Physiol. Cell Physiol. 274 (Cell Physiol. 43): C1397–C1410, 1998.—Cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel activity declines rapidly when excised from transfected Chinese hamster ovary (CHO) or human airway cells because of membrane-associated phosphatase activity. In the present study, we found that CFTR channels usually remained active in patches excised from baby hamster kidney (BHK) cells overexpressing CFTR. Those patches with stable channel activity were used to investigate the regulation of CFTR by exogenous protein phosphatases (PP). Adding PP2A, PP2C, or alkaline phosphatase to excised patches reduced CFTR channel activity by 100% but did not abolish it completely. PP2C caused weak deactivation, whereas PP1 had no detectable effect on open probability (Po). Interestingly, the time course of deactivation by PP2C was identical to that of the spontaneous rundown observed in some patches after excision. PP2C and PP2A had distinct effects on channel gating: Po declined during exposure to exogenous PP2C (and during spontaneous rundown, when it was observed) without any change in mean burst duration. By contrast, deactivation by exogenous PP2A was associated with a dramatic shortening of burst duration similar to that reported previously in patches from cardiac cells during deactivation of CFTR by endogenous phosphatases. Rundown of CFTR-mediated current across intact T84 epithelial cell monolayers was insensitive to toxic levels of the PP2A inhibitor calyculin A. These results demonstrate that exogenous PP2C is a potent regulator of CFTR activity, that its effects on single-channel gating are distinct from those of PP2A but similar to those of endogenous phosphatases in CHO, BHK, and T84 epithelial cells, and that multiple protein phosphatases may be required for complete deactivation of CFTR channels.

Cystic fibrosis; protein phosphatase; channel rundown; cystic fibrosis transmembrane conductance regulator

REGULATION of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel by protein kinases has been extensively studied during the past several years (4, 7, 12, 13, 16, 17, 35). Phosphorylation by protein kinase A (PKA) increases open probability (Po) (35), bursting rate (20, 23), apparent ATP affinity (20, 23), and ATP hydrolysis rate (20). Phosphorylation by protein kinase C (PKC) is also required for CFTR to respond to PKA (17). These actions of kinases are antagonized by protein phosphatases, which probably vary among different cell types. CFTR channel activity declines rapidly when patches are excised from stimulated Chinese hamster ovary (CHO) or airway epithelial cells into bath solution lacking PKA [−10 s at 37°C (35); 100 s at 22°C (2)]. This spontaneous decline in channel activity, or rundown, is slower or absent when channels are studied in patches from guinea pig myocytes (15), transfected fibroblasts (4), or Hi-5 insect cells (39). Characterization and molecular identification of the phosphatases regulating CFTR has become a priority because they are potential therapeutic targets in the treatment of cystic fibrosis (2).

Serine and threonine phosphatases are functionally classified into two types, protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2). The latter is subclassified into protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B), and protein phosphatase 2C (PP2C) (see Refs. 24, 34). PP1 preferentially dephosphorylates the β-subunit of phosphorylase kinase and is sensitive to inhibitors 1 and 2, whereas PP2 preferentially acts on the α-subunit of phosphorylase kinase and is sensitive to these inhibitors. PP2A activity does not require particular ions or cofactors, in contrast to PP2B, which requires Ca2+ and calmodulin, and PP2C, which requires relatively high levels of Mg2+ (EC50, ~1.5 mM; Ref. 8). PP1 and PP2A are both sensitive to okadaic acid and calyculin A but can be distinguished by using appropriate concentrations of these inhibitors (24, 34). PP2B can be identified by its sensitivity to inhibitors such as deltamethrin, cyclosporin, or FK-506 (10, 34). No specific inhibitors of PP2C are available. Rundown and CFTR dephosphorylation are both inhibited by phenylimidazoazahides (2, 3), but at much higher concentrations than are needed to inhibit alkaline phosphatase.

CFTR channel rundown in excised patches is relatively insensitive to okadaic acid (2, 35), suggesting regulation by a robust membrane-associated protein phosphatase other than PP1 and PP2A. These results do not exclude regulation of CFTR by PP2A on the cell, however, since any cytosolic PP2A would be lost from patches after excision. Indeed, Reddy and Quinton (29) showed that okadaic acid (10−8 M) inhibits deactivation of CFTR currents in permeabilized sweat ducts. In guinea pig cardiac myocytes, ~40% of the deactivation after forskolin washout was blockable by okadaic acid or microcystin (15). Exogenous PP1 and PP2B are not effective in regulating CFTR currents in excised patches from fibroblasts (4), although there is evidence that CFTR activity can be stimulated in these cells by the PP2B inhibitors cyclosporin A or deltamethrin, suggesting that PP2B can regulate CFTR (11). By default, PP2C has been proposed as a CFTR phosphatase because it is insensitive to okadaic acid and microcystin (15, 35). PP2C dephosphorylates CFTR and deactivates macroscopic CFTR Cl− currents (38). Deactivation by
PP2C and other phosphatases has not been studied at the single-channel level.

The goal of this study was to characterize the effects of PP1, PP2A, PP2B, and PP2C on single CFTR channels exposed to comparable levels of phosphatase activity. For comparison, the kinetics of CFTR channels were also examined during deactivation by endogenous phosphatases. Finally, we investigated the possible role of PP2A in intact T84 epithelial monolayers by examining the ability of calyculin A to inhibit deactivation of short-circuit current (Isc) after washout of forskolin. The results suggest that CFTR deactivation is mediated primarily by a PP2C-like phosphatase in CHO, baby hamster kidney (BHK), and T84 epithelial cells, although PP2A and PP2B both cause partial deactivation in vitro. These results have been reported in preliminary form (21, 22).

MATERIALS AND METHODS

Cell culture. BHK cells stably expressing wild-type CFTR were plated at low density on glass coverslips 3–5 days before use in patch-clamp experiments. The T84 line was obtained from American Type Culture Collection (Rockville, MD) and studied between passages 77 and 115. T84 cells were plated at a density of 400,000/cm² on porous supports (Millipore, Toronto, ON, Canada), which had been coated with a gel of type I collagen. The growth medium was a 50:50 mixture of DMEM and Ham’s F-12 medium and was supplemented with 15 mM HEPES, fetal bovine serum (5%), penicillin (100 IU/ml), and streptomycin (100 µg/ml). Monolayers were studied 8–12 days after plating, when transepithelial resistance had reached ~1,500 Ω cm⁻².

Phosphatases. Recombinant human PP1γ catalytic subunit was purchased from Calbiochem (La Jolla, CA). PP2AI (smooth muscle phosphatase I) and PP2C (smooth muscle phosphatase II) were prepared from turkey gizzard smooth muscle as described previously (26, 27). PP2A was further purified by sequential chromatography on DEAE-Sephacel, ω-aminooctyl-Sephrose, and an affinity column of thiophosphorylated 20,000 Mᵦ myosin light chains coupled to Sepharose 4B. The PP2C fraction from a Sephacryl S-300 column was chromatographed on DEAE-Sephascel and on the affinity column mentioned above. PP2C bound to the column in the presence of Mg²⁺ and was selectively eluted using EDTA. Bovine brain PP2B was purchased from Boehringer Mannheim (Laval, QC, Canada). Purity of the protein phosphatase preparations was assessed by SDS-PAGE (19) in a 12.5% Microslab gel and then stained with Coomassie blue. For comparison with the four protein phosphatases, some patch-clamp experiments were also carried out using bovine intestinal alkaline phosphatase type VII-S (Sigma, St. Louis, MO). The activity of this enzyme was 2,000–3,000 U/mg enzyme [where 1 unit hydrolyzes 1.0 µmol of p-nitrophenyl phosphate (PNP)/min at 37°C]. Alkaline phosphatase was used at a final concentration of 80 U/ml.

Enzyme activities were determined by measuring release of [32P]orthophosphate from phosphorylated myosin light chains as described previously (28). Assays were carried out under four conditions: 1) high-salt solution used during patch-clamp experiments, 2) low-salt solution (same as 1 but lacking 150 mM NaCl), 3) standard Tris-dithiothreitol (DTT) solution, and 4) Tris only solution (same as 3 but lacking DTT). These and other solutions are listed in Table 1. The substrate used in all phosphatase assays was prepared as described previously (33) and had a specific activity of 3,736 cpm/pmol. After adding phosphatase to the reaction mixture, aliquots were taken at timed intervals, and the reaction was terminated by addition to 100 µl of 17.5% TCA and 100 µl of 6 mg/ml BSA. The resulting solutions were chilled and centrifuged at 15,000 rpm for 1 min. Aliquots of the supernatant (200 µl) were counted by liquid scintillation (Beckman LS 7800) to determine the amount of 32P released.

Patch-clamp studies. BHK cells were placed in a recording chamber (200 µl vol), containing (in mM) 150 NaCl, 2 MgCl₂, and 10 TES (pH 7.4). In most experiments, this solution also contained 0.5 mM MgATP and 100 nM PKA catalytic subunit (prepared in laboratory of Dr. M. P. Walsh, University of Calgary). Patch-clamp experiments were carried out at room temperature (22°C). Pipettes were pulled in two stages (PP-83, Narishige Instrumentation Laboratory, Tokyo, Japan) and had resistances of 4–6 MΩ when filled with 150 mM NaCl solution. The bath was grounded through an agar bridge having the same ionic composition as the pipette solution. Single-channel currents were recorded from both cell-attached and excised patches; the pipette potential was held at +30 mV. Single-channel currents were amplified (Axopatch 1B, Axon Instrument, Foster City, CA), recorded on videocassette tape by a pulse-coded modulation-type recording adapter (DR384, Neurodata Instrument, New York), and low-pass filtered during playback using an eight-pole Bessel filter (900 LPF, Frequency Devices, Haverhill, MA). Final records were sampled at 0.5 kHz and analyzed using a laboratory microcomputer system and DRSCAN, a PC-LAMP-compatible program developed in this laboratory for analyzing long records.

Table 1. Solutions used in patch-clamp experiments and phosphatase assays

<table>
<thead>
<tr>
<th>Soln:</th>
<th>High Salt</th>
<th>Low Salt</th>
<th>Tris-DTT</th>
<th>Tris Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP:</td>
<td>1, 2A</td>
<td>2B</td>
<td>2C</td>
<td>1, 2A</td>
</tr>
<tr>
<td>Na⁺</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>154</td>
<td>158</td>
<td>162</td>
<td>4</td>
</tr>
<tr>
<td>Tris</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TES</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>DTT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

When used for protein phosphatase (PP) assays, solutions (Soln) also contained 1 mM phosphorylated myosin light chains (³²P labeled, 3,736 cpm/pmol). DTT, dithiothreitol. All solutions were pH 7.4. Concentrations are mM except calmodulin, for which units are µg/ml.
Channel cycle time was used to calculate the mean burst duration \( t_o \) as

\[
t_o = \left[ \frac{(N P_o \cdot T)}{b} \right]
\]

where \( N \) is the number of channels, \( P_o \) is the probability of each channel being in the open state (i.e., above a threshold set at half open-channel amplitude), \( T \) is the duration of the segment analyzed, and \( b \) is the number of bursts observed during the segment (14). This method of calculating \( t_o \) is useful because it does not require determination of \( N \), relying instead on \( NP_o \) which can be measured regardless of the number of channels as

\[
NP_o = \sum_{i=1}^{n} t_i / T
\]

where \( t_i \) is the time spent above a threshold “i” set at 0.5, 1.5, 2.5, . . . times the single-channel current amplitude. To evaluate changes in channel gating, \( t_o \) was calculated for each 5-s segment in the record. Because each of the \( \sim 200 \ t_o \) values is essentially a sample estimate \( \bar{X} \) of the mean \( (\mu) \) and SD \( (\sigma) \), the sampling distribution of \( t_o \) has the form \( Z = (\bar{X} - \mu)/\sigma \sqrt{n} \), which is closely approximated by a Gaussian distribution when \( n > 30 \) observations.

Calculation of \( P_o \) and mean interburst duration do require estimates of \( N \). \( P_o \) was determined by dividing \( NP_o \) by \( N \), estimated as the largest number of channels open simultaneously during long recordings when the channels were stimulated (i.e., when \( P_o \) was high, before addition of phosphatase). This method tends to underestimate \( N \) and therefore overestimate \( P_o \); however, the error is small for wild-type CFTR channels (<10%) (23). If the method for calculating \( t_o \) were sensitive to \( N \), any error should be similar for all phosphatases and therefore would not explain their different effects on burst duration. Finally, data were obtained from the same patches before and after adding phosphatases; therefore correction for any underestimate of \( N \) would, if anything, tend to increase differences between phosphatase effects. We therefore corrected for any underestimate of \( P_o \) by \( t_o \) (Fig. 1) before addition of phosphatase. We did not attempt to calculate the mean interburst duration, \( \tau_o = [(N - N \cdot P_o) \cdot T]/(n - 1) \). Interburst duration is an absolute measurement that requires an accurate determination of \( N \). This could not be obtained by adding AMP-PNP at the end of each recording (23), since channels were not locked open after phosphatase exposure.

Transepithelial experiments. T84 cell monolayers were studied in water-jacketed Ussing-type chambers (Vanguard International, Neptune, NJ) that had been fitted with Teflon adaptors for holding cell culture inserts. The control solution contained (mM) 115 NaCl, 2.5 K2HPO4, 1.5 CaCl2, 1 MgSO4, 10 glucose, and 25 NaHCO3 and was gassed with 95% O2-5% CO2 at 37°C. Forskolin (10 μM) inhibited 50% of the PP2A activity but had no effect on PP2C or vice versa (data not shown). This was confirmed by functional assays; okadaic acid (0.01 nM) inhibited 50% of the PP2A activity but had no effect on PP2C preparation. Representative activity curves of PP2C using phosphorylated myosin light chains as substrate under different conditions are shown in Fig. 2. Orthophosphate release was linear during the first 75 s under all conditions and during the first 2 min under the high-salt conditions used in patch-clamp experiments. Phosphatase activities calculated from the initial linear release rates are summarized in Table 1. Orthophosphate release was linear during the first 75 s under all conditions and during the first 2 min under the high-salt conditions used in patch-clamp experiments. Phosphatase activities calculated from the initial linear release rates are summarized in Table 1. Under the high-salt conditions used in patch-clamp experiments, phosphatase activities were inhibited by 80-90% (Table 1).

RESULTS

Characterization of protein phosphatase preparations. Figure 1 shows an SDS-polyacrylamide gel of the four protein phosphatase preparations used in patch-clamp experiments, stained with Coomassie blue. The PP1 and PP2B preparations did not contain detectable contamination. The PP2A had 8% contaminant with M, of ~90,000, whereas the PP2C profile shows two impurities (M, ~30,000), constituting <3% of the protein. Western blot analysis with specific antibodies revealed that the PP2A preparation did not contain detectable PP2C or vice versa (data not shown). This was confirmed with functional assays; okadaic acid (0.01 nM) inhibited 50% of the PP2A activity but had no effect on the PP2C preparation. Representative activity curves of PP2C using phosphorylated myosin light chains as substrate under different conditions are shown in Fig. 2. Orthophosphate release was linear during the first 75 s under all conditions and during the first 2 min under the high-salt conditions used in patch-clamp experiments. Phosphatase activities calculated from the initial linear release rates are summarized in Table 1. Under the high-salt conditions used in patch-clamp experiments, phosphatase activities were inhibited by 80-90% (Table 1). Orthophosphate release was linear during the first 75 s under all conditions and during the first 2 min under the high-salt conditions used in patch-clamp experiments. Phosphatase activities calculated from the initial linear release rates are summarized in Table 1. Under the high-salt conditions used in patch-clamp experiments, phosphatase activities were inhibited by 80-90% (Table 1). Under the high-salt conditions used in patch-clamp experiments, phosphatase activities were inhibited by 80-90% (Table 1).
Purification of detergent extracts and composition of solutions.

To assess alterations in single-channel activity, we tested the effects of exogenous phosphatases (solutions 1–3, Table 1). PP1 (final activity 1.6 pmol/min in 1 ml bath buffer) did not affect $P_0$ ($P > 0.38$), consistent with a previous study (4). By contrast, addition of PP2A (0.78 pmol/min) under the same conditions caused a slow decline in $P_0$ from 0.46 to 0.12 within 6.2 ± 0.6 min ($P < 0.005$; Fig. 6A). PP2B (0.4–0.8 pmol/min) caused some decrease in $P_0$, but this just reached statistical significance ($P = 0.04$) after a 10-min exposure in solution 2 containing Ca$^{2+}$ and calmodulin. PP2C was the most potent phosphatase tested, causing $P_0$ to decline rapidly from 0.48 to 0.11 within 3.4 ± 0.2 min (final activity, 0.16–0.32 pmol phosphate transferred/min; $P < 0.0004$; Fig. 6B). Alkaline phosphatase at much higher levels (80 µmol phosphate transferred/min when assayed using PNP as substrate) caused slow deactivation ($P < 0.002$; Fig. 6C).

Two channel behaviors suggest that phosphatase activity is unevenly distributed. CFTR channel activity was usually observed on BHK cells after incubation with 20 µM forskolin for 5–10 min. Channel activity usually persisted for >5 min after patches were excised into bath solution containing 0.5–1 mM MgATP (Fig. 3, A–C); however, activity did decline rapidly in about one-fourth of the excised patches (Fig. 3, D–F). These results contrast with our previous observations with CHO and epithelial cells, in which CFTR channel activity always declined rapidly (2, 35). We tentatively attribute the variable rundown to an uneven distribution of phosphatase activity in the plasma membrane of BHK cells (perhaps exacerbated by a very high level of CFTR expression), since other factors such as time after plating and recording conditions were kept constant. Channel activity declined to 5–10% of the starting value within 100 s in those patches that exhibited rundown and could be restimulated by adding PKA as in previous studies of CHO, T84, and airway epithelial cells (2, 35, 36).

Burst duration during spontaneous rundown in excised patches. To assess alterations in single-channel kinetics during rundown, we calculated that $t_\infty$ values during each 5-s interval after $P_0$ had declined by at least 90% and compared them with values obtained from patches having stable channel activity (Fig. 3, C and F). The distributions of open burst durations in both groups of patches could be fitted with Gaussian functions having similar means (n = 7 patches with rundown, n = 5 patches without rundown; $P = 0.24$; see Fig. 4). Thus, when membrane-associated phosphatase activity was present in a particular patch, it reduced CFTR channel activity by >90% without significantly altering $t_\infty$.

Effect of calyculin A on CFTR channels from BHK cells. Channel activity declined slowly in 20 of 84 patches excised in the presence of low (50–100 nM) PKA activity and 0.5 mM MgATP. The contribution of membrane-associated PP1/PP2A was assessed in some of the patches that displayed rundown by addition of the potent PP2A inhibitor calyculin A while rundown was in progress. Calyculin A (100–1,000 nM) did not block further rundown in 10 of 12 patches, nor did it enable the low PKA activity present in the bath solution to restimulate channels in any of the patches (n = 10). Figure 5 shows a representative experiment in which CFTR channel activity was high in the cell-attached configuration during forskolin stimulation (indicated by letter a) and declined spontaneously when excised into bath solution containing 100 nM PKA (compare with b and c). Rundown continued after the addition of 0.1 and 1.0 µM calyculin A (d and e, respectively) but was reversed by raising the PKA concentration from 0.1 to 0.3 µM (f). These results confirm that a fraction of membrane patches from BHK cells contain robust phosphatase activity resembling that in CHO cells (35). This predominant phosphatase is insensitive to calyculin A and independent of Ca$^{2+}$ and calmodulin and therefore unlikely to be PP1, PP2A, or PP2B. However, because calyculin A did slow rundown in 3 of 10 patches, excised membrane patches from BHK cells can apparently contain some PP1 or PP2A activity that mediates a small fraction of the deactivation.

Effect of phosphatases on NP$\alpha$. Patches that had stable CFTR activity after 5-min exposure to low PKA (50–100 nM) were used to test the effects of exogenous phosphatases (solutions 1–3, Table 1). PP1 (final activity 1.6 pmol/min in 1 ml of bath buffer) did not affect $P_0$ ($P < 0.005$; Fig. 6A). PP2B (0.4–0.8 pmol/min) caused some decrease in $P_0$, but this just reached statistical significance ($P = 0.04$) after a 10-min exposure in solution 2 containing Ca$^{2+}$ and calmodulin. PP2C was the most potent phosphatase tested, causing $P_0$ to decline rapidly from 0.48 to 0.11 within 3.4 ± 0.2 min (final activity, 0.16–0.32 pmol phosphate transferred/min; $P < 0.0004$; Fig. 6B). Alkaline phosphatase at much higher levels (80 µmol phosphate transferred/min when assayed using PNP as substrate) caused slow deactivation ($P < 0.002$; Fig.
The time of excision (for those patches displaying spontaneous rundown; Fig. 7B) and phosphatase addition (for those patches with stable channel activity after excision) are shown aligned in Fig. 7, so that time courses of deactivation by endogenous and exogenous phosphatases can be compared. Maximal deactivation by PP2C was reached within 3.4 ± 0.8 min and was well fitted by a single exponential function having $\tau =$
0.69 min (Fig. 7F). This decline was similar to that observed during spontaneous rundown after excision (τ = 0.66 min, Fig. 7B) and was faster than the deactivation induced by PP2A (τ = 5.03 min, Fig. 7D). By contrast, the decline induced by alkaline phosphatase began after a considerable delay (~6 min) despite higher unit activity.

Effect of PP2A and PP2C on burst duration. To study phosphatase effects on channel gating, we used patches from forskolin-stimulated BHK cells having stable channel activity after excision. Phosphatases were added after recording channel activity for several minutes (Fig. 8). Representative control data under these conditions are shown in Fig. 3A, and the mean control values are given in Fig. 4A. Forskolin stimulation before excision was the only stimulus used to activate CFTR in these experiments, since exposure of excised patches to both phosphatase and PKA could complicate interpretation of gating effects. Both PP2A (Fig. 8A) and PP2C (Fig. 8B) caused deactivation of CFTR channels under these conditions, although the effect of PP2A was slower, in qualitative agreement with the results obtained when low PKA activity was also present (compare Fig. 7).

To examine rundown more precisely, the response to PP2A and PP2C was divided into two phases: phase i) a rapidly declining phase immediately after addition of PP2A or PP2C and phase ii) a stable phase, which was achieved after channel activity had declined by >90% (see Fig. 8, A and B). τ_i was analyzed in 5-s segments during each phase. Deactivation by PP2A was accompanied by a striking decline in burst duration between phases i and ii (Fig. 8A; compare τ_i in phases i and ii). No burst lasting >0.8 s was observed during 30 min of recording with PP2A present (3 patches, 10 min each). By contrast, PP2C induced rapid deactivation without altering burst duration significantly (n = 3; Fig. 8B). The distribution of τ_i in the presence of PP2C (Fig. 9B) yielded a τ_i during phase ii that closely resembled the one observed during spontaneous rundown (see Fig. 4B). The overall τ_i was shorter during exposure to PP2A, reflecting the loss of a population of long bursts (Fig. 9A). By contrast, long bursts were frequently observed during phase ii when channels were exposed to PP2C. The τ_i values in the presence of PP2A and PP2C are summarized in Fig. 10.

The above results indicate that both exogenous PP2A and PP2C can deactivate CFTR channels, but the rates of deactivation and effects on single-channel kinetics are different. PP2A causes slow deactivation and shortening of bursts, whereas PP2C causes rapid deactivation with no change in burst duration. The rate of decline in channel activity during exposure to PP2C closely matches that during spontaneous rundown, further suggesting that PP2C is primarily responsible for rundown in patches excised from BHK cells.

Effect of Mg²⁺ on CFTR rundown in excised patches from CHO cells. Unlike other protein phosphatases, PP2C activity strongly depends on free Mg²⁺ concentration ([Mg²⁺]). We examined the effect of lowering total [Mg²⁺] from 2 to 0.5 mM on the rundown of CFTR channels in patches excised from CHO cells (Fig. 11).
This represents a decrease in free [Mg\(^{2+}\)] from 1.45 to 0.36 mM, which is expected to reduce PP2C activity by 60–70% according to previous biochemical studies (8). CHO cells were used for these experiments because all patches excised from CHO cells displayed rapid rundown under control conditions. Channel activity was increased approximately fourfold during the first 5 min of exposure to 0 mM Mg\(^{2+}\), consistent with the Mg\(^{2+}\)-dependent phosphatase activity.

Effect of calyculin A on CFTR rundown in T84 cell monolayers. If soluble phosphatases are lost from excised patches, studying rundown might underestimate the importance of cytosolic PP2A in intact cells. We therefore examined the effect of calyculin A on \(I_{sc}\) across intact (unpermeabilized and undialyzed) T84 monolayers. Previous studies have shown that \(I_{sc}\) in this preparation provides a measure of net Cl\(^-\) secretion under various conditions (9) and that Cl\(^-\) secretion is mediated by CFTR channels (6, 37). Calyculin A (20 nM–100 nM) had no effect on \(I_{sc}\) when it was added to both sides (Fig. 12). Subsequent addition of 10 \(\mu\)M forskolin to the serosal side increased \(I_{sc}\) from 3 to 43 \(\mu\)A/cm\(^2\) within ~8 min. The rate and magnitude of the forskolin stimulation were not affected by calyculin A (Fig. 12). \(I_{sc}\) declined exponentially back to the baseline level when forskolin was washed out. The rate of this decline was also unaffected by 20 nM calyculin A (Fig. 12A); \(I_{sc}\) after forskolin washout was well fitted by a single exponential having the same time constant in the absence (\(\tau = 3.6 \pm 0.2\) min) or presence (\(\tau = 3.5 \pm 0.1\) min) of calyculin A. Complete deactivation of \(I_{sc}\) was observed after forskolin removal despite the presence of 20 nM calyculin A. Similar results were obtained using T84 monolayers at three different passages.

When forskolin was removed and 100 nM calyculin A was added simultaneously, \(I_{sc}\) increased slightly (8%) and then declined rapidly to the same low level as in control monolayers. Calyculin A (100 nM) had no effect on the rate or extent of deactivation at concentrations that were 10- to 500-fold higher than the \(IC_{50}\) for PP2A.
Activation by forskolin was inhibited somewhat at 1 µM (data not shown), presumably a nonspecific effect of high calyculin A. Nevertheless, even at this toxic concentration of calyculin A, the decline in membrane-associated phosphatase (G; n = 4) was added to bath. PP1 did not cause induced deactivation under these conditions. PP2A, PP2C, and alkaline phosphatase deactivated CFTR channels with different time courses. PP2B caused some deactivation that reached statistical significance after 10 min. For comparison with spontaneous rundown occurring in ~25% patches, excisions of patches with rundown are aligned in B with time at which exogenous phosphatases were added in other panels to patches having stable P_o. Fast decline after PP2A (time constant t = 5.03 min) or PP2C (t = 0.69 min) was fitted by single exponential functions (dotted lines in D and F, respectively). Spontaneous decline in P_o in patches with rundown was very similar to that induced by addition of exogenous PP2C (t = 0.69 min).

DISCUSSION

The goal of this study was to compare the ability of PP2C and other protein phosphatases to downregulate CFTR and assess their relative importance in deactivating CFTR channels in the epithelial cell line T84. By adding exogenous phosphatases to BHK patches having stable channel activity, we found that PP2A and PP2C both deactivated CFTR channels which had been activated in vivo (by forskolin) or in vitro (by PKA catalytic subunit). The protein phosphatase preparations were assayed biochemically with a common substrate and patch-clamp solution, so that comparable levels of PP activity could be used when recording channel activity.

Evidence that PP2C is the primary phosphatase regulating CFTR in BHK and T84 cells. Several results in this study suggest that endogenous PP2C regulates CFTR in the cells studied: 1) deactivation induced by exogenous PP2C occurred at the same rate as that mediated by endogenous, membrane-associated phosphatase; 2) burst duration did not change during deactivation by exogenous PP2C or during spontaneous rundown after excision, whereas exogenous PP2A caused burst shortening; 3) rundown in excised patches and deactivation after forskolin washout from cell monolayers were both insensitive to calyculin A; and 4) spontaneous rundown was Mg2+-dependent within the millimolar range, consistent with the known properties of PP2C. Recent studies (38, 40) indicate that PP2C is expressed in most tissues, including T84 cells. Our
conclusions regarding the role PP2C in regulating epithelial CFTR are in agreement with recent results of Travis et al. (38), who found that okadaic acid did not inhibit deactivation of Cl⁻ current across permeabilized human airway and T84 cell monolayers. They also found that macroscopic (CFTR-mediated) current in excised patches was reduced by ~80% after exposure to recombinant PP2Cα. PP2A may play a larger role in regulating CFTR in cardiac (15) and sweat duct epithelial cells (29). Distinct effects of PP2A and PP2C on the gating of single CFTR channels have not been described previously.

Properties of protein phosphatase preparations. Analysis of the phosphatases by SDS-PAGE indicated that the PP2A and PP2C preparations contained some impurities (8 and <3%, respectively). However, Western blot analysis with specific antibodies did not reveal any cross-contamination, i.e., no PP2A in the PP2C preparation or PP2C contamination in the PP2A preparation. Moreover, okadaic acid did not affect phosphatase activity of the PP2C preparation but abolished that of the PP2A preparation, and the PP2C preparation exhibited no activity in the absence of Mg²⁺. We conclude that the PP2A and PP2C preparations used were functionally homogeneous. PP2A from a commercial supplier had ~1,000-fold lower activity than the PP2A preparation used here when it was assayed under the same conditions. Commercially prepared PP2B was more satisfactory, having low phosphatase activity in the absence of Ca²⁺ and calmodulin that was stimulated 30- to 40-fold by addition of these cofactors.

Dephosphorylation of myosin light chains by PP1 and PP2C was linear during the first 1–2 min under four assay conditions (high salt, low salt, Tris-DTT, and Tris only). Protein phosphatase activities were determined as picomoles of phosphate released from phos-
phorylated myosin light chains per minute per microliter at 30°C (Table 2). All four protein phosphatases were partially inhibited by the standard high-salt (150 mM NaCl) solution used during patch-clamp experiments. This inhibition was greatest for PP1 (72%) and least for PP2C (20%). Although freeze-thaw cycles were avoided with routine handling of the phosphatases, in control experiments, PP1 activity declined fourfold within 3 h even when kept on ice.

Phosphatases were assayed in patch-clamp solutions and using the same substrate, so that comparable phosphatase activities could be tested in patch-clamp experiments. However, activities could only be approximately matched, since they depend somewhat on the particular substrates used in the assays. We used myosin light chains as the substrate for this standardization rather than phospho-CFTR because of the difficulty in preparing sufficient quantities of purified, full-length CFTR. When another phosphatase substrate, phosphorylase kinase, is used instead of myosin light chains, PP2A is about twofold more effective than PP2C. Thus more PP2A would have been indicated in this study if the phosphatase activities had been matched using phosphorylase kinase. However, during patch-clamp experiments, we tested PP2A activities that were 2.4- to 4.8-fold higher than those of PP2C and we still observed slower deactivation; therefore, PP2C is indeed more efficacious. This issue of substrate specificity is of less concern when PP2C is compared

![Diagram](image-url)
with PP1 and PP2B, which are five- and threefold less potent in dephosphorylating myosin light chains, respectively, since compensating for the use of myosin light chains in assays would only strengthen our conclusion that PP1 and PP2B are less effective in deactivating CFTR.

Effect of PP2A on burst duration. Exogenous PP2A caused a remarkable shortening of burst duration when added to BHK patches. Burst shortening did not occur during the spontaneous deactivation induced by endogenous phosphatase activity or after addition of exogenous PP2C, but has been reported previously for cardiac CFTR where it is a major mechanism by which $P_o$ is downregulated (16). The different results would be reconciled if cardiac cells had more membrane-associated PP2A activity than the cells used here. Higher PP2A activity would explain both burst shortening and the more pronounced inhibition of rundown in cardiac cells by okadaic acid (15).

In contrast to the present results, deactivation of macroscopic CFTR conductance in isolated, permeabilized sweat ducts is prevented by okadaic acid (29). This could reflect higher PP2A and/or lower PP2C expression in sweat duct epithelium, or it may be due to the experimental conditions used. Sweat ducts were treated with $a$-toxin to permeabilize the basolateral membrane to small solutes such as ATP, cAMP, and phosphatase inhibitors. The free $[\text{Mg}^{2+}]$ of the bath solution (exposed to permeabilized membrane) was strongly buffered to ~0.1 mM by high concentrations of ATP, gluconate, and EGTA. Most PP2C would have been inactive if intracellular $[\text{Mg}^{2+}]$ approached this same low level, which may explain the absence of an okadaic acid-insensitive component. In a whole cell patch-clamp study of cardiac cells, in which >50% of the deactivation was okadaic acid-insensitive, Hwang et al. (15) used pipette solution containing 0.92 mM $[\text{Mg}^{2+}]$. In our studies of excised
patches, okadaic acid-insensitive rundown was routinely observed with solutions containing 1–3 mM [Mg²⁺]. Thus the relative contribution of PP2C reported for different preparations correlates with [Mg²⁺], although other explanations for the differences are also possible. Cytoplasmic free [Mg²⁺] has not been measured in the cells used here, but recent estimates for the bulk cytoplasm of other cells are in the 0.5–1.1 mM range (e.g., for review, see Ref. 32).

Calyculin A did not affect spontaneous rundown of CFTR channel activity when added to most patches from BHK cells, although average $P_0$ was higher in 3 of 10 BHK patches continuously exposed to calyculin A, suggesting that some patches have membrane-associated PP2A (or PP1) activity. More significantly, in intact T84 epithelial cells, which presumably have physiological [Mg²⁺] and normal phosphatase activities, calyculin A did not inhibit deactivation of CFTR after forskolin was removed. This extends previous studies of CFTR deactivation in this cell line and suggests that CFTR channels are downregulated primarily by a calyculin A-insensitive phosphatase in T84 cells (see also Ref. 38).

Other phosphatases: PP1, PP2B, and alkaline phosphatase. PP1 is a ubiquitous protein phosphatase that is known to regulate other ion channels. Exogenous PP1 (1.6–3.2 U/ml) failed to deactivate CFTR Cl⁻ channels when added to patches from BHK cells. This result agrees with that of Berger et al. (4), who found that PP1 (5 U/ml) did not deactivate CFTR when patches were excised from NIH/3T3 cells and is also consistent with the insensitivity of $I_{sc}$ deactivation in T84 monolayers exposed to calyculin A (present study) and okadaic acid (38). Insensitivity to calyculin A in T84 cells excludes a role for other isoforms of PP1 (PP1β or PP1γ), since they would also have been inhibited.

Exogenous PP2B did not fully deactivate the channel even under optimal conditions of Ca²⁺ and calmodulin, although there was a small decrease in $P_0$ that reached statistical significance after a 10-min exposure. The normal rundown of CFTR channels in nominally calmodulin- and Ca²⁺-free solution (35) suggests that PP2B plays a little role in deactivating CFTR. Although we found only weak effects of PP2B in the present study, the data are at least compatible with some regulation of CFTR by PP2B, particularly when it is considered that regulation might not be fully recapitulated in excised patches (e.g., if regulation is indirect or requires a cytosolic factor or ancillary protein in addition to Ca²⁺ and calmodulin). Macroscopic CFTR currents in patches from NIH/3T3 cells were completely insensitive to PP2B (4), although the PP2B inhibitors cyclosporin and deltamethrin can stimulate CFTR channel activity in cell-attached patches on the same cells (11). Further studies to determine PP2B expression in NIH/3T3 cells and the specificity of PP2B inhibitors under cell-attached recording conditions may resolve those apparently contradictory findings.

Exogenous alkaline phosphatase deactivated CFTR channels after some delay, consistent with previous studies from this laboratory (2, 35; but see Ref. 4). Becq et al. (1) showed that a polyclonal antibody which recognizes the regulatory region of alkaline phosphatase inhibits rundown, as do phenylimidazothiazoles such as bromotetramisole (2), which are potent alkaline phosphatase inhibitors. Bromotetramisole activated wild-type and mutant CFTR channels in cell-attached patches and inhibited rundown, but the concentration required was several orders of magnitude higher than needed to inhibit alkaline phosphatase in biochemical assays. Both alkaline phosphatase and PP2C are Mg²⁺-dependent enzymes, and it is possible that CFTR is downregulated by a protein phosphatase which is distinct from alkaline phosphatases but which shares some pharmacological and structural similarities. The effect of bromotetramisole on PP2C activity has not yet been reported.

PP2A and PP2C act at functionally distinct phosphorylation sites. The results in this study indicate that CFTR deactivation in several cell lines, including T84 epithelial monolayers, is mediated primarily by an okadaic acid- and calyculin A-insensitive phosphatase (35). These properties and the Mg²⁺ dependence of
rundown point to PP2C; however, a specific inhibitor of PP2C is not presently available. About 10% of the PP2Ca in human HL-60 cells is membrane associated (25). Approximately 33% of PP2Ca activity in BHK, CHO, T84, and Calu-3 cells is in the particulate (membrane) fraction (40). A membrane-bound form of PP2C has also been reported in Paramecium (18). Although PP2A appears to mediate little, if any, deactivation in these cells under normal conditions (i.e., with physiological levels of Mg2+), neither PP2C nor PP2A was able to completely deactivate CFTR channels in the present study. Between 5 and 10% of the initial channel activity persisted in the presence of each exogenous phosphatase. These results suggest that multiple phosphatases may be required for complete deactivation, analogous to previous studies (15). Alternatively, PP2C may need to be associated with CFTR in the membrane to be fully effective. The qualitatively different effects of PP2A and PP2C on gating imply that they dephosphorylate functionally distinct sites on CFTR. Deactivation by PP2A was accompanied by a reduction in \( \xi_v \). By contrast, deactivation by PP2C was not associated with burst shortening, although it caused a similar decline in \( \xi_b \). The inability of PP2C to alter burst duration was not due to weaker phosphatase activity, since PP2C caused more rapid deactivation than did PP2A, and raising the concentration of PP2C by fourfold still did not result in shorter bursts (Luo, unpublished observation). As discussed above, the PP2A and PP2C preparations used in patch-clamp experiments had comparable phosphatase activities when assayed for their ability to dephosphorylate myosin light chains. These results suggest that burst and interburst durations are regulated independently, presumably by distinct phosphorylation sites.

In this paper, we have focused on the acute regulation of CFTR activity by dephosphorylation of PKA sites. This regulation is relatively rapid (usually complete within \( \sim 3 \text{ min} \)) and corresponds to the rapid rundown that is observed immediately after excision and can be fully reversed by PKA (35). It has recently been shown that constitutive PKC phosphorylation is also required for CFTR to respond to PKA (17). We found that, once channels were deactivated (due to rapid dephosphorylation of PKA sites), their ability to respond to exogenous PKA gradually declined unless PKC was also present in the bath. Moreover, once CFTR channels became refractory to PKA stimulation (attained after \( \sim 10 \text{ min without PKC} \)), their responsiveness could be fully restored by PKC exposure. The present experiments were not designed to characterize dephosphorylation of the permissive PKC sites. If membrane-associated PP2C also mediates regulation of those sites, the relatively slow decline in responsiveness to PKA implies that PKC sites are less efficiently dephosphorylated.

It has been proposed that CFTR possesses modulation (\( P_2 \)) sites, which control \( P_o \) and are dephosphorylated by an okadaic acid-insensitive phosphatase, and activation (\( P_j \)) sites, which convert the channel from an inactive to an active state (15). The effects of exogenous phosphatases in the present study support the notion of regulation by particular sites but imply a somewhat different scheme. Our data suggest that one PKA site or group of sites, which we call PKA\(_1\) site(s), controls interburst duration (i.e., opening rate) and is susceptible to both PP2A and PP2C. Another PKA site or group of sites, which we refer to as PKA\(_2\) site(s), controls burst duration (closing rate) and is susceptible to PP2A but not PP2C. The PP2C-sensitive sites mediating rapid rundown of \( P_o \) and the PP2A-sensitive sites mediating rundown and burst shortening should be identified and their relationship to \( P_1 \) and \( P_2 \) sites established. The differential effects of PP2A and PP2C on burst duration described here could provide a tool for identifying the site (or sites) that control the rate of closing from bursts.

We thank Jie Liao for excellent technical assistance. J. Luo was supported by a Canadian Cystic Fibrosis Foundation studentship. J. W. Hanrahan is a Medical Research Council (Canada) scientist. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grants to J. W. Hanrahan and J. R. Riordan.

Address for reprint requests: J. W. Hanrahan, Dept. of Physiology, McGill University, 3655 Drummond St., Montreal, QC, Canada H3G 1Y6.

Received 14 October 1997; accepted in final form 29 January 1998.

REFERENCES


