Regulation of Recombinant Cardiac Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channels by Protein Kinase C

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ABSTRACT We investigated the regulation of cardiac cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels by protein kinase C (PKC) in *Xenopus* oocytes injected with cRNA encoding the cardiac (exon 5−) CFTR Cl^- channel isoform. Membrane currents were recorded using a two-electrode voltage clamp technique. Activators of PKC or a cAMP cocktail elicited robust time-independent Cl^- currents in cardiac CFTR-injected oocytes, but not in control water-injected oocytes. The effects of costimulation of both pathways were additive; however, maximum protein kinase A (PKA) activation occluded further activation by PKC. In oocytes expressing either the cardiac (exon 5−) or epithelial (exon 5+) CFTR isoform, Cl^- currents activated by PKA were sustained, whereas PKC-activated currents were transient, with initial activation followed by slow current decay in the continued presence of phorbol esters, the latter effect likely due to down-regulation of endogenous PKC activity. The specific PKA inhibitor, adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMPS), and various protein phosphatase inhibitors were used to determine whether the stimulatory effects of PKC are dependent upon the PKA phosphorylation state of cardiac CFTR channels. Intraoocyte injection of 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA) or pretreatment of oocytes with BAPTA-acetoxymethyl-ester (BAPTA-AM) nearly completely prevented dephosphorylation of CFTR currents activated by cAMP, an effect consistent with inhibition of protein phosphatase 2C (PP2C) by chelation of intracellular Mg^{2+}. PKC-induced stimulation of CFTR channels was prevented by inhibition of basal endogenous PKA activity, and phorbol esters failed to stimulate CFTR channels trapped into either the partially PKA phosphorylated (P_1) or the fully PKA phosphorylated (P_1P_2) channel states. Site-directed mutagenesis of serines (S686 and S790) within two consensus PKC phosphorylation sites on the cardiac CFTR regulatory domain attentuated, but did not eliminate, the stimulatory effects of phorbol esters on mutant CFTR channels. The effects of PKC on cardiac CFTR Cl^- channels are consistent with a simple model in which PKC phosphorylation of the R domain facilitates PKA-induced transitions from dephosphorylated (D) to partially (P_1) phosphorylated and fully (P_1P_2) phosphorylated channel states.

INTRODUCTION

Cardiac protein kinase A (PKA)-regulated Cl^- channels play an important role in the regulation of action potential duration and resting membrane potential (Harvey and Hume, 1989; Bahinski et al., 1989). It has become apparent that many biophysical and pharmacological properties of these channels resemble those of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels in epithelium (see Gadsby et al., 1995), and recent molecular studies have confirmed that these channels are due to cardiac expression of an alternately spliced isoform (exon 5−) of the epithelial CFTR Cl^- channel (Horowitz et al., 1993; Hart et al., 1996). Exon 5 represents a 30-amino acid segment in the first cytoplasmic loop of CFTR, the functional significance of which is currently unknown. Outside of the exon 5 region, cardiac and epithelial CFTR cDNA exhibit ~91% nucleotide sequence homology with numerous putative PKA and PKC phosphorylation sites highly conserved in the two isoforms. However, the fact that exon 5 contains two putative PKC phosphorylation sites suggests the possibility that there may be characteristic differences in the PKC regulation of the cardiac (exon 5−) and epithelial (exon 5+) isoforms. It is also unclear at this time whether other differences outside the exon 5 spliced region of the cardiac or epithelial isoforms may be important for channel regulation or expression. For example, it has been reported that an engineered epithelial exon 5− CFTR isoform fails to express functional Cl^- channels in Hela cells because of defective intracellular processing (Delaney et al., 1993), but may be functionally expressed in intracellular membranes in 293 HEK cells with a reduced single-channel conductance (Xie et al., 1996). Molecular studies of mammalian cardiac cells in nearly all nonprimate species yet examined reveal exclusive expression of the exon 5− transcript (Horowitz et al., 1993; Hume and Horowitz, 1995), and yet single-channel studies in sarcolemmal membrane patches from most cardiac myocytes examined (Ehara and Ishihara, 1990; Nagel et al., 1992) reveal CFTR single-channel properties nearly identical to those reported for the exon 5+ epithelial isoform.

In native cardiac cells, PKC has been shown to stimulate Cl^- currents, which exhibit macrosopic properties similar to (Zhang et al., 1994; Shuba et al., 1996) or distinct from

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(Walsh, 1991; Walsh and Long, 1994) those of CFTR. The possibility that PKC-activated Cl^- currents in heart might be mediated by the same population of PKA-regulated CFTR Cl^- channels is suggested by previous findings that recombinant epithelial CFTR Cl^- channels are activated by PKC phosphorylation (Tabcharani et al., 1991; Berger et al., 1993), as well as by the report that PKC and PKA activate unitary Cl^- channels in native cardiac cells with nearly identical conductance and rectification properties (Collier and Hume, 1995).

Xenopus oocytes have previously been used as an expression system for functional studies of epithelial CFTR Cl^- channels (Bear et al., 1991; Drumm et al., 1991), and the recent demonstration of robust expression of cardiac (exon 5−) CFTR Cl^- channels in *Xenopus* oocytes (Hart et al., 1996) suggests that this may be a useful expression system for examination of protein kinase regulation of cardiac CFTR Cl^- channels. The purposes of the present study were 1) to compare the regulation of cardiac (exon 5−) CFTR Cl^- channels by PKA and PKC, 2) to determine whether PKC regulation of cardiac CFTR Cl^- channels is dependent upon the PKA phosphorylation state of CFTR channels, and 3) to determine whether mutations at two R domain putative PKC phosphorylation sites, S686 and S790, in the cardiac isoform of CFTR alter the functional response of expressed CFTR Cl^- channels to PKA or PKC stimulation. A preliminary report of these results has been published as an abstract (Yamazaki et al., 1997).

MATERIALS AND METHODS

cRNA preparation and injection of oocytes

cRNA encoding the full-length CFTR (exon 5−) coding sequence from rabbit ventricle was prepared as previously described (Hart et al., 1996); cRNA encoding the full-length human epithelial CFTR (exon 5+) coding sequence was kindly provided by Dr. Johanna Rommens (Hospital for Sick Children, Toronto, Ontario, Canada). Adult *Xenopus laevis* were anesthetized by immersion in a solution of tricaine methanesulfonate (1 g/liter), and oocytes were surgically removed. To remove follicular cells, the oocytes were incubated in ND 96 solution (mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5.0 HEPES; pH 7.4) containing collagenase (Sigma, type 1A) at room temperature for 2–3 h with gentle shaking, and then stored in ND 96 solution containing 2.5 mM sodium pyruvate and gentamycin (1 mg/ml). Injection pipettes with tips ~20 μm in diameter were baked at >150°C for 3–4 h to destroy RNases. The pipettes were mounted in a Drummound Nanoject autoinjector (Drummound Scientific, Broomall, PA), and oocytes were injected with 46 nl of cRNA of cardiac or epithelial CFTR, or distilled water. Oocytes were then stored in ND 96 plus pyruvate and gentamycin at 18°C.

Site-directed mutagenesis

The serine at position 686 and/or 790 was modified by polymerase chain reaction-based site-directed mutagenesis (Jones and Howard, 1991) to alanine to create S686A, S790A, and S686 + 790A cardiac CFTR cDNA. The mutations were confirmed by nucleotide sequencing of both strands of the mutated cDNA.

Whole-cell current recording from oocytes

Membrane currents were recorded at room temperature from oocytes 3–5 days after injection, using a two-microelectrode voltage clamp system (TEV-200; Dagan, Minneapolis, MN). Microelectrodes were filled with 3 mol/liter KCl and had resistances of 0.5–5 MΩ. Voltages are reported with reference to the bath. Cl^- currents were recorded in either nominally Ca^{2+}-free ND96 solution or Ca^{2+}-containing (1.8 mM) ND96 solutions including 100 μM niflumic acid to prevent the activation of endogenous Cl^- channels. Membrane currents were filtered at 2.0 kHz, digitized on-line, and stored on a computer. Data analysis was performed with pCLAMP 5.5.1 (Axon Instruments, Foster City, CA). In some experiments, Cl^- dependence was determined by replacing bath NaCl with Na aspartate (total $[Cl^-]$ was 24 mM). In 0 mM Ca^{2+} solution, Ca^{2+} was replaced with Mg^{2+} (total $[Mg^{2+}]$ was 2.8 mM), and 1 mM EGTA was added. The external solution used to elevate cAMP (cAMP cocktail (1×)) included 8-bromo-cAMP (100 μM), forskolin (1 μM), and 3-isobutyl-1-methylxanthine (IBMX) (500 μM) to activate PKA and adenylate cyclase and inhibit phosphodiesterase activity, respectively. cAMP cocktail (5× and 10×), which contained a 5 and 10 times higher concentration of each component, was used to maximally stimulate CFTR Cl^- channels. The flow rate was usually 1.5 ml/min.

PDBu, 4α-phorbol, DiC8, niflumic acid (Sigma, St. Louis, MO), 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid-acetoxymethyl-ester (BAPTA-AM), staurosporine, bisindolylmaleimide, microcystin LR, and okadaic acid (Calbiochem, La Jolla, CA) were prepared as stock solutions in dimethylsulfoxide (DMSO). Forskolin (Sigma) was prepared as a stock solution in polyethylene glycol. These stock solutions were diluted to the desired final concentration immediately before use. The final concentration of DMSO and polyethylene glycol was less than 0.1%, which, by themselves, did not affect Cl^- currents. Adenosine 3′,5′-cyclic monophosphothioate Rp-isomer (Rp-cAMPS) (Calbiochem) was dissolved in distilled water. All other compounds were purchased from Sigma. Some oocytes were superfused with BAPTA-AM (100 μM) in nominally Ca^{2+}-free solutions.

In some experiments, okadaic acid, microcystin LR, calmidazolium chloride, cyclosporin A, Rp-cAMPS, BAPTA tetrapotassium salt, and $MgSO_4$ were directly injected into oocytes (Drummond Nanoject autoinjector) with glass pipettes (25 μm diameter) after the pH was adjusted to 7.2–7.3. The intracellular concentration of these compounds was estimated from the injection volume (~46 nl) and the approximate volume of the oocytes (1000 nl). Most other compounds were applied directly to the perfusion solution.

Data analysis

The concentration-response curve for PDBu was analyzed by fitting the logistic equation $R = ((R_{max} - R_{min}) \times A^n)/(EC_{50}^n + A^n) + R_{min}$, where R is the amplitude of the current, R_{max} is the maximum current, R_{min} is the minimum current, A is the concentration of PDBu, EC_{50} is the dose of PDBu giving the half-maximum current, and n is the slope factor.

Data are expressed as arithmetic means ± SEM. Statistical analysis between two groups was made by unpaired Student's or Welch's t-test. When values before and after treatments were compared, a paired t-test was used. In the case of more than three groups, one-way analysis of variance (ANOVA) and post hoc Bonferroni's multiple t-tests were performed. When values of three treatments in each preparation were compared, one-way ANOVA with repeated measures and the *post hoc* multiple t-test were used. $p < 0.05$ was considered to be statistically significant.

RESULTS

PKA- and PKC-induced Cl^- currents in cardiac CFTR-injected oocytes

To compare the regulation of cardiac CFTR chloride channels by PKA and PKC, we examined the effects of a cAMP

cocktail (1×) and a PKC activator, phorbol-12,13-dibutylate (PDBu) or 1,2-dioctanoyl-*sn*-glycerol (DiC8). To abolish endogenous Ca^{2+}-activated Cl^- currents and Ca^{2+}-inactivated Cl^- currents (Weber et al., 1995), niflumic acid (100 μM) was included in the superfusion solutions. Before the addition of any drugs, background currents were small over the potential range of −110–70 mV examined. The cAMP cocktail elicited typical time-independent Cl^- currents in oocytes injected with cRNA encoding the cardiac CFTR exon 5− splice transcript (Hart et al., 1996), but not in water-injected control oocytes (Fig. 1 *A*). After washout of the cAMP cocktail, a subsequent application of PDBu (100 nM) elicited similar but smaller time-independent currents in cardiac CFTR-injected oocytes. The current-voltage relationship for PDBu-induced currents exhibited some slight outward rectification under these asymmetrical Cl^- conditions and exhibited a mean reversal potential (−14.8 ± 4.0 mV, $n = 5$) similar to that of cAMP-induced Cl^- currents (−17.8 ± 5.6 mV, $n = 5$; Fig. 1 *B*). PDBu did not alter the background current in water-injected oocytes.

Fig. 1 *C* shows the concentration-response relationship for PDBu. PDBu caused maximum current activation at 100 nM in CFTR-injected oocytes. The EC_{50} value was estimated as 10.5 nM. The PDBu (100 nM)-induced currents were smaller in amplitude than cAMP cocktail-induced currents; the current ratio (PDBu/cAMP) was constant over the potential range from −110 to 70 mV: 66.3 ± 7.7% (−110 mV), 74.4 ± 8.1% (−50 mV), and 63.9 ± 5.1% (70 mV, $n = 4$). In eight oocytes sequentially exposed to the cAMP cocktail and then to PDBu after washout, the cAMP- and PDBu-induced currents during the voltage pulses to 70 mV were 5.1 ± 0.5 μA and 3.4 ± 0.5 μA, respectively. DiC8 (100 μM), a diacylglycerol analog, mimicked PDBu in activating time-independent currents in cardiac CFTR-injected oocytes (Fig. 1 *D*). cAMP cocktail- and DiC8-induced currents at 70 mV were 6.3 ± 1.6 μA and 1.6 ± 0.3 μA, respectively ($n = 3$). Although the DiC8 effect appeared to be smaller than that of PDBu (DiC8/cAMP was 25.2% at 70 mV), DiC8-induced currents also exhibited slight outward rectification, and the current-voltage relationship exhibited a mean reversal potential of −20.3 ± 0.7 mV ($n = 3$). An inactive analog, 4α-phorbol (100 nM), or 0.1% DMSO, the solvent for PDBu and DiC8, had little effect on the background currents in cardiac CFTR-injected oocytes (data not shown).

The experiments in Fig. 2, *A* and *B*, examined the Cl^- sensitivity of the PDBu-induced currents. Outward PDBu-induced currents were reduced upon as external Cl^- was changed from 104 to 24 mM. The reversal potentials were −19.3 ± 1.7 and +10.5 ± 2.8 mV in 104 mM Cl^- and 24 mM Cl^- bath solutions ($n = 4$), respectively, a shift of ~30.5 mV, close to the predicted 35-mV shift of the estimated Cl^- equilibrium potential under these conditions. Fig. 2 *C* shows the inhibition of the PDBu-induced current by the nonspecific protein kinase inhibitor, staurosporine (1 μM), and by a specific PKC inhibitor, bisindolylmaleimide

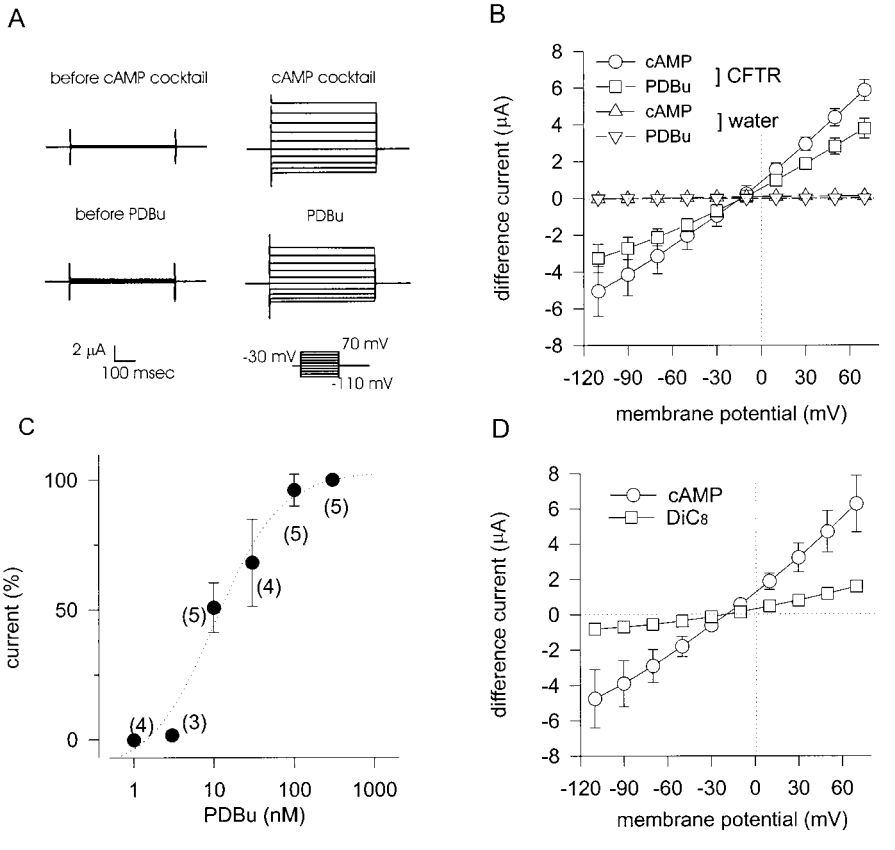

FIGURE 1 Effect of cAMP and PKC activators on membrane currents in oocytes injected with cRNA encoding the rabbit cardiac CFTR exon 5− transcript. (*A*) Raw membrane currents evoked by 500-ms voltage steps from −30 mV to potentials ranging from −110 to 70 mV (20-mV increments) before and after exposure to cAMP cocktail (1×; forskolin, IBMX, and 8-bromo-cAMP) or PDBu (100 nM). (*B*) *I-V* relations of cAMP- and PDBu (100 nM)-induced currents in cardiac CFTR- or water-injected oocytes. Difference currents were obtained by subtracting the control current from the peak current after each treatment. Each data point represents mean ± SEM ($n = 4$). (*C*) Concentration-response relationship for PDBu in CFTR-injected oocytes. The currents are normalized with that evoked by 300 nM PDBu. The data were fitted to a logistic function (*dotted curve*). The estimated EC_{50} was 10.5 nM, and *nH* was 1.1. The numbers in parentheses indicate the number of oocytes tested at each concentration. (*D*) *I-V* relations of cAMP- and DiC8 (100 nM)-induced currents in cardiac CFTR-injected oocytes. Difference currents were obtained by subtracting the control current from the peak current after each treatment. Each data point represents a mean ± SEM ($n = 3$).

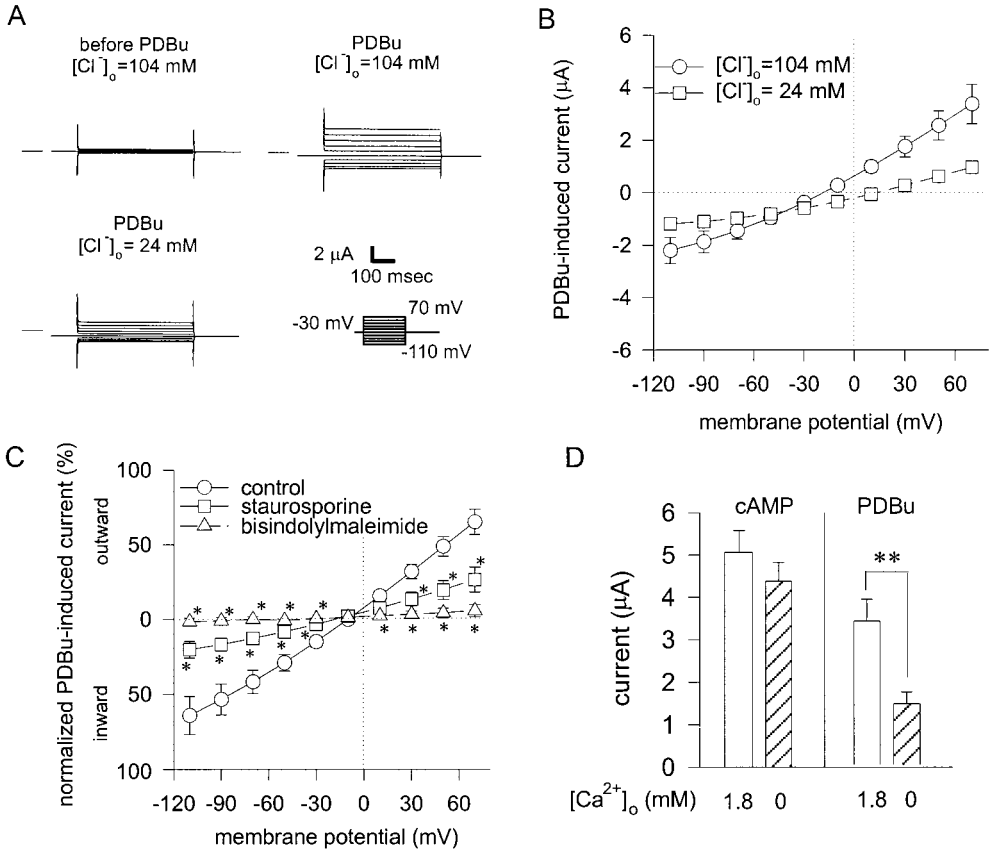

FIGURE 2 Dependence of the PDBu-induced currents on external Cl^- and Ca^{2+}, and effects of kinase inhibitors in cardiac CFTR-injected oocytes. (*A*) Raw membrane currents evoked by 500-ms voltage steps from −30 mV to potentials ranging from −110 to 70 mV (20-mV increments) before and after exposure to PDBu (100 nM) in 104 mM or 24 mM external $[Cl^-]$ ($[Cl^-]_o$). The oocytes were initially exposed to the 104 mM Cl^--containing solution. Thereafter, the solution was changed to 24 mM Cl^--containing solution. (*B*) *I-V* relationships for the PDBu-induced currents with $[Cl^-]_o$ = 104 mM and 24 mM. The currents were measured during 500-ms voltage steps from −30 mV to potentials ranging from −110 to 70 mV (20-mV increments) in the 104 mM Cl^- solution followed by 24 mM Cl^- solution. PDBu-induced currents were obtained by subtracting the current in the absence of PDBu from the current in the presence of PDBu. Each data point represents a mean ± SEM (n = 4). No corrections were made for small changes in junction potential expected during these solution changes. (*C*) *I-V* relations of the PDBu-induced currents before and after exposure to staurosporine (1 μM) or bisindolylmaleimide (1 μM). Current amplitudes were normalized to the maximum current activated by cAMP cocktail (1×) at 70 mV. Each data point represents a mean ± SEM (n = 3–5). *, $p < 0.05$ (ANOVA). (*D*) Comparison of the peak currents at 70 mV evoked by cAMP cocktail (1×) or PDBu (100 nM) in cardiac CFTR-injected oocytes, which were perfused with 1.8 mM Ca^{2+}-containing external solution (□) or which were perfused with Ca^{2+}-free external solution including EGTA (1 mM) (▨). Each data point represents a mean ± SEM (n = 8–20). **, $p < 0.01$ (unpaired t-test).

(1 μM). Staurosporine significantly inhibited the PDBu-induced current by 60% at 70 mV. Bisindolylmaleimide significantly inhibited the PDBu-induced current by over 90% at 70 mV. These results suggest that PKA or PKC stimulation activates time-independent Cl^- currents in oocytes expressing cardiac CFTR, but not in water-injected control oocytes.

Ca^{2+} is known to be required to activate some isozymes of PKC, in addition to diacylglycerol or phorbol ester (Nishizuki, 1988). We therefore tested whether the ability of PKC stimulation to activate CFTR Cl^- currents in oocytes was Ca^{2+} dependent. We compared the effects of the cAMP cocktail and PDBu in cardiac CFTR-injected oocytes (Fig. 2 *D*) that were perfused with either 1.8 mM Ca^{2+}-containing external solutions (*open columns*) to those that were perfused with Ca^{2+}-free external solutions containing EGTA (1 mM) (*hatched columns*). Whereas the mean amplitude of the cAMP-activated current at 70 mV was almost the same under these conditions, the PDBu-induced Cl^- currents were significantly smaller in oocytes in Ca^{2+}-free external solutions compared to oocytes in solutions containing 1.8 mM Ca^{2+}. These results suggest a possible role of some Ca^{2+}-dependent step in the PKC pathway regulating cardiac CFTR Cl^- channels in oocytes.

To confirm that both PKA and PKC activate the same population of cardiac CFTR Cl^- channels, we examined the additivity of currents activated by both pathways. If the same channels are activated by both pathways, it is expected that submaximum concentrations of either agonist should be additive, but a supramaximum concentration of either agonist should occlude the response to the other agonist. Fig. 3 *A* shows typical time courses of the outward currents at 70 mV during stimulation by PKA alone, or in combination with stimulation of PKC (mean current normalized with the

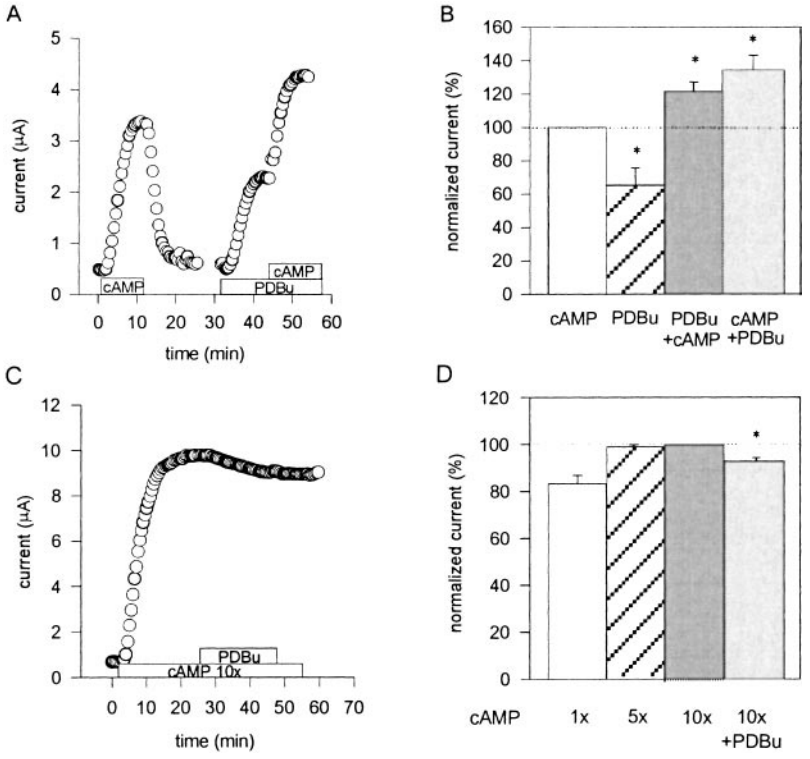

FIGURE 3 Effect of costimulation by cAMP and PDBu on cardiac CFTR Cl^- currents. (*A*) The submaximum effect of the cAMP cocktail (1×) on the peak PDBu-induced current was examined. The time course of the effect of the cAMP cocktail (1×) and 100 nM PDBu on Cl^- currents at 70 mV is shown. (*B*) Currents at 70 mV after exposure of PDBu, PDBu plus cAMP cocktail (1×), or cAMP cocktail (1×) plus PDBu were normalized with peak current evoked by cAMP cocktail (1×) alone. Each data point represents mean ± SEM ($n = 4$). *, $p < 0.05$ (ANOVA with repeated measures) compared with the cAMP-induced current. (*C*) The effect of 100 nM PDBu on Cl^- currents at 70 mV in the continued presence of maximum cAMP cocktail concentration (10×) was examined. (*D*) Concentration response for cAMP cocktail (1×, 5×, and 10×; see Materials and Methods) and the effects of 100 nM PDBu on Cl^- currents maximally activated by cAMP cocktail (10×). Currents at 70 mV during exposure to different concentrations of cAMP cocktail were normalized with peak current evoked by cAMP cocktail (10×). cAMP cocktail (5× and 10×) appears to give the maximum activation of CFTR Cl^- channels. Each data point represents a mean ± SEM ($n = 3$, 3, and 6 for cAMP 1×, 5×, and 10×, respectively; $n = 4$ for cAMP (10×) + PDBu). *, $p < 0.05$ (paired *t*-test).

response to cAMP cocktail). The PDBu-induced current alone was 65.3 ± 10.3% ($n = 4$) of the cAMP cocktail (1×)-induced current (Fig. 3 *B*). Subsequent addition of cAMP cocktail during the peak of the PDBu-induced current further increased the Cl^- current by 21.6 ± 5.5% more than the cAMP cocktail-induced current alone. In Fig. 3 *B*, it can also be seen that PDBu also further increased the Cl^- current activated by cAMP cocktail (1×) by 34.9 ± 8.6% ($n = 4$). These results suggest that the stimulatory effects of submaximum concentrations of either agonist are additive with respect to the amplitude of Cl^- current activated.

We next tested whether supramaximum stimulation by the cAMP cocktail would occlude the stimulatory effects of PDBu. cAMP cocktail (10×) gave the same degree of activation of Cl^- currents as cAMP cocktail (5×), which was ~20% greater than the effect of cAMP cocktail (1×; Fig. 3 *D*), suggesting that cAMP cocktail (5× and 10×) maximally activates the available Cl^- channels. As shown in Fig. 3, *C* and *D*, PDBu failed to increase Cl^- current further once they were maximally activated by cAMP cocktail (10×); instead it decreased the currents slightly. These results are consistent with the interpretation that the effects observed with PKA and PKC stimulation are both mediated by the same population of recombinant cardiac CFTR Cl^- channels.

Biphasic response of CFTR Cl^- channels to PKC stimulation

The time courses and duration of activation of cardiac CFTR channels by cAMP cocktail, PDBu, and DiC8 are compared in Fig. 4 *A*. The PKA-activated Cl^- currents usually reached a steady-state level after ~12 min and were well sustained for more than 30 min. In contrast, the PDBu and DiC8-induced currents exhibited a biphasic time course. PDBu- or DiC8-induced Cl^- currents usually reached their maximum level after 7.5 ± 1.6 min ($n = 4$), and prolonged exposure resulted in a dramatic decline of the currents in the continued presence of either compound. Such a decline was observed at all membrane potentials examined. Fig. 4 *B* summarizes these data for normalized current amplitudes evoked by PKA and PKC stimulation as a function of time of exposure. Currents activated by both PKA and PDBu were nearly at maximum (~90%) within 10 min, and the cAMP-induced currents were stable for more than 30 min in the continued presence of the cAMP cocktail (100.1 ± 1.9% at 30 min, $n = 4$). In contrast, the PDBu-induced currents were reduced to 17.3 ± 11.0% ($n = 4$) of their maximum value within 30 min in the continued presence of PDBu. Such a biphasic response to PDBu was also observed in oocytes bathed in Ca^{2+}-free solutions.

The most obvious explanation for the observed decline in PDBu- or DiC8-induced Cl^- currents in the maintained presence of either agonist is possible desensitization of some component of the PKC pathway. Alternatively, the observed current decline might reflect PKC-induced modulation, resulting in channel dephosphorylation or channel inactivation. In the case of inactivation, one might expect that PKC would also alter subsequent channel activation by other intracellular pathways, for example, the activation by PKA. In Fig. 5 *A* (*left*), the cAMP cocktail was perfused initially to obtain the control response to PKA activation

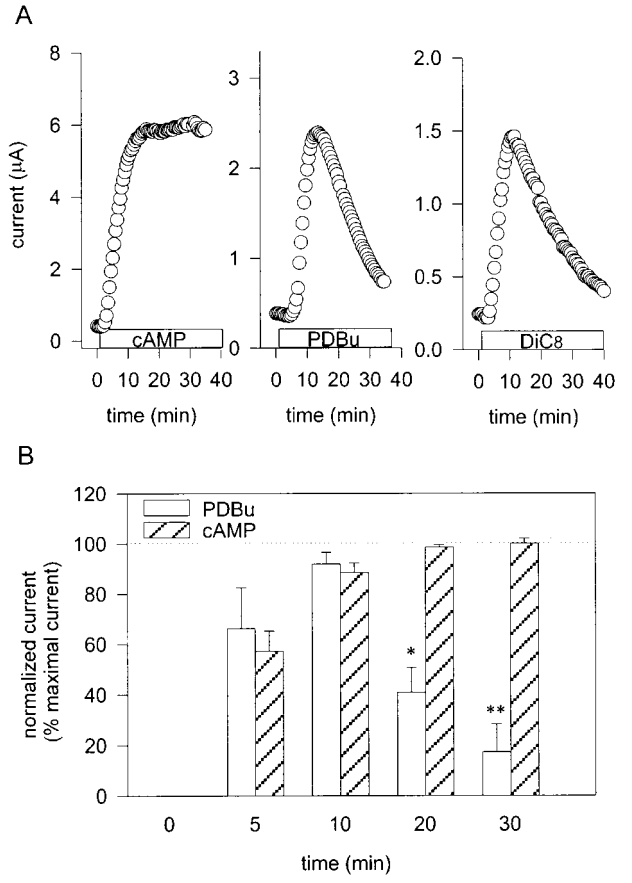

FIGURE 4 Effects of prolonged exposure of cardiac CFTR-injected oocytes to cAMP, PDBu, and DiC8 A; sustained activation by cAMP cocktail (1×); and transient activation by PDBu (100 nM) and DiC8 (100 μM). Currents were evoked by applying repetitive 500-ms voltage steps to 70 mV. (*B*) Time course of the mean currents activated by cAMP and PDBu. The current was evoked by applying 500-ms voltage steps to 70 mV. The amplitudes of currents were normalized with that of the maximum current evoked by the corresponding treatment for 30 min. Each data point represents a mean ± SEM ($n = 4$). *, $p < 0.05$; **, $p < 0.01$ (unpaired *t*-test).

(the first response). After washout of cAMP cocktail, PDBu (100 nM) was perfused, and the PDBu-activated currents were allowed to decline to ~20% of the peak PDBu response (~30 min). Immediately after PDBu-induced current decline, in the continued presence of PDBu, the response to PKA stimulation was retested. A significantly smaller response to the cAMP cocktail was observed compared to the control (Fig. 5 *A, right*; 34.5 ± 9.9%, $n = 4$). In the absence of PDBu, repeated application of the cAMP cocktail after a similar interval of 60 min failed to reveal any significant change in the amplitude of the PKA-induced current (Fig. 5 *A, right*). Thus prior inactivation of cardiac CFTR Cl^- channels by PKC stimulation reduces the ability of PKA to subsequently activate the channels. We also verified that the PDBu-induced inhibition of Cl^- channels can be attributed to PKC stimulation. In oocytes pretreated with the specific PKC inhibitor bisindolylmaleimide (5 μM), PDBu failed to cause an increase in Cl^- current and failed to inhibit Cl^- currents activated by subsequent exposure to cAMP cocktail (data not shown).

To investigate the possibility of functional differences in PKC regulation between cardiac and epithelial isoforms of CFTR Cl^- channels, the protocol that was employed in Fig. 5 *A* was performed with epithelial (exon 5+) CFTR-injected oocytes. cAMP cocktail (1×) and PDBu (100nM) elicited Cl^--dependent outwardly rectifying currents (under asymmetrcial Cl^- conditions) in epithelial (exon 5+) CFTR-injected oocytes, but not in water-injected control oocytes. Like cardiac (exon 5−) CFTR-injected oocytes, the peak PDBu-induced current amplitude at 70 mV was 55.7 ± 10.5% ($n = 4$) of the peak cAMP-induced current amplitude in epithelial (exon 5+) CFTR-injected oocytes. As shown in Fig. 5 *B* (*left*), cAMP cocktail was initially perfused to obtain the response to PKA stimulation. Prolonged perfusion of PDBu resulted in an initial current activation followed by inactivation. As in the case of cardiac CFTR channels, epithelial channels inactivated by exposure to PDBu exhibited a significantly smaller subsequent response to PKA stimulation, compared to the control cAMP response (Fig. 5 *B, right*). It appears, therefore, that epithelial (exon 5+) CFTR Cl^- channels respond to PKC stimulation in a manner similar to that of cardiac (exon 5−) CFTR Cl^- channels: an initial transient activation, followed by slow inactivation.

Role of endogenous protein kinases and phosphatases

It is well known that the activity of CFTR Cl^- channels is regulated by phosphorylation of its regulatory domain (R domain) by PKA. Thus channels are believed to require PKA phosphorylation and ATP hydrolysis to open (Gadsby et al., 1995; Foskett, 1998); however, the role of PKC phosphorylation is less clear. To investigate whether PKC modulation of cardiac CFTR channels involves basal PKA activity in oocytes, we examined the effect of a specific PKA inhibitor, Rp-cAMPS on PDBu-induced Cl^- currents. Injection of Rp-cAMPS (5 μM) was effective in attenuating the cAMP-induced Cl^- conductance when it was injected during perfusion with cAMP cocktail (Fig. 6 *A*). Prior injection of oocytes with Rp-cAMPS greatly attentuated the ability of PDBu (100nM) to stimulate cardiac CFTR currents (Fig. 6 *B*). Fig. 6 *C* shows the time course of PDBu-induced currents at 70 mV. Rp-cAMPS (5 and 10 μM) inhibited the peak amplitude of the PDBu-induced CFTR currents from the control level of 58.8 ± 8.1% of the peak cAMP-induced current to 30.2 ± 6.6% and 16.8 ± 6.5%, respectively. Thus the ability of PDBu to stimulate cardiac CFTR channels seems to be highly dependent upon basal PKA activity, suggesting that PKC phosphorylation per se does not open CFTR Cl^- channels.

We also tested the hypothesis that endogenous PKC activity may regulate CFTR activation by PKA (Jia et al., 1997). Oocytes were first exposed to the cAMP cocktail to

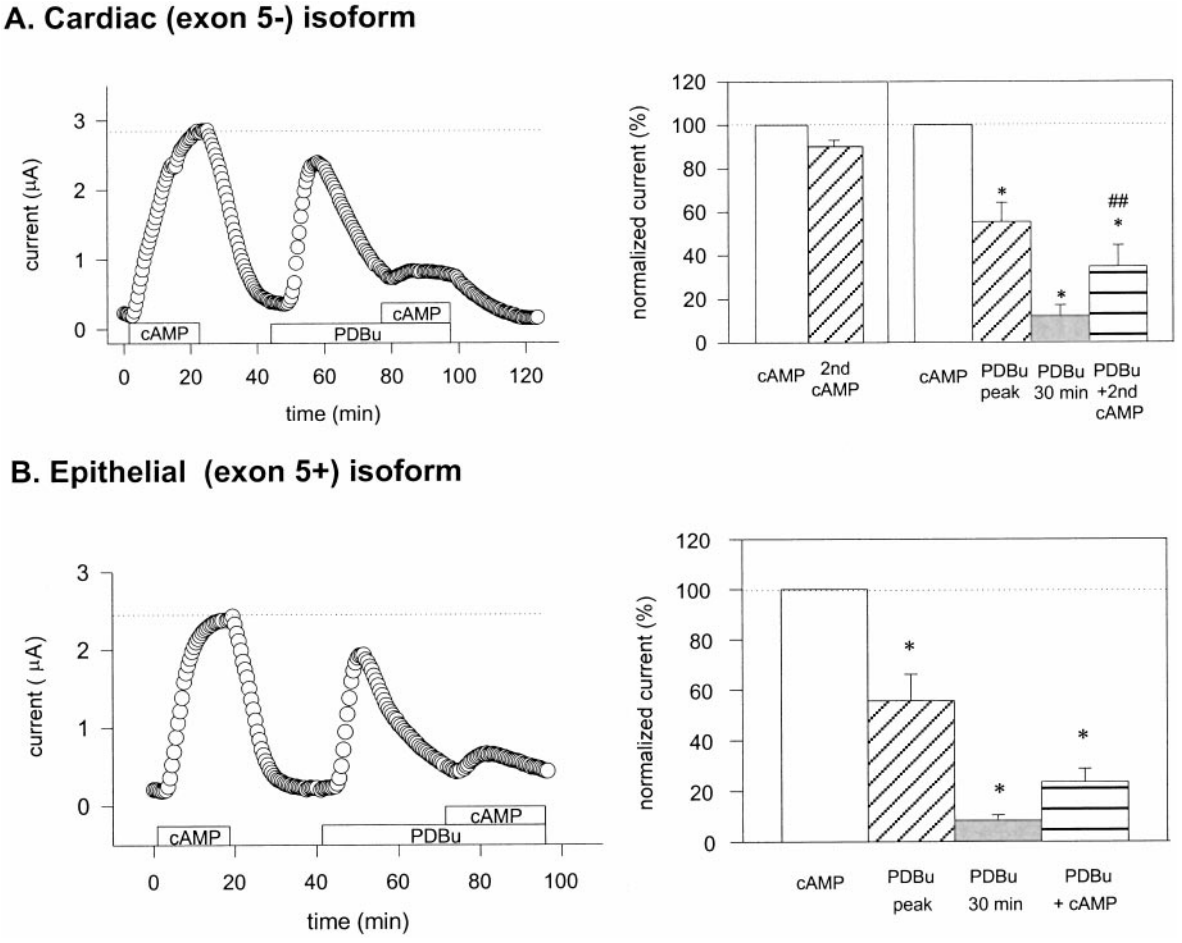

FIGURE 5 Prolonged PDBu exposure inhibits cAMP activation of cardiac and epithelial CFTR Cl^- currents. (*A*) Effects of cAMP and PDBu on membrane currents in oocytes injected with cRNA encoding the cardiac (exon 5−) CFTR transcript. (*Left*) cAMP cocktail (1×) was perfused in the continued presence of PDBu (100 nM). Shown is the time course of the outward Cl^- currents elicited by applying 500-ms voltage steps to 70 mV from a holding potential of −30 mV. The peak amplitude of the current induced by cAMP cocktail (1×) alone was initially estimated. After washout, PDBu caused a transient increase in current, followed by a decline. In the continued presence of PDBu, cAMP cocktail (1×) caused a much smaller activation. (*Right*) currents at 70 mV were normalized with that of the peak current evoked by the first cAMP (1×) exposure. Mean currents elicited by the second application of cAMP cocktail 80 min after its first application are shown in the absence of PDBu (second cAMP). Peak PDBu-induced currents, after 30 min, and peak currents elicited by a second exposure to cAMP in the continued presence of PDBu are shown. (*B*) Effects of cAMP and PDBu on membrane currents in oocytes injected with cRNA encoding the epithelial (exon 5+) CFTR transcript. (*Left*) Experimental protocol as in *A*. (*Right*) Current amplitudes at 70 mV were normalized to the maximmu current evoked by cAMP cocktail (1×) alone. Mean PDBu-induced current amplitudes (peak), PDBu-induced current amplitudes after 30 min of exposure, and amplitudes elicited by a second cAMP exposure in the continued presence of PDBu are shown. Each data point represents a mean ± SEM ($n = 4$). *, $p < 0.05$ (ANOVA with repeated measures) compared with the corresponding cAMP-induced current. ##, $p < 0.01$ (unpaired *t*-test) compared with the current evoked by the second cAMP stimulation in the absence of PDBu.

obtain the control response to PKA activation. After washout of cAMP cocktail, the specific PKC inhibitor bisindolylmaleimide (5 μM) was perfused, and then the response to the cAMP cocktail was retested in the continued presence of bisindolylmaleimide. As illustrated in Fig. 6 *D*, the subsequent response to PKA stimulation was significantly attenuated after inhibition of endogenous PKC activity by bisindolylmaleimide. In three oocytes, the subsequent response to PKA stimulation was reduced to 53.7 ± 10.2% of the control response after exposure to bisindolylmaleimide (*inset*). These data support the hypothesis that PKC phosphorylation of CFTR significantly potentiates PKA activation of CFTR (Jia et al., 1997; Middleton and Harvey, 1998).

Although the PKC-mediated decline in CFTR Cl^- currents may be due to PKC desensitization or PKC-induced channel inactivation, other possible explanations remain. It is possible that the stimulatory effects of PKC are due to a direct phosphorylation of CFTR, whereas the inhibitory effects might be due to dephosphorylation of CFTR channels, possibly from PKC stimulation of an endogenous protein phosphatase. To examine the role of endogenous phosphatases in PKC-induced inactivation, we first investigated the effects of okadaic acid (OA), a protein phosphatase 1 and 2A (PP1 and PP2A) inhibitor, on the peak amplitude and rate of decline of the PDBu-induced current. The rate of deactivation of Cl^- current upon removal of the

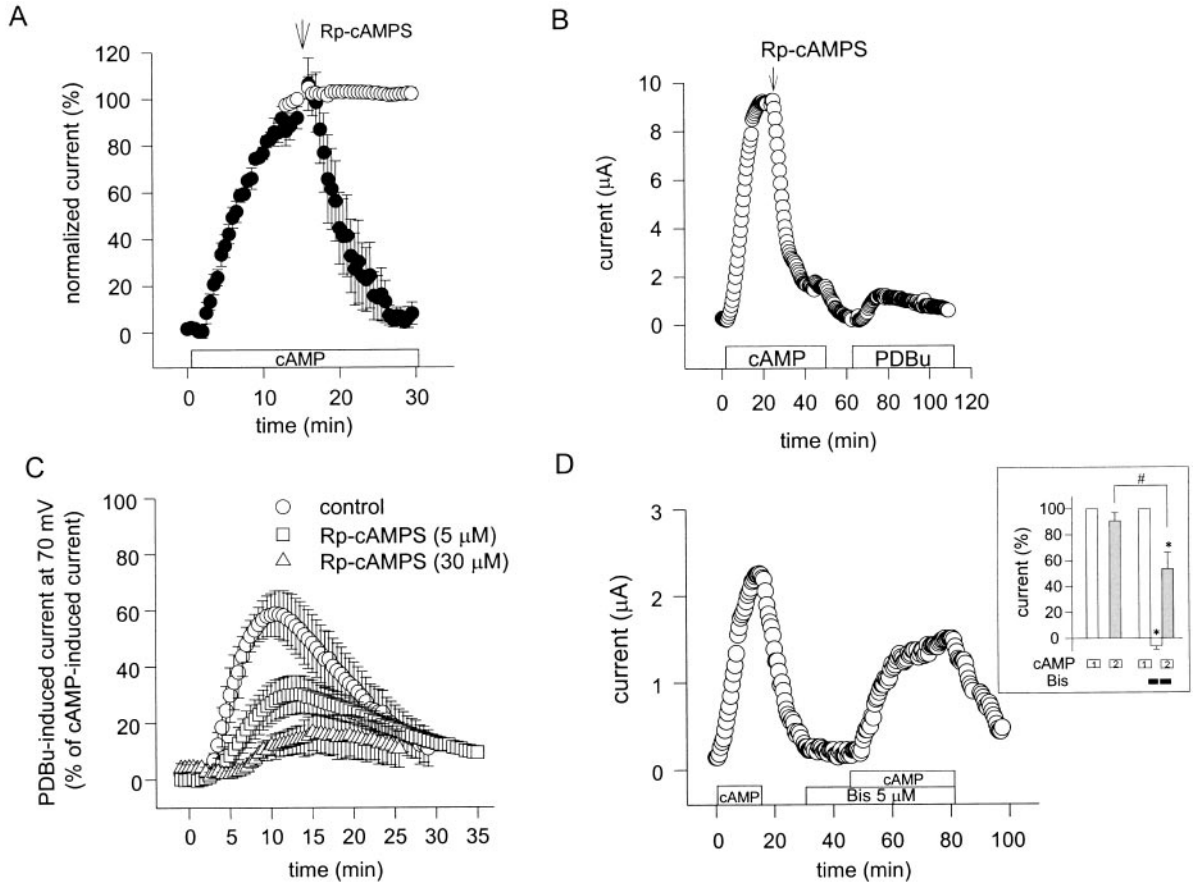

FIGURE 6 Role of basal PKA and PKC activity. (*A*) Blockade of cAMP cocktail (1×)-induced currents by intraoocyte injection of the specific PKA inhibitor, Rp-cAMPS (*closed circles*; approximate concentration, 5 μM) in cardiac CFTR-injected oocytes. The time course of membrane currents elicited by repetitive voltage steps at 70 mV is shown. Open circles were obtained after injection of water in a series of separate control experiments. (*B*) Rp-cAMPS (5 μM) was injected in the continued presence of cAMP cocktail after current activation. After washout of the cAMP cocktail, PDBu (100 nM) was superfused. (*C*) Time courses of PDBu (100 nM)-induced CFTR Cl^- currents at 70 mV in the presence or absence of Rp-cAMPS (mean ± SEM, $n = 4$). Current amplitudes were normalized to the maximum currents activated by cAMP cocktail in the absence of Rp-cAMPS. (*D*) Effect of bisindolylmaleimide (5 μM) superfusion on cAMP cocktail (1×)-induced currents. Time course of the outward Cl^- currents elicited by applying 500-ms voltage steps to 70 mV from a holding potential of −30 mV. The peak amplitude of the current induced by cAMP cocktail (1×) alone was initially estimated. After washout, bisindolylmaleimide was superfused, and in the continued presence of bisindolylmaleimide, cAMP cocktail (1×) caused a smaller activation of current. The inset shows a comparison of the effect of cAMP cocktail with or without bisindolylmaleimide ($n = 3$–5). *, $p < 0.05$ (ANOVA with repeated measures) compared with the first response to cAMP. #, $p < 0.05$ (unpaired *t*-test) compared with the current evoked by the second cAMP application alone.

cAMP cocktail is believed to reflect the rate of dephosphorylation of CFTR channels by endogenous phosphatases (Gadsby et al., 1993), and in native cardiac cells, differential sensitivity to phosphatase inhibitors revealed that PKA regulation of CFTR involves phosphorylation of the R domain at at least two distinct sites: P_1, which is essential for channel activation, and P_2, which controls the open probability of activated channels (Hwang et al., 1993). The okadaic acid (OA)-sensitive protein phosphatase 2A (PP2A) is believed to dephosphorylate the activation site P_1, whereas an okadaic acid-insensitive protein phosphatase (possibly PP2C) is believed to dephosphorylate the modulatory site P_2 (Hwang et al., 1993; see Fig. 11).

Fig. 7 *A* shows an experiment in which intraoocyte injection of OA (~1 mM) alone elicited some small activation of basal Cl^- current, which could be further enhanced by exposure to the cAMP cocktail (1×). The rate of current deactivation after withdrawal of the cAMP cocktail in oocytes injected with OA or superfused with OA (100 nM and 1 μM) was significantly prolonged with $\tau_{1/2}$ of deactivation changed from 7.4 ± 1.3 min to 12.0 ± 1.6 min ($n = 4$) and to 15.3 ± 1.9 min, respectively ($n = 3$; Fig. 7 *B*). Furthermore, in the continued presence of OA, PDBu still elicited a biphasic response (Fig. 7 *C*). The amplitude of the PDBu-induced currents was not significantly different in the presence or absence of okadaic acid (100 nM or 1 μM; Fig. 7 *D*), and the $\tau_{1/2}$ for PDBu-induced current inactivation was not significantly different in the absence (10.88 ± 0.97 min, $n = 4$) or presence (100 nM, 8.00 ± 0.74 min, $n = 4$; 1 μM, 8.83 ± 1.45 min, $n = 3$; $p > 0.05$) of okadaic acid. These results indicate that OA, as in native cardiac myocytes (Gadsby et al., 1993), can inhibit endogenous PP1 and 2A, which causes some prolongation of the rate of dephosphorylation of cAMP-activated CFTR currents in this expression

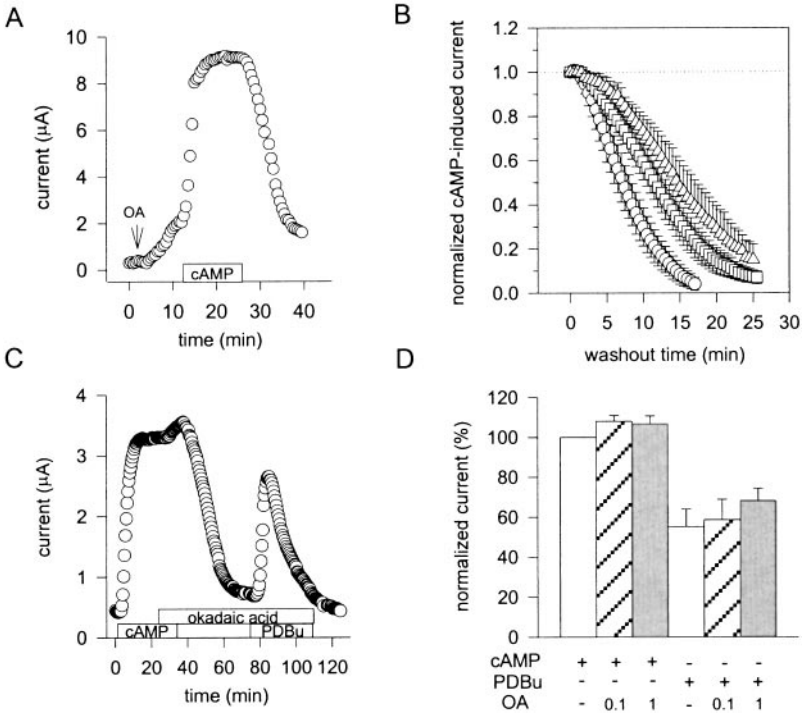

FIGURE 7 Effects of okadaic acid on PKA- and PKC-induced cardiac CFTR Cl^- currents. (*A*) Time courses of Cl^- currents during voltage steps at 70 mV activated by "cAMP cocktail" (1×) after intraoocyte injection of okadaic acid (OA) (approximate final internal concentration of 1 mM). (*B*) Time course of mean cAMP-induced currents at 70 mV, which were normalized with the peak value at time 0. At time 0, cAMP cocktail started to be washed out in the absence of OA (○) or during superfusion of OA (□, 100 nM; △, 1 μM). (*C*) Effects of OA on PDBu-induced CFTR currents. Outward Cl^- currents were elicited by applying 500-ms voltage steps to 70 mV from a holding potential of −30 mV. After the cAMP cocktail (1×)-induced current stabilized, cAMP cocktail was washed out for 20–25 min in the presence of okadaic acid (100 nM). Subsequently, PDBu was perfused in the presence of okadaic acid. (*D*) Effect of okadaic acid (OA) on the peak current activated by cAMP cocktail or PDBu. The data with different concentrations of OA (0, 100 nM, and 1 μM) were obtained from different groups of oocytes. No statistically significant difference was observed in the presence or absence of OA.

system, but phosphatases 1 and 2A are unlikely to be involved in the PKC-mediated inactivation of cardiac CFTR Cl^- channels.

In native cardiac myocytes, both OA-sensitive and OA-insensitive dephosphorylation of CFTR Cl^- channels has been demonstrated, and it has been proposed that the OA-insensitive phosphatase involved may be protein phosphatase 2C (PP2C) (Gadsby and Nairn, 1994). Although PP2C has recently been directly shown to dephosphorylate epithelial CFTR channels in cell-free membrane patches (Travis et al., 1997; Luo et al., 1998), there are no known effective inhibitors of PP2C. Another OA-insensitive phosphatase, the Ca^{2+}-dependent protein phosphatase 2B (PP2B), has recently been shown to dephosphorylate epithelial CFTR channels in NIH 3T3 fibroblasts (Fischer et al., 1998). To initially test whether a Ca^{2+}-sensitive phosphatase, possibly protein phosphatase 2B (PP2B), might also be involved in regulating the dephosphorylation of cardiac CFTR channels in oocytes, we examined the effects of intracellular chelation of Ca^{2+}, by injection of BAPTA tetrapotassium salt (1.25 mM) or pretreatment of oocytes with BAPTA-AM (100 μM), on the rate of deactivation of Cl^- current upon removal of the cAMP cocktail. As shown in Fig. 8 *A*, injection of BAPTA alone caused a small activation of CFTR Cl^- current, which could be further enhanced by exposure to the cAMP cocktail (1×). Interestingly, in contrast to OA, BAPTA injection appeared to nearly completely prevent deactivation of CFTR channels upon washout of the cAMP cocktail. A similar effect was observed in oocytes pretreated with BAPTA-AM (Fig. 8 *B*). Deactivation of CFTR channels was nearly completely prevented for periods of 30 min to 1 h after washout of the cAMP cocktail. This striking effect suggests that a Ca^{2+}-sensitive PP (PP2B) may be a potent regulator of CFTR dephosphorylation in oocytes. However, injection of two known inhibitors of PP2B, calmidazolium and cyclosporin A (Gietzen et al., 1981; Liu et al., 1991), at a concentration of 1 μM, failed to modify the deactivation of CFTR channels upon washout of the cAMP cocktail (data not shown). Because this concentration effectively inhibits PP2B in 3T3 cells (Fischer et al., 1998), it would appear that the ability of BAPTA injection or BAPTA-AM pretreatment to prevent deactivation of CFTR after cAMP washout may be unrelated to inhibition of PP2B. Because the concentrations of cAMP cocktail used in these experiments (1×) appear to activate at least 80% of the maximum CFTR Cl^- current available under control conditions (cf. Fig. 3 *D*), and BAPTA appears to prevent channel dephosphosphorylation, it is possible that channels activated under these conditions become locked into the fully phosphorylated (P_1P_2) state (Hwang et al., 1993; Fig. 11). It was previously shown that under normal conditions, CFTR channels activated by cAMP cocktail immediately dephosphorylate in response to injection of the specific PKA inhibitor Rp-cAMPS (Fig. 6 *A*). If, in fact, BAPTA treatment causes CFTR channels to become locked into the fully phosphorylated (P_1P_2) state, then channels under these conditions would not be expected to dephosphorylate in response to inhibition of PKA by Rp-cAMPS. In agreement with this prediction, CFTR Cl^- currents activated by cAMP cocktail in BAPTA-AM-pretreated oocytes were nearly completely insensitive to injection of Rp-cAMPS (Fig. 8 *C*).

Although many serine/threonine PPs are known to require metals for catalytic activity, PP2C may be a potential

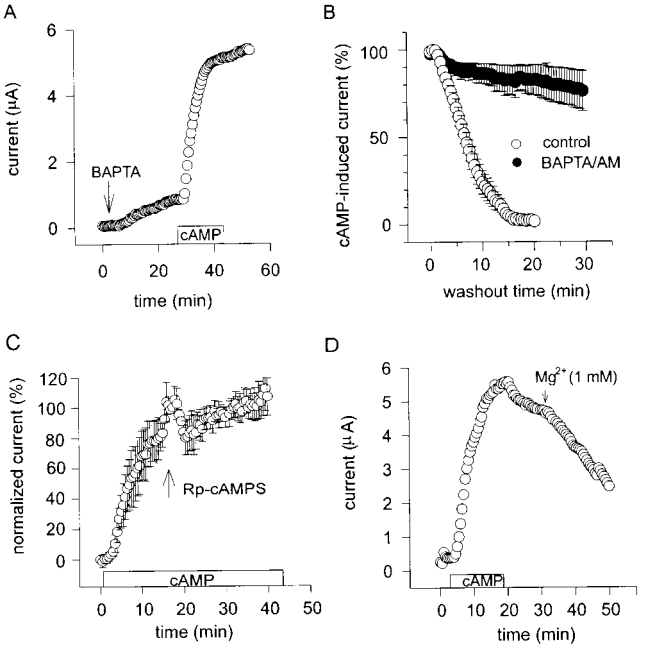

FIGURE 8 Effects of BAPTA on cAMP-induced cardiac CFTR Cl^- currents. (*A*) Time courses of Cl^- currents during voltage steps to 70 mV activated by "cAMP cocktail" (1×) after intraoocyte injection of BAPTA tetrapotassium salt (approximate final internal concentration of 1.25 mM). (*B*) Mean values of the decline of cAMP-induced Cl^- currents after washout of cAMP cocktail (at time 0), recorded at 70 mV in BAPTA/AM-pretreated (●) or untreated control (○) oocytes (n = 3–5). Currents are normalized with the maximum current just before washout of cAMP. (*C*) Effects of Rp-cAMPS on "cAMP cocktail" (1×)-induced currents in BAPTA/AM-treated oocytes expressing cardiac CFTR Cl^- channels. Rp-cAMPS was injected into oocytes to make an approximate intracellular concentration of 5 mM. Currents were normalized with the maximum currents at 70 mV in the absence of Rp-cAMPS. In contrast to control oocytes (Fig. 6 *A*), injection of Rp-cAMPS failed to affect cAMP-activated CFTR currents in BAPTA-AM pretreated oocytes. (*D*) Intraoocyte injection of Mg^{2+} (approximate final internal concentration of 1 mM) reverses the effects of BAPTA-AM on CFTR Cl^- channel dephosphorylation.

target for BAPTA, because PP2C, in contrast to other PPs, requires high (mM) Mg^{2+} for activity (Stemmer and Klee, 1991), and the ability of PP2C to directly dephosphorylate epithelial CFTR channels has been shown to require cytoplasmic Mg^{2+} in the millimolar range (Travis et al., 1997). To test whether the ability of BAPTA-AM pretreatment to nearly completely prevent CFTR channel dephosphorylation might be due to chelation of $[Mg^{2+}]_i$ and subsequent inhibition of endogenous PP2C activity, we tested whether direct intraoocyte injection of Mg^{2+} might reverse the effects of BAPTA-AM. As shown in Fig. 8 *D*, intraoocyte injection of Mg^{2+} (estimated final intracellular concentration 1 mM) effectively stimulated dephosphorylation of CFTR channels after washout of cAMP cocktail in an oocyte pretreated with BAPTA-AM. Similar results were observed in three additional BAPTA-AM-treated oocytes, suggesting that BAPTA-AM pretreatment prevents CFTR channel dephosphorylation by chelation of $[Mg^{2+}]_i$ and possibly inhibition of endogenous PP2C activity. Although additional studies are certainly required to unequivocally establish exactly which PP activity might be inhibited by BAPTA, there is little doubt that in BAPTA-treated oocytes, strong cAMP stimulation irreversibly activates CFTR Cl^- channels, which become locked into a fully phosphorylated state.

CFTR channels locked into partially or fully PKA-phosphorylated states are unresponsive to stimulation by PKC

We first tested whether locking CFTR channels into a partially phosphorylated (P_1) state would affect their response to PKC stimulation. Channels activated by unmasking basal PKA activity by inhibition of PP1 and 2A are expected to partition into both the partially (P_1) and fully (P_1P_2) phosphorylated states, in contrast to channels activated by strong PKA stimulation, which are expected to become more fully phosphorylated (Hwang et al., 1993; Fig. 11). Injection of oocytes with the membrane-impermeant PP1 and 2A inhibitor microcystin (5 μM) alone caused activation of cardiac CFTR channels, presumably by unmasking basal PKA activity (Fig. 9 *A*). These effects were not observed in water-injected control oocytes. Under this condition, subsequent exposure to PDBu still elicited the usual stimulatory and inhibitory effects. However, if some of the channels activated by microcystin are forced to dephosphorylate from the fully phosphorylated state (P_1P_2) by subsequent injection of Rp-cAMPS, then all channels are expected to become trapped into the partially phosphorylated (P_1) state. Remarkably, channels trapped in the P_1 state under these conditions failed to be stimulated by 100 nM PDBu (Fig. 9 *B*). These data show that PKC stimulation of cardiac CFTR Cl^- channels is highly dependent upon the PKA phosphorylation state of the channels.

To examine the effects of PDBu on channels in the fully (P_1P_2) phosphorylated state, oocytes were pretreated with BAPTA-AM and initially exposed to cAMP cocktail (1×) to irreversibly activate CFTR channels. After the washout of cAMP cocktail, when CFTR channels fail to dephosphorylate and currents are sustained, exposure to PDBu (100 nM) caused inactivation but produced little or no stimulation of CFTR currents (Fig. 9, *C* and *D*).

Functional effects of mutagenesis of two PKC phosphorylation sites in the R domain

Epithelial CFTR protein has previously been shown to be directly phosphorylated by PKC (Berger et al., 1993). Serines 686 and 790 are believed to represent two major consensus PKC phosphorylation sites in the R domain, and these have been shown to be phosphorylated by agents that activate PKC (Picciotto et al., 1992; Dulhanty and Riordan, 1994). However, the effects of mutations of these sites on functional regulation of CFTR Cl^- channels by PKC have not yet been examined in cardiac or epithelial CFTR isoforms.

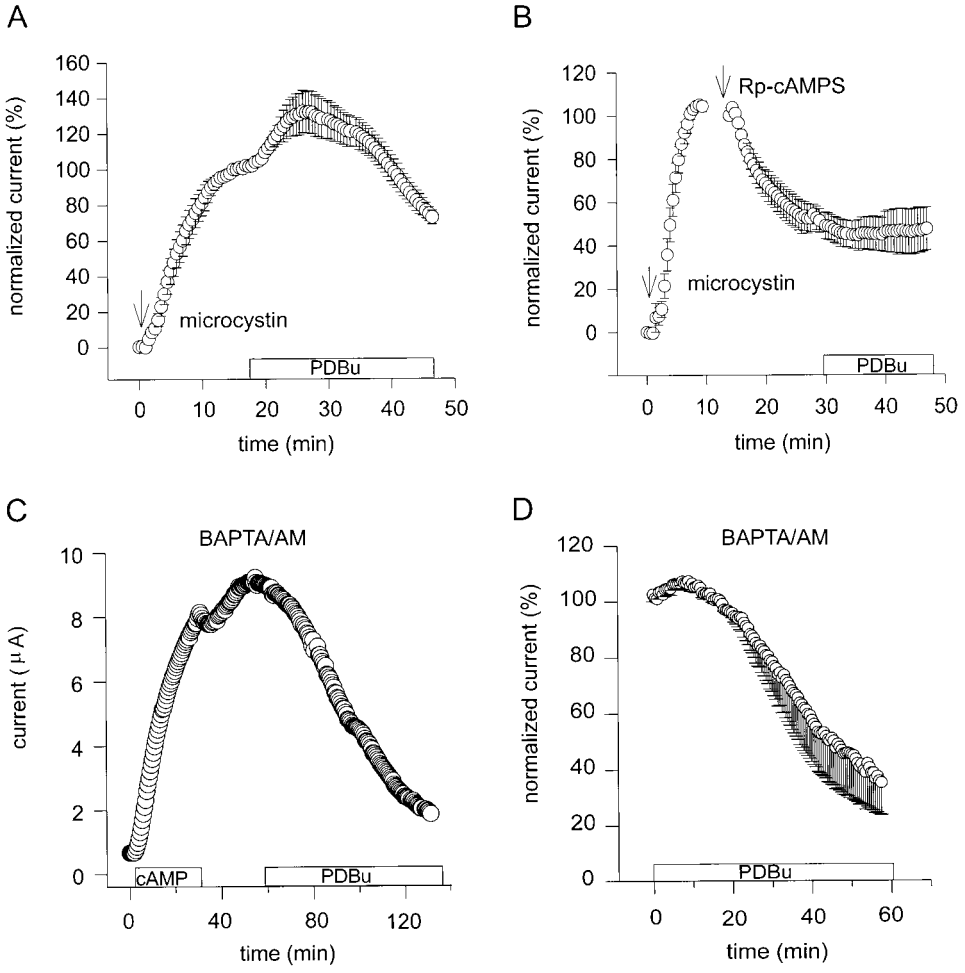

FIGURE 9 Effects of PDBu on cardiac CFTR channels locked into partially or fully PKA-phosphorylated states. (*A*) Time course of Cl^- currents was obtained by repetitive application of 500-ms voltage steps to 70 mV. Initial microcystin LR (approximate final concentration, 5 μM) injection activated CFTR currents, and PDBu (100 nM) was subsequently superfused for the time period indicated. (*B*) Microcystin injection activated CFTR currents, which were allowed to stabilize, and then Rp-cAMPS (30 μM) was injected to lock channels into a partially phosphorylated (P_1) state. Injection of Rp-cAMPS caused partial dephosphorylation, and then oocytes were superfused with PDBu. Currents amplitudes were normalized to the maximum current elicited in response to microcystin alone. Each data point represents a mean ± SEM ($n = 3$). (*C*) Effect of PDBu (100 nM) on the sustained currents after washout of cAMP cocktail in a BAPTA/AM-treated oocyte. Cl^- currents were obtained by repetitive application of 500-ms voltage steps to 70 mV. (*D*) Inhibitory effects of PDBu (100nM) on the sustained Cl^- currents (after washout of cAMP cocktail) at 70 mV in BAPTA/AM-treated oocytes. Currents were normalized to the maximum current just before the addition of PDBu. Individual points represent mean ± SEM ($n = 3$).

We examined cAMP and PDBu regulation of cardiac (exon 5−) CFTR channels in oocytes injected with mRNA encoding three mutant constructs: S686A, S790A, and the double mutant S686A-S790A. Fig. 10, *A* and *B*, shows representative currents at 70 mV for the S686A and S686A-S790A mutants during exposure to the cAMP cocktail, PDBu, and then a subsequent cAMP cocktail in the continued presence of PDBu. In these mutants, cAMP cocktail (1×) stimulated the Cl^- conductance, as well as in the wild-type cardiac (Fig. 5 *A*) or epithelial CFTR (Fig. 5 *B*). PDBu (100 nM) still activated CFTR currents in these mutants, although the amplitude appeared to be less than in the wild-type channels. Fig. 10 *C* shows a comparison of the absolute current amplitudes activated by cAMP in *Xenopus* oocytes injected with 47 ng of mRNA encoding S686A(card), S790A(card), S686A-S790A(card), wild-type cardiac (card), or wild-type epithelial CFTR. There were no significant differences in cAMP-induced current amplitudes in the three mutants and two CFTR wild-type channels. This is consistent with a previous report that showed no significant differences in PKA activation sensitivity for the S686A mutant in epithelial CFTR channels (Wilkinson et al., 1996). In contrast, the relative peak current amplitude elicited by 100 nM PDBu was significantly less in the three mutants compared to either wild-type channel (Fig. 10 *D*). Normalized PDBu-induced current amplitudes were 55.2 ± 8.8% ($n = 4$) for wild-type channels, 27.6 ± 5.5% ($n = 5$) for S686A, 29.0 ± 3.6% ($n = 4$) for S790A, and 25.1 ± 2.4% ($n = 4$) for the S686A-S790A double mutant.

Finally, to assess the degree and extent of PDBu-induced inactivation of CFTR currents in the mutant channels, current amplitudes elicited by a second exposure to cAMP

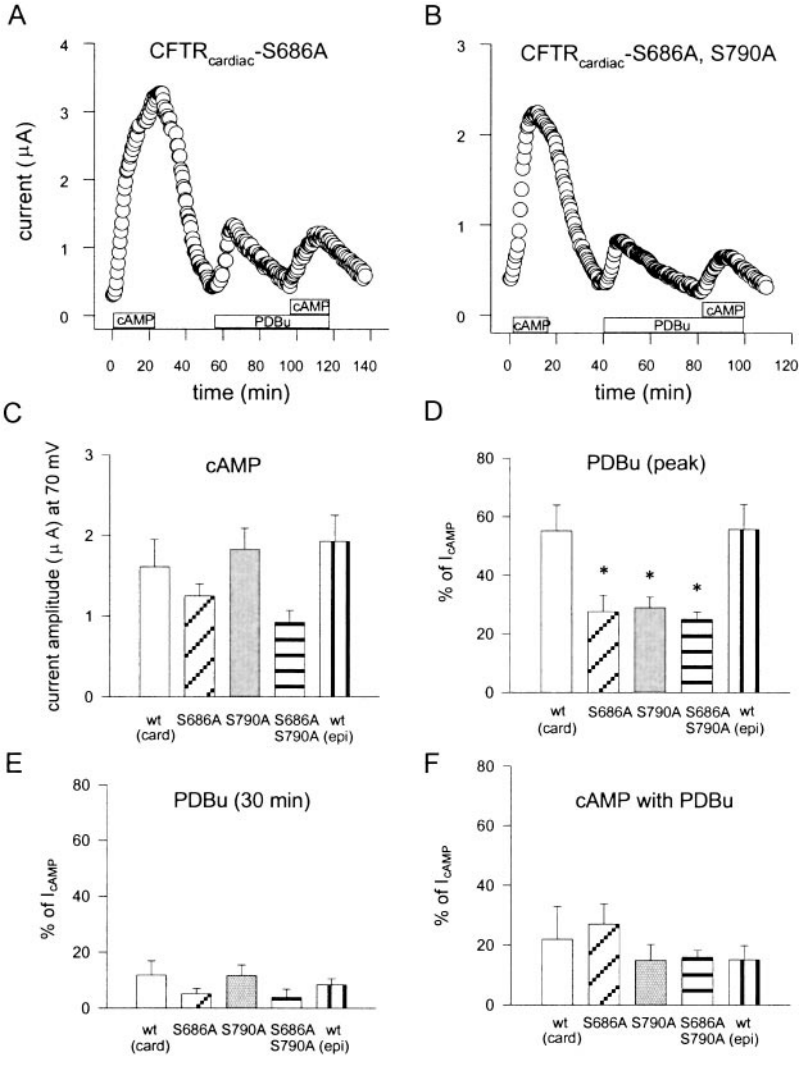

FIGURE 10 Effects of cAMP and PDBu on cardiac S686A and S790A mutant CFTR channels. (*A* and *B*) The effects of cAMP cocktail (1×) and PDBu (100 nM) on cardiac CFTR-S686A and CFTR-S686A, S790A were examined using the same protocol as in Fig. 5 *A*. Outward Cl^- currents were elicited by applying repetitive 500-ms voltage steps to 70 mV from a holding potential of −30 mV. (*C–F*) Comparison of absolute amplitude of cAMP cocktail (1×)-activated Cl^- currents (*C*), relative peak amplitude of PDBu-activated currents (*D*), relative amplitude of the currents in the continued presence of PDBu for 30 min (*E*), and relative amplitude of cAMP-induced currents in the presence of PDBu (*F*) among wild-type and mutant CFTR-injected oocytes. The data were obtained by using the protocol shown in *A* and *B*. Current amplitudes in *D–F* were normalized to the maximum current activated by cAMP alone. Responses in *F* represent the difference currents obtained by subtracting the current 30 min after the beginning of PDBu perfusion from the peak current obtained during the second exposure to cAMP in the continued presence of PDBu. Each data point represents a mean ± SEM (n = 4–5). *, $p < 0.05$ (ANOVA) compared with wild-type cardiac CFTR.

cocktail, after PDBu-induced current inactivation in the continued presence of PDBu, were measured and compared to the control cAMP-induced current amplitudes (Fig. 10, *A* and *B*). There were no apparent differences in the extent of PDBu-induced inactivation of CFTR, measured this way, for the three mutants and two CFTR wild-type channels. These results suggest that the stimulatory effects of PKC on cardiac CFTR Cl^- channels are significantly reduced by mutations in the two R domain consensus PKC phosphorylation sites, S686 and S790, but these mutations have very little effect on the extent of PKC-induced slow inactivation of CFTR Cl^- channels.

DISCUSSION

The present experiments provide direct evidence that stimulation of PKC results in modulation of the cardiac isoform (exon 5−) of CFTR (Hart et al., 1996). This evidence includes the following: 1) Both PDBu and cAMP cocktail activated Cl^- current in cardiac CFTR-injected oocytes, but not in water-injected oocytes. 2) PDBu-induced currents were time independent and exhibited a linear current-voltage relationship (with symmetrical Cl^-) similar to that of endogenous cAMP-activated Cl^- channels in native cardiac myocytes (Harvey et al., 1990; Collier and Hume, 1995). 3) The shift of reversal potential of the PDBu-induced currents, when external Cl^- was reduced, was close to the predicted shift of the estimated Cl^- equilibrium potential, indicating a significant Cl^- permeability, similar to PKA-activated Cl^- currents in cardiac CFTR-injected oocytes (Hart et al., 1996). 4) PDBu-induced currents were inhibited by the kinase inhibitor staurosporine and by a specific PKC inhibitor, bisindolylmaleimide. 5) The effects of PDBu were mimicked by the diacylglyerol analog DiC8, but not by the inactive analog 4a-phorbol. Finally, the findings that submaximum concentrations of PDBu and cAMP cocktail were additive in terms of the overall magnitude of current activated, whereas maximum stimulation by one agonist occluded the effects of the other agonist, support the conclusion that stimulation by PKA and PKC results in activation of the same population of CFTR Cl^- channels.

In oocytes perfused with Ca^{2+}-containing solutions, PDBu-activated CFTR Cl^- current amplitudes were ~55–65% of those of PKA-activated currents. However, in oo-

cytes perfused with Ca^{2+}-free solutions, PDBu-activated CFTR Cl^- current amplitudes were only 20–30% of those of PKA-activated currents. This Ca^{2+} dependence seems specific for CFTR currents activated by PKC, because the presence or absence of Ca^{2+} had no significant effect on the magnitude of currents activated by PKA stimulation. This Ca^{2+} dependence of PDBu-induced CFTR Cl^- currents can be explained by endogenous expression of Ca^{2+}-dependent PKC isozymes in oocytes (Ohno et al., 1988; Nishizuki, 1988). It is noteworthy that even in the absence of Ca^{2+}, PDBu still caused both activation and inactivation of CFTR currents, suggesting the presence of Ca^{2+}-independent PKC isozymes in this preparation as well. That expression patterns of Ca^{2+}-dependent and -independent PKC isozymes vary considerably between different cell types (Newton, 1996) may account for some of the variability reported for the efficacy of phorbol esters in activating CFTR in both native cells (Zhang et al., 1994; Walsh and Long, 1994; Shuba et al., 1996; Oleksa et al., 1996), as well as heterologous expression systems (Tabcharani et al., 1991; Berger et al., 1993). Other factors as well may be expected to influence the efficacy of activation of CFTR Cl^- channels by PKC stimulation. Our results indicate that the ability of phorbol esters to activate cardiac CFTR channels is highly dependent upon the level of basal PKA activity in oocytes. Thus preexposure of oocytes to the specific PKA inhibitor Rp-cAMPS nearly completely eliminates the ability of phorbol esters to subsequently activate the channels (cf. Fig. 6). Therefore, PKC phosphorylation alone seems unable to open CFTR channels, but requires basal PKA activation. The ability of PP inhibitors to cause substantial activation of CFTR channels expressed in oocytes (cf. Fig. 11) also indicates that there is a considerable level of basal PKA activity in this preparation, which may explain the ability of PDBu alone to cause robust activation of these channels in oocytes. However, the basal level of endogenous PKA and PP activities may be expected to vary considerably between different cell types and experimental conditions. This may account for some of the differences reported in the observed sensitivity of CFTR channels to PKC stimulation in different tissues.

Possible differences in the regulation of cardiac and epithelial CFTR channels by PKC phosphorylation were examined, because two putative PKC phosphorylation sites on exon 5 are missing in the rabbit cardiac isoform (Horowitz et al., 1993; Hart et al., 1996). However, functional analysis of both expressed CFTR channel isoforms in our experiments failed to reveal any significant differences in the general pattern of regulation by phorbol esters, suggesting that the two PKC phosphorylation sites on exon 5 play little, if any, role in the normal regulation of the channel by PKC phosphorylation. Site-directed mutagenesis of two serine residues (S686 and S790) in the cardiac CFTR isoform did reveal, however, the importance of two putative PKC phosphorylation sites in the R domain for channel regulation by phorbol esters. PDBu-induced CFTR current amplitudes were reduced by approximately half in the S686A, S790A, and the double mutant S686A-S790A constructs examined. These results provide the first functional confirmation of the importance of these PKC phosphorylation sites, which were predicted from earlier phosphorylation studies (Picciotto et al., 1992; Dulhanty and Riordan, 1994). The fact that these mutations failed to completely eliminate CFTR activation by phorbol esters suggests that other PKC phosphorylation sites, in addition, are also important for CFTR channel regulation.

A characteristic difference in the effects of the cAMP cocktail and PDBu on cardiac recombinant CFTR channels was the slow decay of currents observed in the continued presence of PDBu or DiC-8, compared to those activated by cAMP, which were well maintained in the continued presence of cAMP cocktail. Both current activation and current decay induced by phorbol esters can be attributed to PKC stimulation, because they were both prevented by the specific PKC inhibitor bisindolylmaleimide. A slow phase of PKC-induced down-regulation of L-type Ca^{2+} channels and Na/K ATPase expressed in oocytes has been reported and attributed to phorbol ester activation of endocytosis, triggering internalization of membrane proteins (Vasilets et al., 1990; Bourinet et al., 1992). However, we failed to detect any changes in oocyte membrane capacitance over the time period of PDBu-induced current decline with concentrations of PDBu as high as 500 nM (data not shown).

The most likely explanation for the PKC-induced current decline is desensitization of some component of the PKC pathway (Mond et al., 1991; Parker et al., 1995. This explanation is supported by the recent report that phorbol esters cause a similar slow inhibition of minK-KvLQT1 channels expressed in *Xenopus* oocytes (Lo and Neumann, 1998). In these experiments, the stimulatory effects of phorbol esters on I_{Ks} could be separated from their inhibitory effects by using lower doses and briefer exposure times, which were attributed to PKC phosphorylation at separate, distinct sites on the channel protein. However, the present experiments, which show a very similar slow, inhibitory effect of phorbol esters on a completely different membrane protein, CFTR, also expressed in oocytes, strongly suggest that this may be due to an alteration of the intracellular PKC signaling pathway, rather than to phosphorylation of a distinct site on the channel protein. This interpretation is also supported by our mutagenesis experiments that showed that mutations of S686 and S790 significantly reduced PKC-induced stimulation of CFTR channels but failed to affect PKC-induced CFTR channel decay. Because PKC phosphorylation appears to dramatically influence the PKA sensitivity of CFTR channels and may even be required for acute activation by PKA (Dechecchi et al., 1992; Jia et al., 1997; Middleton and Harvey, 1998), desensitization of the PKC pathway, in this way, could also be expected to reduce the responsiveness of CFTR channels to activation by PKA. Future studies are required to definitively interpret this effect.

In native cardiac cells, differential sensitivity to phosphatase inhibitors was the basis for the sequential PKA phos-

phorylation model of CFTR proposed by Hwang et al. (1993). In the present experiments, we used various PP inhibitors and the specific PKA inhibitor Rp-cAMPS to directly assess the effects of PKC stimulation on CFTR channels partially (P_1 state) or fully (P_1P_2 state) phosphorylated by PKA (Fig. 11, *top*). The regulation of CFTR Cl^- channels by PKC phosphorylation can be explained within the context of this model. Channels can exist in the PKA-dephosphorylated (D) state, a partially PKA-phosphorylated (P_1) state, or the fully PKA-phosphorylated (P_1P_2) state. Channels in the D state or in either of the PKA-phosphorylated states can be further phosphorylated by PKC (designated by *asterisks*). The role of endogenous PPs in dephosphorylation of PKC sites is unknown (?). Because PKC stimulation alone does not lead to channel activation (cf. Fig. 6), we assume that channel openings require PKA phosphorylation of distinct sites on the R domain, whereas ATP hydrolysis of the two nucleotide binding domains is directly coupled to channel gating (Gadsby and Nairn, 1994; Gadsby et al., 1995).

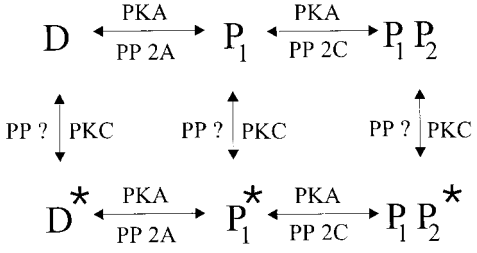

FIGURE 11 Model of CFTR channel regulation by PKA and PKC phosphorylation.

Exposure of oocytes to the PP1 and 2A inhibitors okadaic acid or microcystin, is expected to cause channels to eventually accumulate into the partially phosphorylated P_1 state, and under these conditions, PDBu still activated CFTR currents (Figs. 7 *C* and 9 *A*). Yet channels under these conditions are not really locked into the P_1 state, because some probably reside in the P_1P_2 state as well (depending upon the mode of activation, i.e., exogenous cAMP cocktail or endogenous basal PKA activity). Furthermore, channels in the P_1 state, once phosphorylated by PKC into the P_1^* state, can escape into the $P_1P_2^*$ state, because of the presence of rather large endogenous basal PKA activity in oocytes. Therefore, to examine the effects of PDBu on channels, in which only $P_1 \rightharpoonup P_1^*$ transitions are allowed, PP1 and 2A need to be inhibited and endogenous PKA needs to be inhibited to prevent any possible $P_1^* \rightharpoonup P_1P_2^*$ transitions. The results of such experiments are illustrated in Fig. 9 *B*. CFTR channels were first activated by intraoocyte injection of microcystin, and at the peak of the response, Rp-cAMPS injection caused channels to dephosphorylate and become trapped in the P_1 state. Subsequent exposure to PDBu, which should only allow $P_1 \rightharpoonup P_1^*$ transitions to occur, failed to cause further channel activation.

In our studies of cardiac CFTR channels expressed in oocytes, we found that injection of BAPTA or preincubation of oocytes in BAPTA-AM significantly attenuated deactivation of currents on removal of cAMP cocktail, suggesting that BAPTA may be a potent inhibitor of endogenous phosphatases. Because the effects of BAPTA were not mimicked by two known inhibitors of Ca^{2+}-sensitive phosphatases, calmidazolium and cyclosporin A, BAPTA must be exerting its inhibitory effects on another PP. Because the deactivation of CFTR currents in BAPTA-treated oocytes was much more significantly attenuated than in the presence of OA or microcystin alone, the effects of BAPTA may instead involve the inhibition of an OA-insensitive phosphatase, possibly PP2C. Although many serine/threonine PPs are known to require metals for catalytic activity, PP2C seems to be a likely target for BAPTA, because PP2C, in contrast to other PPs, requires high (mM) Mg^{2+} for activity (Stemmer and Klee, 1991; Travis et al., 1997). BAPTA is known to exhibit high selectivity for Ca^{2+} over Mg^{2+}, but its K_d for Mg^{2+} has been reported to be ~17 mM (Tsien, 1980). It therefore seems likely that injection of millimolar concentrations of BAPTA into oocytes or prolonged exposure of oocytes to BAPTA-AM may inhibit PP2C by direct chelation of intracellular Mg^{2+}. Consistent with this interpretation, intraoocyte injection of Mg^{2+} did reverse the effects of BAPTA and stimulated channel dephosphorylation (Fig. 8 *D*).

Alternatively, the ability of BAPTA to chelate intracellular Mg may interfere with Mg as a cofactor for ATP hydrolysis at nucleotide binding domain 2 (NBD2), which would cause channels to deactivate very slowly, like CFTR channels with mutations in the Walker motif in NBD2 (Wilkinson et al., 1996). Although Dousmanis et al. (1996) showed that such a slowing in channel closure can be effected by chelation of Mg, chelation of Mg also prevented channel activation by interfering with ATP hydrolysis at NBD1 as well. If the effects of BAPTA that we observe are due to interference with the Mg dependence of ATP hydrolysis at the NBDs, then BAPTA should also prevent or greatly attenuate the activation of CFTR by cAMP, an effect clearly not seen in our experiments, in which CFTR channel activation remained robust (or was even accentuated) in the presence of BAPTA (Fig. 8). In addition, if the effects of BAPTA are due to interference with Mg as a cofactor for ATP hydrolysis at nucleotide binding domain 2 (NBD2), channels would still be expected to dephosphorylate in response to PKA inhibition. Exposure of BAPTA-pretreated oocytes to the cAMP cocktail caused strong activation and forced nearly all CFTR channels to become locked in the fully phosphorylated P_1P_2 state, as evidenced by the nearly complete prevention of channel dephosphorylation after removal of the cAMP cocktail (Fig. 8), and these sustained currents were insensitive to Rp-cAMPS.

CFTR channels locked into the fully phosphorylated P_1P_2 state failed to be further activated by exposure to PDBu. These results, along with the lack of effect of phorbol esters on channels locked into the partially phosphorylated P_1 state

(Fig. 9 *B*), suggest that the stimulatory effects of PKC phosphorylation only occur when $P_1^* \rightarrow P_1P_2^*$ transitions are allowed. We interpret these results to mean that the stimulatory effects of PKC phosphorylation on CFTR Cl^- channels can be attributed to facilitation of PKA-mediated phosphorylation and channel transitions from $P_1^* \rightarrow P_1P_2^*$. Thus PKC phosphorylation of sites on the R domain may cause a conformational change that facilitates phosphorylation of the R domain by PKA. It is also possible that PKC phosphorylation may also facilitate PKA-mediated transitions from $D \rightarrow P_1$ ($D^* \rightarrow P_1^*$), which would be consistent with the observed attenuated amplitude of cAMP-activated CFTR currents in oocytes in which endogenous PKC was inhibited by 5 μM bisindolylmaleimide (Fig. 6 *D*), and with similar observations in native cardiac myocytes (Middleton and Harvey, 1998) and detached membrane patches from Chinese hamster ovary cells expressing epithelial CFTR channels (Jia et al., 1997).

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