PKC regulation of cardiac CFTR Cl⁻ channel function in guinea pig ventricular myocytes

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**Middleton, Lisa M., and Robert D. Harvey.** PKC regulation of cardiac CFTR Cl⁻ channel function in guinea pig ventricular myocytes. Am. J. Physiol. 275 (Cell Physiol. 44): C293–C302, 1998.—The role of protein kinase C (PKC) in regulating the protein kinase A (PKA)-activated Cl⁻ current conducted by the cardiac isoform of the cystic fibrosis transmembrane conductance regulator (cCFTR) was studied in guinea pig ventricular myocytes using the whole cell patch-clamp technique. Although stimulation of endogenous PKC with phorbol 12,13-dibutyrate (PDBu) alone did not activate this Cl⁻ current, even when intracellular dialysis was limited with the perforated patch-clamp technique, activation of PKC did elicit a significant response in the presence of PKA-dependent activation of the current by the β-adrenergic receptor agonist isoproterenol. PDBu increased the magnitude of the Cl⁻ conductance activated by a supramaximally stimulating concentration of isoproterenol by 21 ± 3.3% (n = 9) when added after isoproterenol and by 36 ± 16% (n = 14) when introduced before isoproterenol. 4α-Phorbol 12,13-didecanoate, a phorbol ester that does not activate PKC, did not mimic these effects. Preexposure to chelerythrine or bisindolylmaleimide, two highly selective inhibitors of PKC, significantly reduced the magnitude of the isoproterenol-activated Cl⁻ current by 79 ± 7.7% (n = 11) and 52 ± 10% (n = 8), respectively. Our results suggest that although acute activation of endogenous PKC alone does not significantly regulate cCFTR Cl⁻ channel activity in native myocytes, it does potentiate PKA-dependent responses, perhaps most dramatically demonstrated by basal PKC activity, which may play a pivotal role in modulating the function of these channels.

**THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) gene encodes a Cl⁻ channel found in epithelial tissue.** An alternatively spliced isoform of the protein has also been identified in cardiac muscle of a number of different mammalian species (16, 35). This cardiac isoform of CFTR (cCFTR) lacks exon 5, which encodes a cytoplasmic region located between the second and third transmembrane segments. Aside from this 30-amino acid deletion, this isoform of the protein isolated from rabbit ventricle is reported to retain >90% homology to human epithelial CFTR (eCFTR) (13). Both isoforms of the channel protein have been shown to contain a high concentration of phosphorylation sites in a region referred to as the regulatory domain, which contains several sites that can be phosphorylated by protein kinase A (PKA) and/or protein kinase C (PKC). PKA phosphorylation sites predominate in the regulatory domain, and PKA-dependent phosphorylation is the well-known activation mechanism for both cCFTR and eCFTR. The role of PKC phosphorylation is much less clear, especially for cCFTR. Although the regulatory domain contains 9 of 10 consensus sites for PKA phosphorylation, it contains only 7 of 29 consensus sites for PKC phosphorylation, and 2 of the 22 PKC phosphorylation sites found outside the regulatory domain are in exon 5 (26). The impact, if any, of the PKC phosphorylation sites that are missing from the cCFTR channel protein is not known but could underlie fundamental differences in the way that cCFTR and eCFTR are regulated.

Studies involving eCFTR have clearly demonstrated that this protein is phosphorylated by PKC (11). However, the functional consequence of PKC-dependent phosphorylation is somewhat complex. In heterologous expression systems, PKC alone stimulates eCFTR activity to a level that is only 10–15% of that activated by PKA (3, 19, 30). Although the response to PKC alone is relatively minor, this kinase also has been shown to potentiate the effect of PKA-dependent stimulation such that channel activity observed in the presence of both kinases has been reported to be 50–200% greater than that activated by PKA alone (19, 30). It has even been demonstrated that basal PKC activity, although it does not sustain channel activity on its own, is actually required for subsequent PKA-dependent stimulation (19). Activation of endogenous PKC and activation of endogenous PKA have also been shown to produce qualitatively similar results in native epithelial cells, which constitutively express eCFTR. In rat pancreatic duct cells, stimulation of PKC alone activated detectable Cl⁻ currents, but activation of PKC did increase the response to PKA-dependent stimulation by 31% (37). Also, in T84 epithelial cells, there is indirect evidence that basal PKC activity is necessary for PKA-dependent stimulation of eCFTR (6).

In contrast to its effect on eCFTR channel function, the effect of PKC on cCFTR channel activity is more controversial. We have previously reported that phorbol ester stimulation of endogenous PKC does not activate a macroscopic Cl⁻ conductance in guinea pig ventricular myocytes (24). Although other groups have described a phorbol ester-activated Cl⁻ current in these same cells (5, 28, 33, 34), the relative magnitude of such PKC-activated currents is unclear. Because the level of cCFTR expression varies significantly from cell to cell (18), the most meaningful estimates require comparison of PKC- and PKA-dependent responses obtained...
from the same cell. Zhang et al. (39) described a PKC- and PKA-activated Cl\(^{-}\) current in cat ventricular myocytes and found that PKC was as effective as PKA in activating that current. They also found that maximal PKC- and PKA-dependent responses were not additive. This suggests that activation of PKC does not potentiate PKA-dependent stimulation of this cardiac Cl\(^{-}\) current. However, the structure of the channel responsible for the Cl\(^{-}\) current in cat myocytes has not been determined. Although similar findings have been reported by Walsh and Long (34) in guinea pig ventricular myocytes, where the PKA-activated Cl\(^{-}\) current is known to be conducted by the alternatively spliced cCFTR (16), the interpretation of the results is complicated by the fact that their experiments were carried out using myocytes dialyzed with exogenous PKC isoforms. Furthermore, no one has yet examined whether basal PKC activity has any effect on PKA-dependent regulation of cCFTR.

Therefore, to clarify the functional role of PKC in regulating cCFTR, the purposes of the present study were 1) to reevaluate the effect of acute activation of endogenous PKC alone on cCFTR, 2) to determine whether there is any interaction between acute activation of endogenous PKC- and PKA-dependent responses, and 3) to examine the question of whether basal PKC activity affects PKA-dependent responses.

**MATERIALS AND METHODS**

Cell isolation. Hearts excised from anesthetized adult guinea pigs of either sex were perfused via the aorta with Krebs-Henseleit buffer (KHB) containing (in mM) 120 NaCl, 4.8 KCl, 1.5 CaCl\(_2\), 2.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 25.0 NaHCO\(_3\), and 11.0 glucose. The buffer pH was maintained at 7.35 by bubbling with 95% O\(_2\)-5% CO\(_2\) at 36°C. Ventricular myocytes were then isolated according to a previously described method (14). After dissection, the heart was first exposed to Ca\(^{2+}\)-free solution containing KHB for 5 min. Perfusion was then switched to Ca\(^{2+}\)-free KHB for another 5 min, following which enough collagenase B (Boehringer Mannheim) was added to the Ca\(^{2+}\)-free buffer to obtain a final concentration of ~0.5 mg/ml. After 30 min of digestion, the ventricles were removed, minced, rinsed free of collagenase, and reintroduced to Ca\(^{2+}\)-containing KHB. Gentle trituration freed individual cells for use in patch-clamp experiments.

Data acquisition and analysis. Except as noted, macroscopic membrane currents were recorded using the conventional whole-cell patch-clamp technique (12). Electrodes were pulled from borosilicate glass capillary tubing (Corning 7052, Garner Glass) and had resistances between 0.5 and 1.5 MΩ. The bath was grounded with a 3 M KCl-agar agar. Currents were recorded using an Axopatch 200 voltage-clamp amplifier (Axon Instruments) and an IBM-compatible computer with a TL-125 interface and pCLAMP software (version 5.7, Axon Instruments). Recording the current elicited by 100-ms voltage-clamp steps to \(+50\) mV every 3 s provided a record of the time course of change in Cl\(^{-}\) current in response to application and removal of drug(s). Once a peak or steady-state response to a particular drug was observed, a current-voltage (I-V) relationship was obtained by recording currents elicited during 100-ms voltage steps to test potentials from \(-120\) to \(+50\) mV.

Data were also analyzed using pCLAMP software. The Cl\(^{-}\) current was defined as the difference current obtained by subtracting currents recorded in the absence of any drug from those recorded in the presence of drug(s). In addition to the PKA-activated Cl\(^{-}\) current conducted by cCFTR, guinea pig ventricular myocytes also possess a swelling-activated Cl\(^{-}\) current (32), and PKC has been reported to stimulate a similar current found in dog atrial cells (7). However, DIDS inhibits the swelling-activated Cl\(^{-}\) current but not the cCFTR Cl\(^{-}\) current (32). Therefore, we verified that the Cl\(^{-}\) current enhanced by the activation of PKC was not inhibited by 250–500 μM DIDS (Sigma; see Fig. 5).

Current traces were measured over a 15-ms span at the end of each 100-ms voltage step. Slope conductances were calculated by linear regression of I-V relationships over the range of membrane potentials positive to the reversal potential. For dose-response relationships, data were fitted to appropriate equations using a nonlinear, least squares curve-fitting routine (SigmaPlot, Jandel Scientific). Results are reported as means ± SE. Statistical tests (SigmaStat, Jandel Scientific) used to evaluate the potential significance of responses to different drug treatments are indicated (see Figs. 3, 6, and 7).

Solutions. For study of the Cl\(^{-}\) current, cells were dialyzed with an internal solution composed of (in mM) 130 glutamic acid, 5.0 HEPES, 5.0 EGTA, 20.0 tetraethylammonium (TEA) chloride, 5.0 MgATP, and 0.1 Tris-GTP; pH was brought to 7.2 with CsOH. Cells were bathed in an external solution of (in mM) 140.0 NaCl, 5.4 CsCl, 2.5 CaCl\(_2\), 0.5 MgCl\(_2\), 5.5 HEPES, and 11.0 glucose; pH was raised to 7.4 with NaOH. All K\(^{+}\) currents were eliminated by using these K\(^{+}\)-free solutions containing Cs\(^{+}\) and/or TEA. L-type Ca\(^{2+}\) currents were eliminated by adding 1 μM nisoldipine (Miles Laboratories) to all external solutions, while Na\(^{+}\) channels were inactivated through the use of a ~30 mV holding potential. For study of delayed rectifier K\(^{+}\) currents, the internal solution was modified by replacing glutamic acid with potassium glutamate, TEA chloride with KCl, and CsOH with KOH; the external solution was modified by replacing CsCl with KCl. For perforated patch experiments, the technique used was as previously described (38). Amphotericin B (Sigma; 240 μg/ml) was added to the internal solution described above. All experiments were conducted at 36–37°C.

Drugs. Cells were exposed to external solution after being placed in a 0.5-ml chamber on the stage of an inverted microscope. Once the whole cell configuration was obtained, each cell was positioned in front of a rapid perfusion system, thereby making it possible to change the solution bathing a cell in <1 s (38). R(-)-isoproterenol-bitartrate (Research Biochemicals International) was dissolved in water. Chelerythrine chloride (Research Biochemicals International), phorbol 12,13-dibutyrate (PDBu; Sigma), 4α-phorbol 12,13-didecanoate (4α-PDD; Sigma), and bisindolylmaleimide (Calbiochem) were initially dissolved in DMSO (Sigma) and further diluted in water. All drug-containing solutions were prepared by diluting stock solutions 1,000-fold. The final concentration of DMSO never exceeded 0.1%. In experiments involving PDBu and 4α-PDD, 0.1% albumin (Sigma) was added to all external solutions to prevent the hydrophobic phorbol esters from adhering to the plastic tubing in our setup (2). Ascorbic acid (Sigma; 50 μM) was present in all isoproterenol-containing solutions to prevent oxidative degradation.

**RESULTS**

Effect of PDBu alone on cCFTR in guinea pig ventricular myocytes. Phorbol ester-dependent activation of endogenous PKC has been reported to elicit a measur-
able macroscopic Cl\textsuperscript{−} current in guinea pig ventricular myocytes (28, 33, 34). However, in a previous study, we saw no evidence for such a response (24). One possible explanation for the discrepancy is that intracellular dialysis associated with the whole cell patch-clamp technique caused the loss of a key component necessary for PKC-dependent activation of such a current. Therefore, we conducted a similar study using the perforated patch-clamp technique to limit intracellular dialysis. Cells were exposed to 100 nM PDBu, which is approximately 10 times the concentration necessary for half-maximal binding to and activation of PKC (23) and twice the concentration reported to effectively stimulate Cl\textsuperscript{−} channel activity in guinea pig ventricular myocytes (5, 33). Figure 1 demonstrates that we were still unable to detect any evidence for PKC-dependent activation of such a current. Subsequent activation of the Cl\textsuperscript{−} activity of eCFTR, it has been clearly demonstrated that activation of PKC alone does have a significant effect on channel activity through the PKA-dependent pathway. Cells were then exposed to 100 nM PDBu in the continued presence of isoproterenol. Figure 2 illustrates a representative experiment demonstrating that, under these conditions, PDBu does exert an effect on channel activity. The addition of PDBu did not affect the time independence, voltage dependence, or reversal potential of the current, consistent with the idea that PDBu had simply enhanced the magnitude of the current already activated by isoproterenol. Furthermore, in similar experiments, the current activated by PDBu in the presence of isoproterenol was not inhibited by DIDS (n = 3). This suggests that the PKC-activated current was conducted by cCFTR.

This stimulatory effect of PDBu was not mimicked by 4a-PDD, a phorbol ester that does not activate PKC (Fig. 3A). Whereas 100 nM PDBu increased the magnitude of the Cl\textsuperscript{−} current by 21 ± 3.3% (n = 9) when added subsequent to channel activation by 100 nM isoproterenol, the magnitude of the Cl\textsuperscript{−} current actually decreased by 6.0 ± 1.2% (n = 5) in the presence of 100 nM 4a-PDD (Fig. 3B). This decrease is most likely due to current rundown, a common phenomenon in which the magnitude of the current activated by an agonist diminishes with time in dialyzed cells (38). This suggests that the magnitude of the PDBu-dependent response is slightly underestimated. These data are also consistent with the idea that the PDBu-induced increase in Cl\textsuperscript{−} current magnitude is due to activation of PKC.

Having seen a response to activation of PKC in the presence of a supramaximally stimulating concentra-
tion (100 nM) of isoproterenol but not in its absence, we next wanted to determine whether the magnitude of the PKC-dependent response was affected by the level of β-adrenergic receptor stimulation. Therefore, we compared the response to activation of PKC in the presence of concentrations of isoproterenol between 1 and 30 nM. All responses were normalized to the magnitude of current elicited by that concentration of isoproterenol alone, in the absence of PDBu. We found that activation of PKC had no effect in the presence of a subthreshold concentration of isoproterenol (1 nM; n = 6), even though subsequent exposure to 1 µM isoproterenol clearly demonstrated that cCFTR channels were present. However, after exposure to 3, 10, and 30 nM isoproterenol, subsequent addition of 100 nM PDBu increased the magnitude of the Cl\textsuperscript{−} current by 17 ± 4.5 (n = 4), 31 ± 5.4 (n = 5), and 28 ± 5.6% (n = 6), respectively. The magnitudes of these PKC-dependent responses were statistically independent of the concentration of isoproterenol that was present. Figure 4 illustrates the fact that PDBu activation of endogenous PKC increases the magnitude of the current without affecting the threshold sensitivity of channel activation.

Fig. 2. Phorbol ester activation of endogenous PKC further stimulates Cl\textsuperscript{−} current after it has been first activated by protein kinase A. A: time course of changes in membrane current recorded during 100-ms voltage-clamp steps to +50 mV applied once every 3 s. A supramaximally stimulating concentration of isoproterenol (100 nM) elicits a Cl\textsuperscript{−} current that is further enhanced by subsequent addition of 100 nM PDBu. B: membrane currents recorded at time points in protocol illustrated in A. Currents were elicited by 100-ms voltage-clamp steps from holding potential of −30 mV to \( V_m \) values between −120 and +50 mV in 10-mV increments. C: \( V_m \) dependence of \( \Delta I \) obtained by subtracting currents recorded under control conditions (a) from currents recorded in presence of 100 nM isoproterenol alone (b), in presence of 100 nM isoproterenol + 100 nM PDBu (c), and after washout of isoproterenol and PDBu (d).

Fig. 3. PDBu-induced increase in isoproterenol-activated Cl\textsuperscript{−} current is due to phorbol ester activation of PKC. A: time course of changes in membrane current recorded during 100-ms voltage-clamp steps to +50 mV applied once every 3 s; 100 nM isoproterenol elicits a Cl\textsuperscript{−} current that is not further enhanced by concurrent exposure to 100 nM 4\alpha-phorbol 12,13-didecanoate (4\alpha-PDD), a phorbol ester that does not activate PKC. B: scatter plot depicting slope conductance (\( g_{Cl} \)) of CFTR Cl\textsuperscript{−} current recorded in presence of phorbol ester + isoproterenol normalized to that recorded in presence of isoproterenol alone, before addition of phorbol ester. Dashed line represents no change in \( g_{Cl} \) on addition of phorbol ester. Solid lines in each column indicate averages for each group. *Statistically significant difference between PDBu and 4\alpha-PDD responses (P < 0.0001; unpaired t-test). n, No. of cells.
Effect of PDBu on subsequent PKA-dependent activation of cCFTR. Metabolic labeling studies have demonstrated that the order in which eCFTR is exposed to PKA and PKC significantly affects the relative degree of protein phosphorylation (4). It was found that more labeled phosphate was incorporated when exposure to PKC preceded exposure to PKA. Assuming that phosphorylation of cCFTR is also subject to this kind of hierarchical effect, we performed experiments to determine whether it might be reflected in a difference in the functional response. For these experiments, cells were first exposed to PDBu alone for 5 min, after which time the response to 100 nM isoproterenol was measured in the continued presence of the phorbol. However, to determine whether the response to isoproterenol had been altered by preexposure to PDBu, it was necessary to precede this protocol with a brief exposure to the same concentration of isoproterenol alone. In this way, the effect of PDBu on isoproterenol-activated current could be compared with the current activated by isoproterenol alone in the same cell. This protocol is depicted in Fig. 5A. We found that the response to isoproterenol observed following exposure to PDBu was significantly greater than the magnitude of the response to isoproterenol alone. On average, the magnitude of the current activated by isoproterenol following exposure to PDBu was 36 ± 16% (n = 14) greater than the magnitude of the current activated by isoproterenol alone in the same cell. Consistent with the previous set of experiments, the current activated by isoproterenol in the presence of PDBu exhibited the same time independence, voltage dependence, and reversal potential as the current activated by isoproterenol alone. In addi-
as expected for the cCFTR Cl− current, this conductance was not blocked by DIDS (n = 4).

To verify that the increase in the magnitude of the response to isoproterenol observed in the presence of PDBu was due to activation of PKC, the same protocol was repeated using 4α-PDD (Fig. 5B). We found that the magnitude of the Cl− current activated by isoproterenol in the presence of 4α-PDD was actually 22 ± 9.7% (n = 9) smaller than that activated by isoproterenol alone. This decrease is most likely due to current rundown. The greater degree of rundown in these experiments is probably due to the longer duration of the protocol (38). To confirm this conclusion, we repeated the same protocol in the absence of any phorbol ester and found that the magnitude of the Cl− current activated by the second exposure to isoproterenol was 11 ± 6.9% (n = 11) smaller than that activated by the same concentration of isoproterenol 5 min earlier. The magnitude of this decrease is not statistically different from that observed in the presence of 4α-PDD, consistent with the idea that the decrease observed in the presence of this inactive phorbol ester is due to current rundown and not a direct effect of 4α-PDD. However, the magnitude of the response to isoproterenol following exposure to 4α-PDD (as well as that in the absence of phorbol ester) is significantly different from the magnitude of the response to isoproterenol observed following exposure to PDBu. These results with 4α-PDD are consistent with the idea that the effect of PDBu is due to activation of PKC. The fact that there was some rundown of the isoproterenol response in the presence of 4α-PDD indicates that the magnitude of the PDBu-dependent response was probably slightly underestimated. The results of individual experiments are illustrated in Fig. 6.

As to whether the order of kinase activation affected the magnitude of the PKC-dependent response, the effect that preactivation of PKC had on the subsequent response to isoproterenol was not statistically different from the magnitude of the response that acute activation of PKC had after the current had already been activated by isoproterenol. However, it should be noted that there was greater variability in the response to isoproterenol following preactivation of PKC (see Fig. 6), with the response to isoproterenol being increased by up to 150% in some cells.

Is PKC activity obligatory for PKA-dependent stimulation of cCFTR? In our hands, activation of endogenous PKC alone elicits no measurable macroscopic Cl− conductance in guinea pig ventricular myocytes (see Fig. 1). However, PKC activation does potentiate PKA-dependent stimulation of the cCFTR Cl− current (see Figs. 2 and 5). This raises the interesting question of whether basal PKC activity might also affect PKA-dependent regulation of cCFTR in native cardiac myocytes, in a manner similar to the effect that basal PKC has been reported to have on eCFTR channel activity in heterologous expression systems (19). To test this hypothesis, we examined the response to isoproterenol in the presence and absence of 10 µM chelerythrine, a highly selective inhibitor of PKC. A protocol similar to that used in Figs. 5 and 6 was followed when investigating the effect of PKC inhibitors. Cells were briefly exposed to 100 nM isoproterenol alone, followed by exposure to the PKC inhibitor alone for 5 min, after which time 100 nM isoproterenol was reintroduced in the continued presence of inhibitor. In this way, the effect of the PKC inhibitor on the isoproterenol-activated current could be compared with the current activated by isoproterenol alone in the same cell. This protocol is depicted in the experiment illustrated in Fig. 7A. After exposure to 10 µM chelerythrine, the magnitude of the isoproterenol-stimulated current was reduced by 79 ± 7.7% (n = 11).

The ability of chelerythrine to inhibit isoproterenol activation of the macroscopic Cl− current in native cardiac myocytes is consistent with the effect that this drug had on PKA-stimulated eCFTR channel activity (19). However, because our experiments relied on β-adrenergic receptor stimulation of cAMP production and because chelerythrine has been reported to increase phosphodiesterase activity (10), we also studied the effect of another highly selective PKC inhibitor, bisindolylmaleimide. After exposure to 30 nM bisindolylmaleimide, the magnitude of the isoproterenol-stimulated current was reduced by 52 ± 10% (n = 8). Although there is no statistical difference between the magnitude of the effects of bisindolylmaleimide and chelerythrine, both effects are significantly different from the average results obtained in the time control experiments (Fig. 7B). These findings are consistent with the idea that basal PKC activity in cardiac myocytes does modulate PKA-dependent activation of cCFTR.

![Graph](http://c298.ajpcell.physiology.org/ajpcell.00654-2006.Fig6.png)

Fig. 6. GCl− observed following a second exposure to isoproterenol (in presence or absence of phorbol ester) is normalized to that observed during an initial exposure to isoproterenol in absence of drug. Dashed line represents no change in normalized GCl− relative to initial exposure of isoproterenol alone. Average of all cells in each group is illustrated by a solid line through data points. There is a statistically significant difference between groups (P < 0.01; one-way ANOVA). *Results obtained in presence of PDBu are statistically different from those obtained in presence of 4α-PDD or absence of any phorbol ester (P < 0.05; Bonferroni t-test). n, No. of cells.
Elicit a measurable macroscopic Cl− current from guinea pig ventricular myocytes by stimulating endogenous PKC activity. This is consistent with our previous work (24), and it is also consistent with the effect that PKC has on eCFTR channel activity. Although exogenous PKC alone has been reported to stimulate activity of eCFTR in heterologous expression systems, the magnitude of such responses is small compared with the effect that PKA has on the same channels (3, 4, 30). Furthermore, activation of endogenous PKC alone has been reported to have no measurable effect on eCFTR constitutively expressed in epithelial cells (37).

The fact that we see no response when activating PKC alone would appear to be inconsistent with the effect that others have reported in cardiac myocytes. We can dismiss the possibility that the concentration of cytosolic free Ca2+ in our experiments did not permit activation of PKC. Although some isozymes of PKC are Ca2+-dependent, they are not prevalent in adult cardiac myocytes (25, 29). Even if they are involved, phorbol ester activation of these isozymes does not necessarily require Ca2+ (22). Furthermore, we failed to see PKC-dependent activation of a Cl− current even when we used the perforated patch technique, which does not disturb cytosolic Ca2+ (see Fig. 1), and when we buffered cytosolic Ca2+ at a level that should have permitted activation of Ca2+-dependent isoforms of PKC (24). Finally, we can infer that PDBu was activating PKC because we found that it did stimulate the delayed rectifier K+ current, an effect that was reversed by the PKC inhibitor bisindolylmaleimide. In addition, PDBu does affect the cCFTR Cl− current in the presence of isoproterenol, an effect that is not mimicked by the inactive phorbol 4α-PDD.

Perhaps the most plausible explanation for the discrepancy between our results with phorbol ester alone and those of others is that basal PKA activity is variable in different preparations. Our present work demonstrates that PKC enhances the cCFTR Cl− current in the presence of submaximal PKA-dependent stimulation of channel activity (see Fig. 4). Therefore, if basal PKA activity is high enough to maintain even a minimal degree of channel activation, then acute stimulation of PKC would be expected to elicit a response. PKC modifies PKA-dependent regulation of cCFTR. Although we do not find any evidence that activation of PKC alone stimulates the cCFTR Cl− current, activation of PKC in the presence of concurrent PKA-dependent stimulation does elicit a significant response. This suggests that PKA and PKC are exerting their effects at distinct phosphorylation sites. Our assumption has been that these sites are on the channel protein itself. However, there are other potential explanations. One possibility is that, in the presence of β-adrenergic receptor stimulation, PKC enhances cAMP production or inhibits cAMP breakdown. However, such a mechanism is not consistent with the fact that activation of PKC enhances the cCFTR Cl− current even in the presence of a supramaximally stimulating concentration of isoproterenol (Fig. 2). It is also not consistent with the fact that activation of PKC does not affect the threshold for β-adrenergic stimulation of the current (Fig. 4).

Another possible explanation for the effects that we have observed is that PKC is facilitating PKA-dependent channel phosphorylation by directly stimulating PKA or by inhibiting phosphatase activity. Although we cannot rule out either of these possibilities, a simpler interpretation might be that PKC is directly phosphorylating the channel protein itself. A recent study has
indicated that some cCFTR PKA phosphorylation sites are stimulatory and others are inhibitory (36). Therefore, it is plausible that PKC phosphorylation may have an allosteric effect on the subsequent phosphorylation of other sites by PKA. That is, PKC phosphorylation may facilitate PKA-dependent phosphorylation of stimulatory sites or suppress PKA-dependent phosphorylation of inhibitory sites. For PKC to affect channel function in this way would suggest that it is causing a conformational change in the tertiary structure of the protein. This question has actually been addressed, and it was determined that PKA, but not PKC, causes a conformational change (9). However, these studies used a construct limited to the regulatory domain, which contains 90% of the consensus sites for PKA phosphorylation but only 24% of those for PKC. In addition, the impact on the protein when exposed to both kinases concurrently was not investigated.

Is PKC obligatory for cCFTR activation? An important aspect of the role of PKC in regulating cCFTR activity relates to the question of whether PKC is mandatory for channel activation, as has been suggested for cCFTR (19). We have found that the PKC inhibitors chelerythrine and bisindolylmaleimide both attenuate isoproterenol-dependent activation of cCFTR currents, which is consistent with the idea that basal PKC activity plays an important role in regulating PKA-dependent responses. However, this interpretation assumes that chelerythrine and bisindolylmaleimide do not directly inhibit PKA activity. These two compounds were chosen on the basis of their selectivity for inhibition of PKC. Both are 200 times more potent as inhibitors of PKC than as inhibitors of PKA. The concentration of chelerythrine that causes 50% inhibition (IC50) of PKC is 0.7 µM, whereas the IC50 for PKA is 170 µM (15). For bisindolylmaleimide, the IC50 for PKC is 0.01 µM, whereas the IC50 for PKA is 2 µM (31). In our experiments, we used concentrations that were 17 and 67 times less than the IC50 values for inhibition of PKA, respectively.

Other alternative explanations for the apparent inhibitory effects of chelerythrine and bisindolylmaleimide must also be considered. For example, it is possible that these compounds can directly block the channel pore. Although there is no specific information about PKC inhibitors directly blocking CFTR Cl− channels, it should be noted that bisindolylmaleimide does not exert such an effect on other cardiac Cl− channels (8). Another possibility is that PKC inhibitors interfere with ATP interactions at one or both of the nucleotide binding domains (NBDs) that are essential for the activity of this channel. This could be a concern for bisindolylmaleimide, which acts as a competitive inhibitor at the ATP binding site of PKC (31). However, ATP binding and hydrolysis are believed to play a unique functional role at each of the NBDs: one NBD is responsible for channel opening and the other effects channel closing (1). Therefore, if bisindolylmaleimide were to interfere with ATP interactions at the NBDs, it might be difficult to predict what the net effect would be. A nonspecific interaction at an NDB is not likely to explain the effect of chelerythrine because this compound inhibits PKC activity independent of ATP binding (15).

Our working hypothesis has been that the mechanism underlying the reduced responsiveness of isoproterenol in the presence of PKC inhibitors and that responsible for the phorbol ester-induced increase in the magnitude of PKA-dependent channel activity are one and the same. Still, a possible interpretation of why PKC inhibitors attenuate the isoproterenol response is that PKC affects cAMP levels, either by stimulating cAMP production or by preventing its degradation. However, PKC does not affect the isoforms of adenylate cyclase present in cardiac muscle (17), and, if anything, PKC has been suggested to stimulate phosphodiesterase activity in adult ventricular myocytes (27). In any event, regardless of how PKC may modulate channel activity, the fact remains that the presence of PKC inhibitors significantly affects the overall cellular response to PKA-dependent activation of the channel.

Although the PKC inhibitors significantly reduced the responsiveness of the cells to isoproterenol, we cannot say that PKC activity was completely inhibited. Therefore, we cannot conclude whether PKC is absolutely obligatory for PKA-dependent activation of cCFTR. We can, however, comment on the difference in the extent of attenuation seen in the presence of bisindolylmaleimide and chelerythrine. One possible explanation is that we used a concentration of chelerythrine that was 14 times the IC50 for inhibition of PKC but a concentration of bisindolylmaleimide that was only 3 times the IC50 for inhibition of PKC. Another possibility is related to differences in the ability of each compound to inhibit specific isoforms of PKC. Bisindolylmaleimide has been shown to be less effective at inhibiting PKCe (21), the predominant isoform in adult ventricular tissue (25, 29). However, chelerythrine, at the same concentration and time of exposure that we used, has been shown to completely abolish effects attributed to PKCe in cardiac myocytes (20). Therefore, if indeed the PDBu-induced potentiation of isoproterenol-stimulated Cl− current is due to activation of PKCe, we might expect chelerythrine to more effectively inhibit the response.

Although we cannot conclude that PKC is absolutely mandatory for channel activation by PKA, the fact that both inhibitors significantly reduce the ability of isoproterenol to activate CCFTR Cl− current in these cells suggests an underlying role for basal phosphorylation by PKC in order for PKA to effectively activate the channel. Further studies characterizing the distribution, function, and inhibitor profiles of the PKC isoforms, as well as those investigating the point in the signaling pathway affected by PKC, may clarify the regulatory role PKC plays in modulating CCFTR activity.

In conclusion, besides suggesting that PKC regulates cCFTR and cCFTR in a similar manner, our results help clarify the role that PKC plays in regulating the
activity of CFTR in cardiac myocytes. We show that, although acute activation of PKC alone does not have a significant effect, endogenous PKC activity does potentiate PKA-dependent responses. Furthermore, our data are consistent with the idea that perhaps the most important role of PKC lies in its basal activity, which regulates subsequent PKA-dependent stimulation of cCFTR Cl− channel function. Therefore, the data presented here provide a functional example of a kinase cross talk mechanism in which cellular responses are modulated by the activity of different kinases.

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