Properties of a Protein Kinase C–Activated Chloride Current in Guinea Pig Ventricular Myocytes

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Cardiac ventricular myocytes from several species, including the guinea pig, possess a cAMP-dependent protein kinase A (PKA)-activated Cl\(^-\) channel. In the present study, the properties of a protein kinase C (PKC)-activated Cl\(^-\) current were studied in isolated guinea pig ventricular myocytes using the whole-cell arrangement of the patch-clamp technique. Intracellular dialysis of ventricular cells with PKC resulted in the activation of a large background current that displayed time-independent kinetics. In the presence of 146 mmol/L external Cl\(^-\) and 71 mmol/L internal Cl\(^-\), the reversal potential \((E_{rev})\) of the background current \((-17 \pm 1 \text{ mV})\) was close to that of the Cl\(^-\) equilibrium potential \((-18 \text{ mV})\), and the current versus voltage relation for the current was outward rectifying in shape. When [Cl\(^-\)]\(_i\) or [Cl\(^-\)]\(_o\) was reduced by substitution of Cl\(^-\) with aspartic acid, \(E_{rev}\) for the background current shifted in a manner expected for a Cl\(^-\)-selective channel. Based on \(E_{rev}\) measurements, the permeability sequence for this PKC-activated Cl\(^-\) channel was determined to be SCN\(^-\) > I\(^-\) > Br\(^-\) = Cl\(^-\).

The PKC-activated Cl\(^-\) current was not inhibited by the Cl\(^-\) channel blocker 4,4'-dinitrostilbene-2,2'-disulfonic acid (100 mmol/L) but could be blocked by anthracene-9-carboxylic acid (1 mmol/L). Activation of the current was abolished in the presence of the PKC inhibitor staurosporine (2.5 mmol/L). Under conditions designed to cause a maximal activation of the Cl\(^-\) channels by PKC, the addition of forskolin (1 mmol/L) to stimulate PKA caused only a slight further increase in the amplitude of the Cl\(^-\) current. Thus, PKC activates a Cl\(^-\) channel in guinea pig ventricular cells with properties similar but not identical to the PKA-activated channel. (Circ Res. 1994;74:121-129.)

**Key Words**: protein kinase C • ventricular myocytes • Cl\(^-\) channels • patch clamp

Protein kinases regulate the electrical activity of various cells through their actions on membrane ion channels.\(^1,2\) In the heart, stimulation of either cAMP-dependent protein kinase (PKA) or protein kinase C (PKC) results in an augmentation of the L-type Ca\(^{2+}\) current \((I_{Ca})\) and the delayed rectifier K\(^+\) current \((I_K)\).\(^8,12\) The presence of consensus sites for PKA- and PKC-dependent phosphorylation on the Ca\(^{2+}\) and delayed rectifier channels has led to speculation that PKA and PKC may act directly on these ion channel proteins.\(^13,14\)

Recently, a \(\beta\)-adrenergic–activated Cl\(^-\) current has been identified in guinea pig ventricular myocytes.\(^15-17\) This Cl\(^-\) channel displays a number of distinctive properties including an outward-rectifying whole-cell current-voltage (I-V) relation when measured with physiological Cl\(^-\) concentrations,\(^15-17\) an inhibition by monocarboxylic acids (eg, 100 \(\mu\)mol/L anthracene-9-carboxylic acid [A-9-C]),\(^18\) and a novel dependence on extracellular Na\(^+\) for activity.\(^17,19,20\) Stilbene derivatives such as 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) have been reported either to inhibit\(^15,17\) or to have no direct effect on this Cl\(^-\) current.\(^16,21\) Of particular relevance to the present study, the current is activated during intracellular dialysis of cells with the catalytic subunit of PKA,\(^19\) suggesting that phosphorylation is required for activation of the Cl\(^-\) channel. In contrast to cardiac Ca\(^{2+}\) and K\(^+\) channels,\(^22\) guanine nucleotide–binding proteins (G proteins) do not appear to have direct actions on this channel.\(^23\)

Phorbol ester compounds, such as phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate (PDB), which stimulate PKC,\(^24\) have also been reported to activate a Cl\(^-\) current in guinea pig\(^25\) and cat\(^26\) ventricular myocytes. The small size of the phorbol ester–activated current in the guinea pig cells\(^25\) has made it difficult to accurately characterize the properties of this channel and to determine whether PKA and PKC act in an additive manner to regulate the Cl\(^-\) channel. To overcome this limitation in the present study, ventricular cells were dialyzed with PKC and then exposed to PDB. Stimulation of PKC under these conditions resulted in the activation of a large whole-cell Cl\(^-\) current, which displayed an outward-rectifying I-V relation and a permeability for SCN\(^-\) and I\(^-\) greater than that of Cl\(^-\).

**Materials and Methods**

**Cell Preparation**

An enzymatic dissociation procedure described previously\(^27\) was used to isolate the myocytes. Briefly, hearts were removed from adult guinea pigs (250 to 400 g), mounted on a Langendorff-type column, and perfused for 10 minutes with a Ca\(^{2+}\)-free Tyrode's solution containing collagenase (0.25 to 0.32 U/mL, type B, Boehringer Mannheim Corp, Indianapolis, Ind) and protease (0.2 mg/mL, type 14 or 25, Sigma Chemical Co, St Louis, Mo). After 20 minutes of perfusion with 0.2 mmol/L Ca\(^{2+}\)-containing Tyrode's solution, the heart was dissected into small pieces, and single cells were obtained by gentle
agitation. Cells were stored at room temperature (22° to 25°C) in normal Tyrode’s solution (see below) and used between 1 and 10 hours after isolation.

Voltage Clamp

The patch-clamp method was used to record whole-cell ventricular currents using a PC-501 (Warner Instrument Corp., Hamden, Conn) and an EPC-7 (List and List Associates, Grell, NY) amplifier. Pipettes were made from Gold Seal Accu-fill 90 Micropets (Clay Adams Inc) and had resistances of 1 to 3 MΩ when filled with CsCl/aspartate internal solution (see below). Series resistance was determined by measuring the time constant of the capacity current and the membrane capacitance. Series resistance ranged from 3.5 to 8.0 MΩ with a mean±SEM of 5.8±0.3 MΩ (n=30 cells). Membrane currents were measured with a 12-bit analog to digital converter (Scientific Associates, Rochester, NY). Unless stated otherwise, data were sampled at 10 KHz, filtered at 2.5 KHz with a low-pass Bessel filter, and stored using a Softek 386 computer.

A reference electrode made from a Ag-AgCl pellet was connected to the bath using an agar salt bridge saturated with Tyrode’s solution. Data were adjusted for liquid junction potentials that are produced when there is an interface between dissimilar salt solutions. In the present study, the membrane potential (V_m) was corrected for junction potentials that arose (1) between the pipette solution and the bath solution (V_j1) and (2) between the bath solution and the reference electrode (V_j2) using the equation: V_m=V_j1+V_j2, where V_j1 was determined previously. In this equation, V was the voltage reading on the patch-clamp amplifier, V_j2 was equal to the offset potential created during the zeroing of the CsCl/aspartate internal solution in the NaCl, Na, NaBr, and NaSCN external solutions, and V_j1 was determined for each of the external solutions (bath solutions) using a 3 mmol/L KCl-filled patch pipette. With the exception of SCN⁻, which produced a 2-mV offset, anion substitution produced little change (<0.5 mV) in V_m.

Recording Solutions

Isolated cells were initially placed in normal Tyrode’s solution consisting of (mmol/L) NaCl, 132; KCl, 5; MgCl2, 2; CaCl2, 1; dextrose, 5; and HEPES, 5 (pH 7.4). After establishment of the whole-cell voltage clamp solution, the clamp was changed to a K⁺,-free external solution containing (mmol/L) NaCl, 140; MgCl2, 1; CaCl2, 1; dextrose, 5; HEPES, 5; and BaCl2, 1; along with 10 to 50 μmol/L tetrodotoxin (pH 7.4, 279±1 mosm, n=5). The internal pipette was eliminated by addition of 200 to 500 mmol/L nisoldipine (Miles Laboratories, West Haven, Conn) or 0.1 mmol/L CdCl2 to the external solution.

Patch electrodes were normally filled with an internal solution consisting of (mmol/L) CsCl, 60; cesium aspartate, 50; MgCl2, 2; CaCl2, 1; EGTA, 11; ATP (K⁺ salt), 5; HEPES, 10; and CsOH, 30 (pH 7.3, 280±1 mosm, n=5). Addition of PKC (see below) to the internal solution increased the total [Cl⁻] to 71 mmol/L. The ratio of EGTA to CaCl2 in the internal solution sets the free [Ca²⁺] to ~10 nmol/L. In some experiments free [Ca²⁺] was raised to 100 nmol/L to determine whether this might enhance the effects of PKC. Some trend toward greater stimulation was observed with higher [Ca²⁺], although these results were not quantified.

Measurement of Icl

Typically, background currents were measured during 40-millisecond voltage steps applied from a holding potential of −40 mV to various potentials. To activate the Cl⁻ current (Icl) in these experiments, purified PKC was dialed in via the micropipette. Although dialysis of PKC alone was capable of activating Icl, it was found that addition of a phorbol ester compound, such as PDB, to the external solution facilitated this activation. This action could be related to the ability of phorbol esters to increase the affinity of PKC for Ca²⁺ nearly 100-fold.

PKC used in this study was obtained from two sources. A mixed isozyme preparation of PKC, containing primarily the α and β isozymes, was generously supplied by Muriel C. Maurer and Dr. Julienne J. Sando, Department of Pharmacology, University of Virginia. This PKC preparation was purified from rat brain using DE-52 cellulose and threonine sepharose chromatography. A single isozyme preparation of PKC, containing the α isozyme, was purchased from GIBCO BRL, Gaithersburg, Md. This preparation was purified from baculovirus-infected insect cells expressing the rabbit brain PKC α isozyme. The enzymes were stored in a buffer solution (mmol/L: Tris-Cl, 20; EGTA, 0.5; and EDTA, 0.5; with 10% [vol/vol] glycerol) and diluted fourfold to fivefold in the dilution enzyme ranged from 1 to 5 nmol inorganic phosphate per minute per milliliter when assayed at 30°C in a buffer containing histone H1 (100 μg/mL), 300 μmol/L CaCl2, 100 μmol/L [γ-32P]ATP (250 cpm/pmol), 5 mmol/L magnesium acetate, 20 mmol/L Tris-Cl, phosphatidylserine (17 μg/mL), and diolene (2 μg/mL). This activity corresponded to an enzyme concentration of 10 to 40 mmol/L.

For internal cellular dialysis of PKC, a solution of either the mixed or single isozyme was included in the internal pipette solution. This was accomplished by backfilling a small amount of the enzyme solution into a pipette containing CsCl/aspartate internal solution. After disruption of the cell membrane, the enzymes move from the pipette into the cell by diffusion. On the basis of theoretical studies, PKC (molecular mass =80 000 D) should reach equilibrium at a slow rate (10 to 14 minutes) after the size of the cells and electrodes used in the present study. This time course closely matches experimental rates determined for augmentation of Icl and Ix (see Fig 1).

Use of this procedure allowed adequate time for the measurement of control and PKC-sensitive current.

One major problem encountered in the present study was the difficulty involved in making high-resistance seals to the cells and correctly breaking into the cell to form the whole-cell configuration. Even with backfilling of the pipettes, the combination of PKC and glycerol reduced the overall success of forming whole-cell recordings to <10%. However, in those cells in which the whole-cell configuration was achieved, activation of Icl by PKC was observed in a large proportion of the myocytes (>50%). As described previously, addition of PDB alone could result in an activation of Icl. The effect of PDB on the background current tended to be small (Icl at +60 mV, 275±35 pA; n=15; see previous study for experimental details) compared with that found with internal PKC dialysis (Icl at +60 mV, 817±120 pA; n=20; present study). Thus, although phorbol esters alone could activate Icl, it was necessary to use exogenous PKC to increase the size of the current so that the properties of the current could be carefully examined.

The reversal potential (Erev) for Icl was defined as the potential where the PKC-sensitive background current was zero. In those cases in which the exact zero current was not recorded, Erev was determined by fitting a straight line through the points on the I/V curve directly above and below this potential. The permeability ratio (P_Ca/P_K) of a test anion (A⁻) to Cl⁻ was determined using the Goldman-Hodgkin-Katz equation:

\[ E_{rev} = -RT/zF \ln \left( \frac{P_L[A^-]}{P_C[aCl^-]} \right) / (P_L[A^-] + P_C[aCl^-]) \]

where [Cl⁻] is 71 mmol/mL, [A⁻] is 6 mmol/mL, [A⁻] is 140 mmol/mL, P_L, P_C, and P_A are the permeabilities, R is the gas constant, T is the temperature (K), z is the valence, and F is Faraday’s constant. The increases in Cl⁻ currents were not detected in the Icl experiments, permeability to Na⁺ and K⁺ was neglected in determining the permeability ratio for A⁻ to Cl⁻.
Drugs and Chemicals
PDB, forskolin, A-9-C, and staurosporine were purchased from Sigma. A-9-C was prepared from a 500 mmol/L stock solution in 1N NaOH. DNDS was obtained from Molecular Probes, Eugene, Ore.

Results
Dialysis of Ventricular Cells With PKC Increases I_K and Activates a Background Current
The success of the present experiments was critically dependent on the ability to dialyze PKC into the ventricular cells. Since I_K is regulated by PKC in the guinea pig cells,9,10 measurement of this current was useful in monitoring cellular PKC responsiveness. The left panel of Fig 1 shows I_K recorded either at 1 minute (■) or 15 minutes (○) after membrane breakthrough of a ventricular cell with a patch pipette containing a KCl internal solution and the PKC enzyme. Dialysis of the myocytes with PKC caused a large increase in I_K (85±10%, n=3), which could be measured both in the time-dependent outward current recorded during the 2-second voltage step to +50 mV and the tail current recorded on return to the −40-mV holding potential (Fig 1).

In addition to the augmentation in I_K, dialysis of the cells with PKC was also associated with an increase in the background current. This effect was most readily observed as an increase in the time-independent outward current measured at +50 mV (see dashed lines in Fig 1). In the right panel of Fig 1, increases in I_K and the background current are plotted as a function of the time after membrane breakthrough with PKC-containing pipettes. Despite variances that existed from one cell to another, augmentation of I_K (time constant, 12 minutes) and the background current (time constant, 13 minutes) followed a very similar time course. Since increases in these currents were not observed in the absence of PKC, these results suggest that both I_K and the background current are responsive to the movement of PKC into the cell.

The PKC-Sensitive Background Current Is I_CL
Since stimulation of PKC with phorbol esters has previously been shown to activate a Cl^− channel in guinea pig23 and cat24 ventricular cells, it was to be determined whether the background current recorded in the presence of exogenous PKC was indeed I_CL. To accomplish this, K^+ currents were eliminated by dialyzing cells with a cesium internal solution and by addition of 1 mmol/L BaCl_2 to the external solution (see “Materials and Methods”). Under these conditions, negligible inward and outward currents are observed during the first few minutes after breakthrough of the cell membrane with a patch pipette containing PKC when 40-millisecond voltage steps are applied at −60 and +60 mV (see sweeps labeled □ in Fig 2). Since effects of PKC are not apparent at this time, these currents serve as appropriate control records. If 10 to 15 minutes is then allowed for PKC to enter the cell from the pipette, addition of the phorbol ester compound PDB (20 mmol/L) to stimulate PKC (see “Materials and Methods”) results in the activation of large inward and outward background currents (● in Fig 2).

The right panel of Fig 2 displays the I-V relation for the PKC-sensitive current recorded over the voltage range of −90 to +60 mV. The amplitude of the PKC-sensitive current was obtained by subtracting control currents recorded 2 minutes after membrane break-
through from currents recorded in the presence of PDB 14 minutes later. As can be seen in Fig 2, with $[\text{Cl}^-]_0$ of 146 mmol/L and $[\text{Cl}^-]$, of 71 mmol/L, the I-V relation displayed outward rectification. Although the magnitude of the outward rectification was sometimes minimal (eg, see right panel of Fig 7), this outward rectification was observed to some degree in all cells examined under these conditions (eg, $[\text{Cl}^-]_0$, 146 mmol/L; $[\text{Cl}^-]$, 71 mmol/L) (n=20). In addition to the outward rectifying properties, the PKC-sensitive current had an $E_{\text{rev}}$ ($-17\pm1$ mV, n=20) close to the Cl$^-$ equilibrium potential ($E_{\text{cl}}, -18$ mV), suggesting that this current might arise from Cl$^-$-selective ion channels in the ventricular cells.

To determine whether the PKC-sensitive background current does result from the activity of Cl$^-$ channels, the effect of reducing either internal or external Cl$^-$ on $E_{\text{rev}}$ was examined by substituting Cl$^-$ with aspartate. The left panel of Fig 3 displays the I-V relation for the PKC-sensitive current recorded with 10 mmol/L internal Cl$^-$. In this experiment, $E_{\text{rev}}$ for the current was $-60$ mV. Overall, in three experiments with $[\text{Cl}^-]_0$, $146$ mmol/L and $[\text{Cl}^-]$, of $10$ mmol/L, $E_{\text{rev}}$ was $-64\pm6$ mV ($E_{\text{cl}}, -67$ mV). In addition to shifting $E_{\text{rev}}$ to more negative potentials, reduction of internal Cl$^-$ caused the inward currents to become greatly attenuated in amplitude. This effect resulted in the appearance of a strongly outward-rectifying I-V relation (Fig 3, left panel). When $[\text{Cl}^-]$, was reduced to $10$ mmol/L with normal internal solution (ie, $[\text{Cl}^-]$, $71$ mmol/L), $E_{\text{rev}}$ shifted to more positive potentials ($32\pm3$ mV, n=3) (Fig 3, right panel) as predicted from the Cl$^-$ equilibrium potential ($E_{\text{cl}}, 49$ mV).

The $\beta$-adrenergic/cAMP-dependent PKA-activated I$_{\text{cl}}$ in guinea pig ventricular cells has been reported to display time-independent kinetics.$^{15-17}$ In contrast, Zigmond and Gibbons$^{35,36}$ have identified a Ca$^{2+}$-activated transient outward I$_{\text{cl}}$ in rabbit myocytes. Fig 4 shows an example of the PKC-sensitive background current recorded during 250-millisecond voltage steps applied to various potentials. As might have been predicted from the currents displayed in Fig 2, the PKC-sensitive

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**Fig 2.** Activation of a background current by protein kinase C (PKC). Left, Currents were recorded during 40-millisecond voltage steps to $-60$ and $+60$ mV measured either at 2 minutes (c) or 14 minutes (e) after membrane breakthrough with a patch pipette containing PKC. Currents measured at 14 minutes after breakthrough were recorded in the presence of 20 nmo/L phorbol 12,13-dibutyrate (PDB). The PKC-sensitive current was $-50$ and 220 pA in amplitude (at $-60$ and $+60$ mV, respectively) just before PDB addition (reversal potential, $-18$ mV at 9 minutes after breakthrough) and increased to $-234$ and 530 pA (at $-60$ and $+60$ mV) in the presence of PDB (as shown). Right, Current-voltage relation is plotted for PKC-sensitive background current. PKC-sensitive current was determined by subtracting currents recorded during the second minute after breakthrough of the pipette into the cell from currents recorded 12 minutes later. $[\text{Cl}^-]_0$ was 146 mmol/L, and $[\text{Cl}^-]$, was 71 mmol/L (Cl$^-$ equilibrium potential, $-18$ mV; reversal potential, $-10$ mV). A curved line was drawn by hand to the current-voltage relation to emphasize the outward rectification. Temperature was maintained at 24°C. Data are for cell B83.

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**Fig 3.** Dependence of protein kinase C (PKC)-activated current on internal and external Cl$^-$. Left, Current-voltage relation for PKC-sensitive background current was recorded with 10 mmol/L internal Cl$^-$ and 146 mmol/L external Cl$^-$ (Cl$^-$ equilibrium potential, $-67$ mV; reversal potential, $-60$ mV). A curved line was drawn by hand to the current-voltage relation. Temperature was maintained at 24°C. Data are for cell C11. Right, Current-voltage relation for PKC-sensitive background current was recorded with 10 mmol/L external Cl$^-$ and 71 mmol/L internal Cl$^-$ (Cl$^-$ equilibrium potential, 49 mV; reversal potential, 38 mV). The points have been connected by straight lines. Temperature was maintained at 23°C. Data are for cell C13.
EGTA and EDTA that were present in the PKC storage buffer (see "Materials and Methods"). Cell swelling induced through increases in intracellular osmolality causes the activation of \( I_{Cl} \) in canine ventricular cells.37 Furthermore, dialysis of the canine atrial cells under equiosmotic conditions for periods longer than 10 minutes may be associated with the activation of a similar \( I_{Cl} \). Since the PKC internal solution had an osmolality approximately two times greater than that of the normal internal solution (500 to 600 mosm), it was important to determine whether activation of \( I_{Cl} \) resulted specifically from PKC and not from nonspecific effects of the PKC solution (ie, increases in cell volume). Fig 5 shows the results of an experiment in which membrane currents were measured at various times after breakthrough of a cell with a "sham" PKC internal solution containing heat-inactivated PKC. Even after 20 minutes of dialysis, no obvious change could be observed in membrane currents recorded between \(-90\) and \(+40\) mV. Small increases in current (\(\approx 100\) pA) were sometimes noted at potentials positive to \(+40\) mV after prolonged periods of dialysis (30 to 40 minutes). However, there were no significant increases in inward current measured in any of the four cells dialyzed with the "sham" solution. To eliminate any nonspecific actions occurring at positive potentials in the present study, dialysis experiments were normally not conducted beyond 30 minutes after patch breakthrough.

As one further test to determine if \( I_{Cl} \) was specifically activated by PKC, experiments were performed in which the myocytes were pretreated with the PKC inhibitor staurosporine. In the presence of 2.5 \( \mu \)mol/L staurosporine, PDB caused almost no change in the inward and outward (Fig 6) \( I_{Cl} \) measured in three PKC-dialyzed cells. This sharply contrasts with the strong activation normally observed under these conditions (see Fig 9).

Permeability of \( I_{Cl} \) to Other Anions

To determine the anion selectivity of the PKC-activated \( I_{Cl} \), I-V relations were obtained both in the

![Diagram](http://circres.ahajournals.org/)

**Fig 4.** Protein kinase C (PKC)–activated \( Cl^- \) current (\( I_{Cl} \)) displaying time-independent kinetics. Shown at the bottom is the PKC-sensitive component of current recorded during 250-millisecond voltage steps (\( \gamma_{step} \)) applied to potentials ranging from \(-90\) to \(+30\) mV as illustrated at the top. The PKC-sensitive current was determined by subtracting currents recorded during the first minute after breakthrough of a pipette containing PKC into the cell from currents recorded 18 minutes later. \([Cl^-]\)_\(e\) was 146 mmol/L, and \([Cl^-]\)_i was 71 mmol/L \( (Cl^- \) equilibrium potential, \(-18\) mV). Temperature was maintained at 24°C. Data are for cell C15.

**Activation of \( I_{Cl} \) Results Specifically From PKC**

In addition to PKC, the CsCl/aspartate internal solution used in this study was supplemented with 4 to 5 mmol/L Tris, 2% to 2.5% glycerol, and small amounts of
presence of 140 mmol/L Cl\(^-\) and after equimolar substitution with a test anion. The relative permeability of the channel was then calculated based on the \(E_{rev}\) of the current in NaCl, NaSCN, NaI, and NaBr external solutions (see Table). As can be seen in the left panel of Fig 7, replacement of external Cl\(^-\) with SCN\(^-\) caused a shift of \(-15\) mV in \(E_{rev}\). This negative shift was accompanied by a slight increase (19±2%, \(n=4\)) in the slope conductance of \(I_{Cl}\) as measured near \(E_{rev}\). These effects of NaSCN were readily reversed on return to the NaCl external solution (results not shown). For the experiment displayed in the right panel of Fig 7, substitution of Cl\(^-\) with I\(^-\) caused \(E_{rev}\) to shift from \(-15\) to \(-24\) mV. Based on these and other replacement experiments, the selectivity sequence of the PKC-activated channel was determined to be SCN\(^-\) > I\(^-\) > Br\(^-\) = Cl\(^-\) (Table). It was also determined in four experiments that the permeability of F\(^-\) for the channel was less than that of Cl\(^-\). However, unlike the other anions, substitution of external Cl\(^-\) with F\(^-\) resulted in an increase in outward background currents under basal conditions. For this reason the data obtained with F\(^-\) were not included in the Table.

**Pharmacology of the PKC-Activated \(I_{Cl}\)**

Pharmacologic agents such as DNDS and A-9-C are known to block Cl\(^-\) channels present in a large number of tissues. Fig 8 shows the I-V relation for the PKC-activated \(I_{Cl}\) recorded in the presence and absence of a 1 mmol/L concentration of A-9-C. In three cells examined, addition of A-9-C resulted in a reduction of 51±8% and 52±9% in the amplitude of \(I_{Cl}\) when measured at \(-60\) and +60 mV, respectively. \(E_{rev}\) was not affected by A-9-C. In contrast to the inhibition caused by A-9-C, the stilbene derivative DNDS caused no significant change in the amplitude of \(I_{Cl}\) when tested at a concentration of 100 μmol/L (change in current, +6±2% at +60 mV; \(n=3\)) (results not shown).

**Effects of PKC and PKA on \(I_{Cl}\) Are Not Strongly Additive**

The results given in Figs 2 through 8 indicate that dialysis of ventricular myocytes with PKC leads to the activation of a time-independent outward-rectifying \(I_{Cl}\). Since stimulation of PKA has been shown to activate a similar current in these cells,\(^{15-17}\) it was important to determine whether PKA and PKC regulate the same Cl\(^-\) channel. Fig 9 (left panel) shows the results of an experiment in which a myocyte was dialyzed with a high concentration of PKC (40 mmol/L) and then treated as usual with PDB (•). When the PKC-activated \(I_{Cl}\) reached a peak amplitude and stabilized (at 20 to 25 minutes after breakthrough), a 1-μmol/L concentration of the diterpene compound forskolin was added to the external solution to stimulate PKA. As shown in Fig 9, forskolin caused only a slight further increase in \(I_{Cl}\) under these conditions. Overall, in five experiments, forskolin increased \(I_{Cl}\) by 0.8±0.2 pA/pF (at +60 mV) (Fig 9, right panel). These results sharply contrast with the large activation in \(I_{Cl}\) normally observed with forskolin in these cells.\(^{18,20,23}\)

One explanation for the lack of effect of forskolin on \(I_{Cl}\) in the above experiment could be that exposure of cells to PDB or dialysis with the PKC buffer solution (eg, Tris and glycerol) inhibits β-adrenergic-stimulated pathways and thus prevents PKA-mediated effects on the channel. To address this concern, a myocyte was dialyzed with the “sham” PKC internal solution (see above) and treated with external PDB. After 20 minutes of dialysis, addition of forskolin caused an eightfold increase in the background current as expected from earlier studies.\(^{18,20,23}\) (results not shown). In five experiments performed under these conditions, stimulation of PKA by forskolin resulted in an increase of 3.6±0.3 pA/pF in the background current (at +60 mV) (Fig 9, right panel).

**Discussion**

**Activation of a Ventricular \(I_{Cl}\) During Dialysis of PKC**

In the present study, dialysis of ventricular cells with PKC was found to cause the activation of an outward-rectifying, time-independent, whole-cell \(I_{Cl}\). Based on experiments demonstrating that background currents were not increased during dialysis with heat-inactivated PKC or when cells were pretreated with the PKC inhibitor staurosporine, it is concluded that activation of \(I_{Cl}\) results specifically from cellular effects of PKC. This conclusion is also supported by the finding that augmentation of \(I_{K}\) and activation of \(I_{Cl}\) followed a very similar time course during dialysis of PKC. Thus, these results are consistent with previous
studies indicating that phorbol ester compounds, by stimulating PKC, can activate a Cl⁻ channel in guinea pig²⁵ and cat²⁶ ventricular myocytes. However, it should be noted that in the previous study from this laboratory,²⁵ treatment of cells with phorbol esters activated an Iₚ that usually displayed a linear I-V relation. Cardiac muscle has been shown to contain predominantly the α isozyme of PKC.⁴⁰ Since the α isozyme was used primarily in the present study, differences between the dialyzed and endogenous enzyme cannot account for the outward-rectifying versus linear I-V relations measured in the two studies. A more plausible explanation for the linear I-V shape previously observed could be related to the use of a K⁺ internal solution in the earlier experiments.²⁵ Decreases in outward K⁺ currents during these experiments may have resulted in an underestimation of the size of the outward Iₚ. Nevertheless, it cannot be ruled out that different Cl⁻ channels are activated by endogenous and exogenous PKC. It should be noted that the phorbol ester-sensitive Iₚ found in cat ventricular myocytes does display outward rectification.²⁶

Similarity of the PKC-Activated Iₚ to Other Cardiac Iₚ$⁸$

Three apparently distinct whole-cell Iₚ$⁸$ have been identified in cardiac myocytes: a transient, Ca⁺⁺-activated current,¹⁰,³⁵,³⁶ a swelling-induced current,³⁵,³⁶ a swelling-induced current,²⁰,³⁵,³⁶ and a cAMP-dependent PKA-activated current.¹⁴,¹⁵,¹⁷ All of these currents would be expected to display an outward-rectifying I-V relation under the conditions of the present study. Thus, it is reasonable to speculate that the PKC-activated Iₚ may represent one of these currents or quite possibly a combination of all three. Based on the ability of the PKC-activated Cl⁻ channels to be active in the presence of low [Ca⁺⁺$^{++}$], (10 to 100 mmol/L), it is unlikely that PKC-sensitive current is related to the Ca⁺⁺$^{++}$-activated channel reported in rabbit atrial and ventricular cells.³⁵,³⁶ It is possible that the transient rise in intracellular Ca⁺⁺, believed to be responsible for the transient nature of this current,³⁵,³⁶ stimulates PKC, which then mediates channel opening through phosphorylation. However, unlike the PKC-activated Iₚ, stilbene compounds such as DIDS and 4-acetamido-4'-'-isothiocyanato-2,2'-disulfonic acid stilbene readily block the Ca⁺⁺$^{++}$-activated Iₚ.³⁵,³⁶

A swelling- and stretch-induced Iₚ has recently been observed in canine ventricular²⁷ and atrial²⁸ myocytes as well as in rabbit atrial and sinoatrial cells.⁴¹ The swelling-induced current displays an outward-rectifying I-V relation²⁷,³⁸,⁴¹ and is relatively insensitive to inhibition by the stilbene compound DNDS³⁸,⁴¹ (5 mmol/L DNDS inhibits). Of particular importance, the stretch-induced Iₚ recorded in sinoatrial cells has an anion selectivity of SCN⁻ > I⁻ > Br⁻ > Cl⁻,⁴¹ which is very close to the selectivity found for the PKC-activated Iₚ (SCN⁻ > I⁻ > Br⁻ = Cl⁻; see the Table). Thus, the swelling-induced Iₚ could represent a likely candidate for the PKC-activated Iₚ if this Cl⁻ channel is capable of becoming activated through PKC-mediated phosphorylation. However, the swelling-induced current is active in the presence of the protein kinase inhibitors 1-(5-isouquinolinesulfonyl)-2-methylpyperazine (H-7)$^{⁴¹}$ and N-(2-[methylamino]ethyl)-5-isouquinolinesulfonamide (H-8).³⁷ Furthermore, in preliminary experiments, we have failed to identify a swelling-induced current in the guinea pig ventricular myocytes using either
a hypsometric external solution (210 mosm) or a hypsometric internal solution (520 mosm) (K.B. Walsh, unpublished results). Thus, a contribution by the swelling-induced Cl⁻ channel to the PKC-activated I₃ₛ does not seem likely.

As shown in Fig 9, stimulation of PKA through addition of forskolin to the external solution, when occurring subsequent to activation of the Cl⁻ channels by PKC, produced only a slight further increase in I₃ₛ. Since the effects of PKA and PKC are not strongly additive on this current, one interpretation of these results is that the two kinases regulate the same Cl⁻ channel in the ventricular myocytes. This hypothesis is supported by the finding that both the β-adrenergic/PKA-activated current¹⁵-¹⁸ and the PKC-activated current share similar physiological properties (e.g., Cl⁻ dependence of ERₑ, outward-rectifying I-V relation, and time-independent kinetics). In addition, both the PKC-activated I₃ₛ and the PKA-activated I₃ₛ in skinned fibers are inhibited by A-9-C₁₈ but are insensitive to the stilbene derivative DNDS.²¹ However, as an alternative interpretation of the results given in Fig 9, exposure of ventricular cells to exogenous PKC could attenuate cellular responsiveness to forskolin and thus prevent activation of the channels by PKA. Stimulation of PKC has previously been shown to desensitize the adenylyl cyclase system in various cell types.²⁴ Despite the similarities between the PKA- and PKC-activated currents, it should be noted that the permeability of the PKC-activated channel for I⁻ was clearly greater than that for Cl⁻ (see the Table). In contrast, the permeability of the PKA-activated channel for I⁻ has been reported to be equal to or less than that of Cl⁻.⁴²,⁴³ In addition, I⁻ appears to greatly reduce the PKA-activated current,⁴⁴ possibly by directly blocking the pore of the channel. The difference in I⁻ permeability would suggest that either PKA and PKC activate different whole-cell I₃ₛ or that activation of the Cl⁻ channel by PKC alters the permeability of the channel for I⁻. Future single-channel studies may provide a more direct approach for studying the selectivity of heart Cl⁻ channels to various anions and determining the nature of the PKC-activated channel. Thus, in conclusion, PKC activates a Cl⁻ channel in guinea pig ventricular cells with properties similar but not identical to the PKA-activated channel.

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