Regulation of Adenosine Triphosphate-Sensitive Potassium Channels from Rabbit Ventricular Myocytes by Protein Kinase C and Type 2A Protein Phosphatase†

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Received December 15, 1994; Revised Manuscript Received March 16, 1995⊗

ABSTRACT: Myocytes from rabbit ventricle were enzymatically dissociated and the effects of protein kinase C (PKC) on the properties of single ATP-sensitive (K_{ATP}) channels were studied using excised inside-out membrane patches. Application of a purified, constitutively active form of PKC (20 nM) to the intracellular surface of inside-out patches caused a 48% ± 4% (n = 18) reduction in the open probability of single K_{ATP} channels. In the presence of the PKC inhibitors peptide PKC(19−31) or chelerythrine chloride, PKC had no effect on K_{ATP} channel properties. Heat-inactivated PKC had no effect on channel properties. K_{ATP} channel activity returned spontaneously after removal of PKC. However, application of okadaic acid, at a concentration (5 nM) appropriate for specific inhibition of type 2A protein phosphatase (PP-2A), after removal of PKC, prevented spontaneous recovery of channel activity. Treatment with purified PP-2A during the PKC-mediated inhibition of K_{ATP} channel activity caused a partial or full restoration of activity. The Hill coefficient for ATP binding was reduced from 2.2 (control) to 1.2 in the presence of PKC. The apparent inhibition constant (K_i) for ATP was unaffected by PKC [K_i(control) = 21 μM; K_i(PKC) = 20 μM]. PKC is, therefore, capable of inhibiting cardiac K_{ATP} channel activity, and the extent to which the channels remain phosphorylated appears to be dependent on membrane-associated PP-2A activity. These enzymes may, therefore, be involved in signal transduction mechanisms which serve to regulate the activity of cardiac K_{ATP} channels.

Potassium channels that are inhibited by intracellular ATP (K_{ATP}) have been found in a variety of different cell types, including cardiac tissue (Noma, 1983). Besides being regulated by ATP, K_{ATP} channel activity can be modulated by other factors such as nucleoside diphosphates, pH, lactate, divalent cations, and G-proteins [for reviews see Nichols and Lederer (1991), Edwards and Weston (1993), and Lazdunski (1994)]. It has also been demonstrated that phorbol esters, which have been shown to activate protein kinase C (PKC),[1] can modulate the activity of K_{ATP} channels in the insulin-secreting cell line RINmF5 (Ribalet et al., 1988; Wollheim et al., 1988; de Weille et al., 1989; Dunne, 1994) and smooth muscle cells (Bonev & Nelson, 1993). PKC can also modulate the activity of sodium (West et al., 1991), calcium (Ono & Fozzard, 1993), potassium (Walsh & Kass, 1991; Busch et al., 1992), and chloride channels (Picciotto et al., 1992; Berger et al., 1993).

The steady-state level of phosphorylation of any protein is dependent on the equilibrium between phosphorylation and dephosphorylation. Thus, if the cardiac K_{ATP} channel is dynamically regulated by PKC, then phosphatase activity must also play an important role in K_{ATP} channel regulation.

The aim of this study, therefore, was to provide information on whether (1) the cardiac K_{ATP} channel is directly regulated by PKC and (2) endogenous protein phosphatase activity reverses the action of PKC. Single cardiac K_{ATP} channels in excised inside-out membrane patches from rabbit ventricular myocytes were utilized along with a variety of biochemical and pharmacological tools.

MATERIALS AND METHODS

Materials. ATP (as K_2ATP, Sigma Chemical Co., St. Louis, MO) was added as required from a 10 mM stock in high-potassium bath solution which was prepared immediately before use. The nonhydrolyzable ATP analogue AMP-PNP (Sigma) was used in the same way as ATP. Glibenclamide (Sigma) was stored as a 10 mM stock solution in dimethyl sulfoxide. Okadaic acid (OA) was generously provided by Dr. Alastair Aitken (NIMR, London, U.K.). Chelerythrine chloride was purchased from LC Services Corp. (Woburn, MA) and stored at −20 °C as a 5 mM stock solution in distilled, deionized water. The PKC inhibitor peptide PKC(19−31) (House & Kemp, 1987) was synthesized using a Beckman Model 990B automated peptide synthesizer and purified by preparative reverse-phase HPLC as described by Litwin et al. (1991). The peptide was shown to be >95% pure by analytical HPLC and its structure was

† This work was supported by grants from the Medical Research Council of Canada (to R.J.F. and M.P.W.). R.J.F. is a Senior Scholar and M.P.W. a Medical Scientist of the Alberta Heritage Foundation for Medical Research (AHFMR). B.G.A. is a Heart and Stroke Foundation of Canada and AHFMR Fellow.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1995.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; OA, okadaic acid; PKC, protein kinase C; PP-2A, protein phosphatase type 2A; AMP-PNP, 5′-adenylyl imidodiphosphate.

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verified by amino acid composition analysis. It was stored at a concentration of 0.97 mM in 20 mM Tris-HCl (pH 7.5) and used at a final concentration of 2 μM.

Cell Isolation. Single rabbit ventricular myocytes were enzymatically dissociated and isolated using the methods described by Giles and Imaizumi (1988).

Single-Channel Recordings. The pipette solution used for all excised, inside-out patch recordings contained the following (in millimolar): NaCl, 140; KCl, 5; HEPES, 10; $CaCl_2$, 1; $MgCl_2$, 1; and glucose, 10, at pH 7.4. The standard bath solution contained (in millimolar): potassium aspartate, 130; KCl, 10; HEPES, 10; EGTA, 1; $MgCl_2$, 1.4; and glucose, 10. EGTA (1 mM) was included in the bath solution to prevent (1) the activation of Ca^{2+}-activated potassium channels and (2) Ca^{2+}-induced run-down of K_{ATP} channels (Findlay, 1988). The pH of the bath solution was adjusted to 7.4 with KOH. To measure single-channel conductance, current−voltage relationships were recorded in separate experiments under quasisymmetric conditions (140 mM K^+) using the standard high-potassium bath solution, in which 1 mM $CaCl_2$ replaced Mg^{2+} and EGTA, as the pipette solution.

Single-Channel Recordings and Data Acquisition. Standard patch clamp recording techniques (Hamill et al., 1981) were used to record single-channel currents in the inside-out patch configuration. Pipettes were pulled from borosilicate glass (PG52151-4, World Precision Instruments Inc., Sarasota, FL), and their shanks near the tip were coated with a silicone resin (Sylgard 184, Corning, NY) and fire-polished. Typical pipette resistance was 3−7 MΩ. After the establishment of a seal (>10 GΩ), the pipette and attached cell were lifted from the base of the chamber and a short, rapid spurt of solution was applied to rip the cell from the pipette, leaving an excised inside-out patch. Patches were then directly exposed to test solutions via a multi-input perfusion pipette with a common outlet at a flow rate of 100−150 μL/min. The time taken to change solutions was less than 2 s. All recordings were carried out at room temperature (20−22 °C).

Single-channel currents were recorded at a holding potential of 0 mV, amplified (Axopatch 200, Axon Instruments Inc., Foster City, CA), digitized (Neuro-corder DR-384, Neuro Data Instruments Corp., New York, NY), and then stored on videotape. Data were replayed through a 4-pole Bessel filter, low-pass filtered at 100 Hz (LPF-100, Warner Instruments Corp., Hamden, CT), and sampled at 250 Hz using a computer interface (Axolab 1100, Axon Instruments Inc.) connected to a Compaq PC (386) for analysis. Data were analyzed using pCLAMP version 5.5 and 6.0 software (Axon Instruments Inc.).

K_{ATP} channel open probability was expressed as NP_o, the product of N, the number of channels in the patch, and P_o, the mean open probability. NP_o was calculated by dividing the mean patch current (over a 20−30-s test period) by the mean unitary current amplitude. Mean current amplitudes were calculated from the difference between peaks in a multiple Gaussian fit to all-points current amplitude histograms which were constructed from data segments 20−30 s in duration. NP_o data were usually expressed in normalized form for each patch, i.e., N_o(test)/NP_o(initial [ATP]), where initial [ATP] = 50 μM unless otherwise stated.

K_{ATP} Channel Run-down. The activity of K_{ATP} channels in most tissues slowly decreases with time after patches are excised into ATP-free solution (see Figure 1). This phenomenon is known as "run-down" (Kakei & Noma, 1984; Findlay, 1987). Patches when excised were first exposed to 0.5−1 mM ATP for 30−60 s, to help slow run-down. Data from patches exhibiting significant run-down (>25%) were discarded.

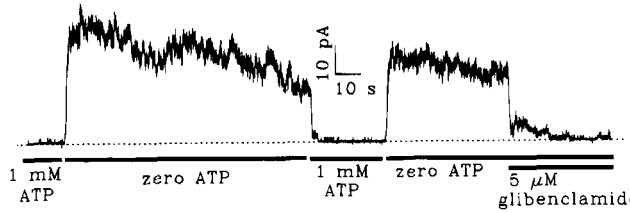

FIGURE 1: Identification of ATP-sensitive potassium channels from rabbit ventricular myocytes. Current trace from a single membrane patch in the excised inside-out configuration at a holding potential of 0 mV. ATP (1 mM) on the internal side of the patch inhibited activity of the K_{ATP} channels. Removal of ATP caused the activation of at least 30 channels, whose activity diminished gradually over a period of several minutes. Treatment with 1 mM ATP (~ 30 s) partially reversed run-down as seen by restoration of channel activity when the ATP was subsequently removed. Application of 5 μM glibenclamide caused a rapid inhibition of channel activity. Dotted line denotes zero current level.

In experiments designed to test the effects of PKC on K_{ATP} channel activity, patches were subsequently exposed to 50 μM ATP continuously unless otherwise stated. This concentration of ATP was chosen to provide enough ATP for phosphorylation by PKC, while allowing sufficient channel activation to occur to assay the effects of PKC. The K_m (ATP) of rat brain PKC was reported to be 6 μM (Kikkawa et al., 1982). In the presence of 50 μM ATP, there was no discernible run-down of K_{ATP} channel activity observed during the time period under which experiments were undertaken.

In experiments designed to test the ATP concentration dependence under various conditions, patches were exposed to a 1 mM ATP solution for 30 s, between test ATP concentrations, to minimize run-down.

Purification and Use of PKC and Protein Phosphatases. A constitutively active, oxidized form of PKC which does not require phospholipid, diacylglycerol, or calcium for activity was used during this study. PKC (a mixture of α, β, γ, and ϵ isoenzymes) was purified from rat brain by homogenization of the tissue in EGTA-containing buffer and sequential chromatography of the supernatant on columns of DEAE-Sephacel, phenyl-Sepharose, and poly(L-lysine)−agarose (Allen et al., 1994). The enzyme was rendered constitutively active by air oxidation at 4 °C and stored at −80 °C at a concentration of 2.0 μM in 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 mM dithiothreitol, 25% (v/v) glycerol, and 0.05% (v/v) Triton X-100. PKC was then used at a final concentration of 20 nM.

Type 2A protein serine/threonine phosphatase was purified from chicken gizzard smooth muscle as previously described (Winder et al., 1992) and stored at −80 °C at a concentration of 0.75 μM in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 20 mM NaCl, and 1 mM dithiothreitol. The phosphatase was used at a final concentraton of 7.5 nM. In several instances, inactivated enzymes were used as negative controls; this was achieved by immersing samples of stock enzyme solutions in boiling water for 5 min immediately before use.

Statistics. Statistical significance was evaluated by Student's paired or unpaired t tests, as appropriate. Differences

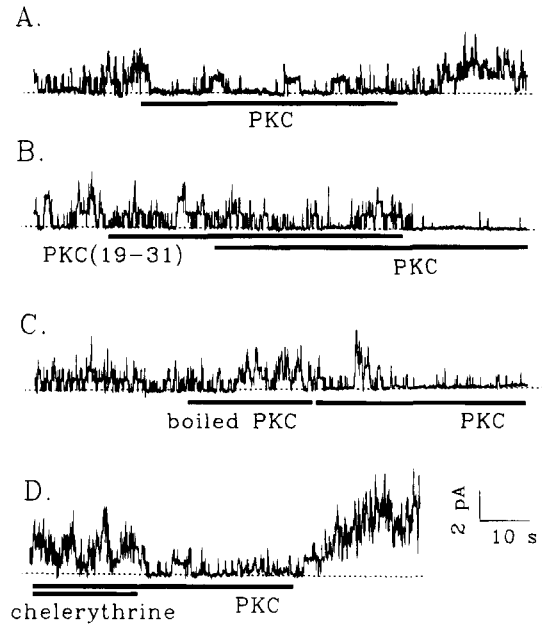

FIGURE 2: Effect of PKC on the spontaneous activity of K_{ATP} channels. Panels **A-D** represent current traces from individual excised inside-out patches. The ATP concentration in all cases was 50 μM. Dotted lines denote zero current levels. (**A**) Application of purified, constitutively active PKC (20 nM) caused an inhibition of channel activity which was reversible upon removal of PKC. (**B**) In the presence of the PKC inhibitor peptide PKC-(19−31) (2 μM), PKC failed to inhibit channel activity. (**C**) Heat-inactivated PKC was unable to inhibit channel activity. (**D**) Application of PKC in the presence of the PKC inhibitor chelerythrine chloride (5 μM) did not affect channel activity.

with values of $p < 0.05$ were considered to be significant. All values in the text are mean ± SEM.

RESULTS

Identification of K_{ATP} Channels. Upon excision of inside-out patches into a high-potassium ATP-free solution, spontaneous channel activity was observed. Subsequent exposure to 500 μM or 1 mM ATP caused elimination of the majority of channel activity. The removal of ATP caused a reactivation of channel activity, which slowly ran down in the absence of ATP (Figure 1). Exposure to 5 μM glibenclamide, a potent blocker of K_{ATP} channels, in ATP-free conditions caused rapid and almost complete inhibition of channel activity (see Figure 1). The single-channel conductance was 70 ± 5 pS ($n = 4$, in symmetrical 140 mM K^+), a value similar to that reported previously in rabbit ventricular myocytes (Han et al., 1993) and for cloned expressed channels from rat ventricle (Ashford et al., 1994). The currents reversed at 0 mV. At positive potentials, the single-channel current exhibited slight inward rectification in the presence of internal Mg^{2+}. The properties of these channels are consistent with their identification as K_{ATP} channels.

PKC Inhibits K_{ATP} Channels in a Reversible Manner. Application of constitutively active PKC to the intracellular surface of the patch significantly inhibited channel activity within 5−10 s (Figure 2, normalized $NP_o = 0.52 \pm 0.040$, $n = 18$, $p < 0.05$). This inhibitory effect of PKC on K_{ATP} channel activity was observed in 47 of 49 patches tested. Application of PKC did not affect the amplitude of single channel currents: at a holding potential of 0 mV, the mean unitary current amplitudes in the absence and presence of

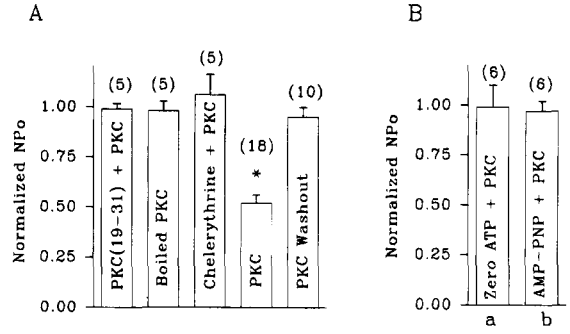

FIGURE 3: (**A**) Pooled data from the sets of experiments represented in Figure 2, panels **A**−**D**. Open probability (NP_o) is expressed in normalized form to the initial NP_o (in 50 μM ATP alone). (**B**) (**a**) In the absence of ATP, PKC has no effect on K_{ATP} channel activity (data normalized to NP_o in the absence of ATP). (**b**) In the presence of 50 μM AMP-PNP, a nonhydrolyzable ATP analogue, PKC does not inhibit K_{ATP} channel activity (data normalized to NP_o in 50 μM AMP-PNP alone). Numbers in brackets represent the number of experiments in each set. The asterisk denotes a significant difference ($p < 0.05$) from the other groups in the histogram (Student's t-test). Error bars are ± SEM.

PKC were 1.48 ± 0.11 pA and 1.46 ± 0.13 pA ($n = 7$), respectively. Subsequent washout of PKC almost completely restored K_{ATP} channel activity within 20−60 s (Figures 2A,D and 3A; normalized $NP_o = 0.95 \pm 0.05$, $n = 10$). In the presence of PKC(19−31), a specific PKC inhibitor peptide (House & Kemp, 1987), PKC exhibited no significant effect on channel activity (normalized $NP_o = 0.99 \pm 0.03$, $n = 5$; see Figure 2B). Upon removal of the inhibitor peptide, PKC was seen to rapidly inhibit K_{ATP} channel activity. PKC in the presence of the PKC inhibitor chelerythrine chloride (5 μM) or heat-inactivated PKC alone (Figures 2C,D and 3A) did not affect K_{ATP} channel activity (normalized $NP_o = 1.06 \pm 0.11$, $n = 4$, and 0.98 ± 0.035, $n = 5$, respectively). After removal of the chelerythrine chloride, or replacement of the heat-inactivated PKC with constitutively active PKC, channel inhibition was once again oberved (Figure 2C,D). In the absence of ATP, the application of PKC had no effect on K_{ATP} channel activity (normalized $NP_o = 0.99 \pm 0.11$, $n = 6$; see Figure 3B). In the presence of 50 μM AMP-PNP, a nonhydrolyzable analogue of ATP, application of PKC did not induce any inhibition of K_{ATP} channel activity (normalized $NP_o = 0.97 \pm 0.05$, $n = 6$; see Figure 3B).

Reversibility of PKC Inhibition Is Dependent on Membrane-Associated Type 2A Protein Phosphatase Activity. In order to test the hypothesis that membrane-associated protein phosphatase activity is responsible for the reversal of PKC-mediated inhibition of K_{ATP} channels, a potent inhibitor of type 1 and type 2A phosphatases, okadaic acid (OA) (Bialojan & Takai, 1988; Haystead et al., 1989), was used at a low concentration (5 nM) to specifically block type 2A protein phosphatase activity present in the excised patches (Figure 4). Application of OA (5 nM) to 9 patches had no or minimal effect on K_{ATP} channel activity (normalized $NP_o = 0.95 \pm 0.016$). PKC, in the presence of OA, again caused a significant inhibition of K_{ATP} channel activity (normalized $NP_o = 0.56 \pm 0.035$). When PKC was then removed but OA was retained, no reversal of PKC-mediated inhibition was observed (normalized $NP_o = 0.59 \pm 0.053$). If OA was subsequently removed and the patch was exposed to control solution, nearly complete recovery of K_{ATP} channel activity occurred within 30−60 s (see Figure 4A) (normalized $NP_o = 0.87 \pm 0.023$).

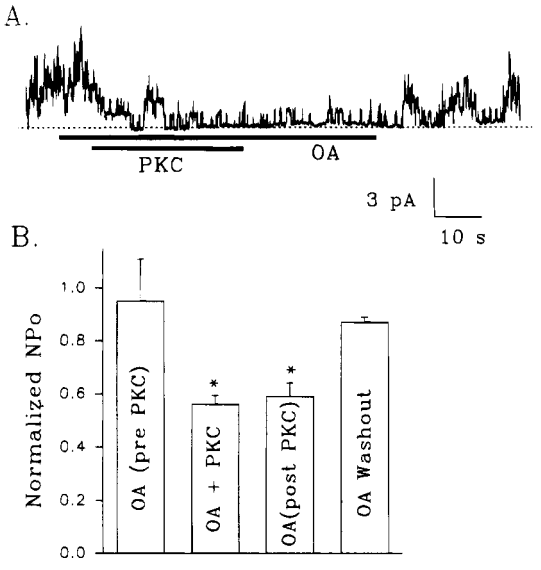

FIGURE 4: Phosphatase inhibitor okadaic acid (5 nM) prevented the reversal of PKC inhibition upon removal of PKC. (**A**) Current trace from a single excised inside-out patch; ATP concentration was 50 μM. Dotted line denotes zero current level. (**B**) Pooled data from nine patches treated under the same conditions as in panel **A**. Open probability is expressed in normalized form as a fraction of the initial NP_o (in 50 μM ATP alone). The asterisk denotes a significant difference ($p < 0.05$) from the OA (pre-PKC) and OA washout groups.

Exogenously Applied Type 2A Protein Phosphatase Prevents PKC-Mediated Inhibition of K_{ATP} Channels. In order to further test the effects of protein phosphatase activity on the PKC-mediated inhibition of K_{ATP} channels, purified type 2A protein phosphatase (PP-2A) was applied to excised inside-out patches. Application of PKC alone again caused a significant inhibition of channel activity (normalized $NP_o = 0.40 \pm 0.078$, $n = 7$). Application of active PP-2A (7.5 nM) during the PKC-mediated inhibition of channel activity caused near complete reversal of inhibition (normalized $NP_o = 0.86 \pm 0.058$, $n = 7$; Figure 5A). Application of heat-inactivated PP-2A in the presence of PKC did not affect the extent of PKC-mediated inhibition of channel activity (Figure 5B,C).

Effect of PKC on the ATP Sensitivity of the K_{ATP} Channel. The [ATP] vs channel open probability relationship was fitted, using a least-squares method of analysis, to the following equation:

$$NP_o = NP_{o(max)}/\{1 + ([ATP]/K_i)^n\}$$

where NP_o is the open probability at varying [ATP], $NP_{o(max)}$ is the open probability in zero [ATP], K_i is the inhibition constant for ATP, and n is the Hill coefficient.

Under control conditions, ATP inhibited K_{ATP} channel activity in a concentration-dependent manner with an apparent K_i of 21 μM and a Hill coefficient of binding of 2.2 $\pm$ 0.2 ($n = 7$; see Figure 6). In the presence of PKC, the apparent K_i was 20 μM and the Hill coefficient was 1.2 $\pm$ 0.3 ($n = 5$; Figure 6). In order to test whether the observed changes in the cooperativity of ATP binding induced by PKC are due to a phosphorylation process rather than a direct interaction of the enzyme with the channel, the following experiments were undertaken: Patches were initially exposed for 1 min to PKC, 1 mM ATP, and okadaic acid (5 nM); PKC was then removed and patches were then exposed to different ATP concentrations in the presence of okadaic acid (to prevent dephosphorylation of the PKC-phosphorylated channels). Under these conditions, the apparent K_i was 22 μM and the Hill coefficient was 1.13 $\pm$ 0.2 ($n = 6$; Figure 6).

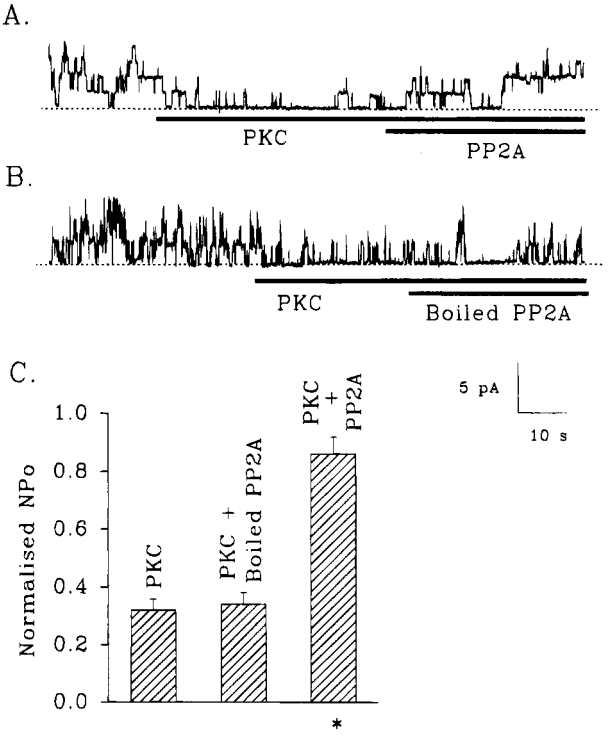

FIGURE 5: Type 2A protein phosphatase (PP-2A) reversed the PKC-mediated inhibition of channel activity. (**A**, **B**) Current traces from single excised inside-out patches: ATP concentration was 50 μM. (**A**) In the presence of PKC, PP-2A reversed the PKC-mediated inhibition of channel activity. (**B**) Heat-inactivated PP-2A had no effect. (**C**) Pooled data from seven patches using the protocols shown in panels A and B. Open probability (NP_o) is normalized by dividing by the initial NP_o (in 50 μM ATP alone). Dotted lines denote zero current levels. The asterisk denotes a significant difference ($p < 0.05$) from the other groups in the histogram.

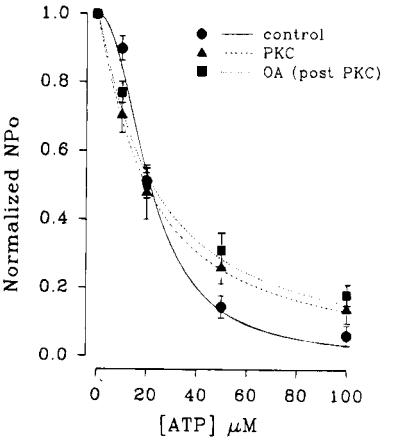

FIGURE 6: ATP concentration versus normalized open probability in the absence and presence of PKC and in the presence of 5 nM okadaic acid after an initial exposure to PKC. Open probabilities were normalized to the NP_o at zero ATP in control ($n = 7$) and OA post-PKC experiments ($n = 6$) or obtained from fits to the data for each patch (see text) in the presence of PKC ($n = 5$). Solid and dashed curves represent fits to the data (see text for equation). The apparent K_is for ATP were 21 μM (control), 20 μM (PKC), and 22 μM (OA post-PKC). The Hill coefficient for ATP binding was reduced from 2.2 (control) to 1.2 (PKC) and 1.13 (OA post-PKC).

In experiments where PKC was present, $NP_{o(max)}$ was estimated by fitting individual NP_o values to the above equation for each patch, as no experimental measurement of channel activity could be made at zero [ATP] due to the ATP requirement of PKC activity.

DISCUSSION

In this study we have demonstrated that (1) PKC inhibits the activity of single cardiac K_{ATP} channels, (2) the extent to which channels remain phosphorylated and inhibited is dependent on the activity of an endogenous, membrane-associated type 2A protein phosphatase, and (3) PKC-catalyzed phosphorylation of the K_{ATP} channel, or perhaps an associated regulatory protein, reduces the cooperativity of ATP-mediated inhibition of the channel.

PKC and K_{ATP} Channel Inhibition. The ability of PKC to phosphorylate and hence regulate the activities of several different ion channels is well documented (see introduction). PKC has also been shown to inhibit K_{ATP} channels from smooth muscle (Bonev & Nelson, 1993), and activation of PKC indirectly by phorbol esters can modulate K_{ATP} channel activity in insulin-secreting cell lines (Ribalet et al., 1988; Wollheim et al., 1988; deWeille et al., 1989; Dunne, 1994). In this study we have shown for the first time that PKC is capable of directly inhibiting single K_{ATP} channels from the heart and that the PKC-mediated phosphorylation does not affect either the unitary channel conductance or the half-inhibition concentration for ATP, but modulates channel open probability by changing the shape of the ATP dose-response relation. PKC activity is required for this effect since the heat-inactivated enzyme was ineffective, and PKC, either in the absence of ATP or in the presence of AMP-PNP (a nonhydrolyzable analog of ATP), had no significant effect on K_{ATP} channel activity. In the presence of either PKC-(19−31) (House & Kemp, 1987) or chelerythrine chloride (Herbert et al., 1990), two selective inhibitors of PKC, PKC had no effect on K_{ATP} channel activity. PKC(19−31) is a synthetic peptide corresponding to the pseudosubstrate domain of PKC which acts as a competitive inhibitor of PKC with respect to the protein substrate. This peptide is ineffective or weakly inhibitory toward other kinases (House & Kemp, 1987). Chelerythrine chloride is a potent and selective inhibitor of PKC which inhibits PKC with an IC_{50} of 0.66 μM and acts as a competitive inhibitor with respect to the phosphate acceptor and a noncompetitive inhibitor with respect to ATP (Herbert et al., 1990). These data confirm that the action of PKC is through direct phosphorylation of the channel or a patch-associated regulatory protein and not via some nonspecific effect.

The concentration of PKC used in this study was 20 nM or 1.6 unit/mL (where 1 unit is the amount of PKC catalyzing the incorporation of 1 nmol of P_i/min at 30 °C into histone III-S), a concentration similar to that found in intact cells (0.62−8.7 units/mL) assuming the tissue to be 10% (w/v) protein (Stabel et al., 1987).

Endogenous Type 2A Protein Phosphatase Activity Is Responsible for the Reversal of PKC-Mediated Inhibition of Channel Activity. Upon removal of PKC, the inhibition of K_{ATP} channel activity was partially or fully reversed. This suggests that there is a mechanism present for the efficient dephosphorylation of the channel, probably catalyzed by an endogenous protein phosphatase. Application of the potent protein phosphatase inhibitor okadaic acid, after removal of PKC, prevented the normal restoration of channel activity. The concentration of OA used in this study was 5 nM, a value well above the IC_{50} for type 2A protein phosphatase (0.2 nM) but 4-fold lower than the IC_{50} for type 1 protein phosphatases; protein phosphatases 2B and 2C are resistant to 1 μM okadaic acid [for reviews, see Cohen (1989) and Hardie (1990)]. Okadaic acid has no effect on PKC (Haystead et al., 1989). Data from this set of experiments, therefore, imply the existence of a membrane- associated type 2A-like protein phosphatase activity which is capable of dephosphorylating the PKC target phosphorylation site(s) on, or associated with, the K_{ATP} channel. Indeed, it has been previously shown in other channel types that application of protein phosphatase inhibitors such as okadaic acid can modulate ion channel activity (Ono & Fozzard, 1993; Frace & Hartzell, 1993; Obara & Yabu, 1993; Murphy et al., 1993). The involvement of a type 2A protein phosphatase in the observed dephosphorylation process was further suggested by the application of a purified type 2A protein phosphatase to patches in the presence of PKC. The inhibitory effect of PKC on K_{ATP} channel activity was reversed in the presence of the type 2A protein phosphatase.

In the 12 patches tested, nine patches showed no effect when type 2A protein phosphatase was applied on its own; however, of the remaining three patches tested, one patch showed a decrease and two patches showed an increase in K_{ATP} channel activity when type 2A protein phosphatase was applied alone (data not shown). This suggests that, at least in some of the patches tested, there was a significant level of channel phosphorylation present which was sensitive to type 2A protein phosphatase and that different phosphorylation sites have opposite functional effects, either activating or inactivating the K_{ATP} channel.

PKC and ATP Sensitivity of the Channel. The apparent K_i for ATP in the presence or absence of PKC was unaffected, suggesting that PKC-mediated phosphorylation of the K_{ATP} channel does not affect the affinity of individual ATP binding sites. However, the reduction of the Hill coefficient from 2.2 in control to 1.2 in the presence of PKC suggests a reduction in the cooperativity of binding of ATP. When, after an initial exposure to PKC, ATP concentration dependence was measured in the presence of okadaic acid (to maintain the channels in a phosphorylated state), a similar reduction in the Hill coefficient was observed. This suggests that phosphorylation *per se* rather than an allosteric effect accounts for the reduction in the cooperativity of ATP binding caused by PKC. Sequence analysis of a cloned renal K_{ATP} channel has shown the existence of consensus PKC phosphorylation sites in the ATP-binding domain of this channel (Ho et al., 1993). Whether phosphorylation at one or more of these sites is responsible for the PKC-mediated inhibition of K_{ATP} channel activity remains unknown but will soon be amenable to experimentation, as a putative cardiac K_{ATP} channel clone has recently been purified (Ashford et al., 1994).

In conclusion, data from this study have shown that PKC can directly inhibit single cardiac K_{ATP} channels in a phosphorylation-dependent manner and that this effect is reversed by dephosphorylation catalyzed by the type 2A protein phosphatase. These enzymes may, therefore, be involved in signal transduction pathways which serve to regulate K_{ATP} channels and hence metabolic energy expenditure in the myocardium.

ACKNOWLEDGMENT

We are very grateful to Dr. W. R. Giles and Dr. H. Tanaka (Department of Medical Physiology, University of Calgary) for generously providing the isolated rabbit ventricular myocytes.

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BI942890Z