Dihydropyridine-sensitive skeletal muscle Ca channels in polarized planar bilayers

3. Effects of phosphorylation by protein kinase C

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ABSTRACT The effects of protein kinase C (PKC) were studied on dihydropyridine (DHP)-sensitive Ca channels from rabbit skeletal muscle T tubule membranes. To determine which channel subunits become phosphorylated under the conditions used for electrophysiological studies, we first performed biochemical studies of phosphorylation. T tubular membranes were fused with vesicles of the lipid mixture used in the planar bilayers, and phosphorylation was assessed using the same concentrations of PKC, adenosine 5'-triphosphate, and buffers as were used in the electrophysiological experiments. The α_1 subunit of the DHP receptors was phosphorylated by PKC to an extent of 1 mol phosphate/mol protein. The β subunit was also phosphorylated but to a significantly lesser extent. The DHP-sensitive Ca channel activity was studied after fusing T tubule membranes with planar bilayers (Ma, J., C. Mundiña-Weilenmann, M. M. Hosey, and E. Ríos. 1991. *Biophys. J.* 60:890–901). The bilayers were held at −80 mV and activated by depolarizing voltage clamp pulses. The observed Ca channels exhibited two open states (τ_{o1} = 5 ms and τ_{o2} = 25 ms). On addition of purified PKC to the intracellular side, the proportion of the longer open state increased threefold. The average open probability during a 2-s, maximally activating pulse ($\bar{P}_{max}$) increased from 10 to 15%. The voltage dependence of activation was not changed by PKC; the Boltzmann parameters were V_t = −20.5 mV and K = 10.5 mV, which were not significantly different from the reference channels. The deactivation (closing) time constant was increased from 7 to 12 ms after PKC. The inactivation time constant during the pulse was slightly increased (from 1.2 to 1.6 s), and the channel availability at the holding potential was decreased from 76 to 71%. Taken together, the results revealed that PKC increased $\bar{P}_{max}$ largely through a shift in the voltage independent open-close equilibrium of the fully activated channels. This is in contrast with the effect of phosphorylation by PKA (Mundiña-Weilenmann, C., J. Ma, E. Ríos, and M. M. Hosey. 1991. *Biophys. J.* 60:902–909), which also increases $\bar{P}_{max}$, but mostly by increasing the availability of channels and slowing inactivation during the pulse.

INTRODUCTION

Protein phosphorylation plays an important role in the regulation of various types of ion channels (Hosey and Lazdunski, 1988). The role of protein kinases in regulating voltage-activated ion channels was first studied most extensively with regard to the effects of cyclic adenosine monophosphate-dependent protein kinase (PKA) on the dihydropyridine (DHP)-sensitive Ca^{2+} channels in cardiac cells (Reuter, 1983; Tsien et al., 1986; Pelzer et al., 1990). Electrophysiological data from many laboratories contributed to the hypothesis that these channels are modulated in a positive manner by PKA via a reaction that involves phosphorylation of the channels or associated regulatory proteins. Biochemical results to support this hypothesis have come from studies of the DHP-sensitive Ca^{2+} channels from skeletal muscle. The α_1 subunit of this multisubunit protein has been shown to be a substrate for PKA in vitro (Hosey et al., 1987; Takahashi et al., 1987; Imagawa et al., 1987; Nastainczyk et al., 1987) and in intact muscle cells (Lai et al., 1990; Mundiña-Weilenmann et al., 1991*a*), and reconstitution experiments have demonstrated that phosphorylation of the skeletal muscle DHP-sensitive Ca^{2+} channels by PKA results in an increase of channel activity (Flockerzi et al., 1986; Nunoki et al., 1989; Mundiña-Weilenmann et al., 1991*a*; Chang et al., 1991). Recently, electrophysiological studies of the skeletal muscle DHP-sensitive Ca^{2+} channels incorporated into planar lipid bilayers have shown that PKA increases the probability of channel opening, shifts the voltage-dependence of activation leftward by a small but significant amount, and slows the rate of inactivation (Mundiña-Weilenmann et al., 1991*b*). Thus, there is good evidence from both biochemical and electrophysiological experiments to support the hypothesis that these channels are regulated by PKA mediated phosphorylation of the channel proteins.

Protein kinase C (PKC) also has been suggested to play a role in regulating voltage-activated Ca^{2+} channels in a variety of cells. Much of the evidence for this type of regulation is indirect, because it has come from studies using activators of PKC such as phorbol esters and diacylglycerol analogues. These agents have been shown to attenuate whole cell Ca^{2+} currents in chick dorsal root ganglion cells (Rane and Dunlap, 1986), to uncover a silent Ca^{2+} current in *Aplysia* bag cell neurons (Strong et al., 1987), and to exert both inhibitory and stimulatory actions on DHP-sensitive Ca^{2+} channels in a smooth muscle derived cell line (Galizzi et al., 1987; Fish et al., 1988) and in cardiac cells (Lacerda et al., 1988). A complication in the interpretation of the aforementioned results is the more recent finding that some effects of diacylglycerol analogs and phorbol esters on Ca^{2+} channels do

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0006-3495/92/09/639/09 $2.00

not appear to involve the participation of PKC (Hockberger et al., 1989).

A more direct approach to understand the role of PKC in the regulation of Ca^{2+} channels is to test effects of purified PKC in biochemical and electrophysiological studies of the channels. The DHP-sensitive Ca^{2+} channels from skeletal muscle have been demonstrated to be phosphorylated in vitro by PKC (Nastainczyk et al., 1987; O'Callahan et al., 1988). The α_1 subunit is the preferred substrate when phosphorylation is carried out in the native T tubule membranes, and the β subunit is phosphorylated to a lesser extent (O'Callahan et al., 1988). In biochemical reconstitution studies, this PKC mediated phosphorylation has been shown to lead to activation of the channels (Chang et al., 1991). To further our understanding of how PKC modulates the properties of these Ca^{2+} channels, we have characterized the effects of PKC on the properties of the channels after incorporation into planar lipid bilayers (Ma et al., 1991). This analysis of the effects of PKC at the level of the single channel provides a detailed description of the effects of PKC on the voltage dependence and kinetics of channel activation, inactivation, and deactivation.

MATERIALS AND METHODS

Preparation of the transverse tubule membranes from skeletal muscle

Transverse (T)-tubule membranes were isolated from frozen rabbit skeletal muscle (Pel-Freeze Biologicals, Rogers, AZ) using the procedures described by Galizzi et al. (1984). Membrane vesicles were stored at a concentration of 5–7 mg protein/ml in 20 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl (pH 7.4) in liquid nitrogen until use. This T tubule membrane preparation routinely contained 20–50 pmol DHP receptors/mg protein, measured by the specific binding of (+)[^{3}H]PN200-110.

Planar bilayer recording of the skeletal muscle DHP-sensitive Ca channels

Planar bilayers were formed across an aperture of 200 μm diameter with a lipid mixture of phosphatidylethanolamine:phosphatidylserine:cholesterol in a ratio of 1:1:0.2; the lipids were dissolved in decane at a concentration of 50 mg lipid/ml. The *cis* (intracellular) solution contained 200 mM KCl, 3 mM ATP-Mg, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes)-Tris (pH 7.4); the *trans* (extracellular) solution contained 50 mM NaCl, 100 mM $BaCl_2$, 10 mM Hepes-Tris (pH 7.4). Bay K 8644 (0.3 μM) was always present in the *cis* solution, because it permitted consistent detection of DHP-sensitive Ca^{2+} channel activity. Single channel currents were recorded with an Axopatch-1C unit (Axon Instruments, Foster City, CA) and filtered at 100 Hz using an 8 pole Bessel filter (902 LPF; Frequency Devices, Haverhill, MA). The procedures of data analysis have been described elsewhere (Ma et al., 1991).

To incorporate the channels, T tubule vesicles (1–3 μl) were added to the *cis* solution. The bilayer was initially held at −80 mV (holding potential; h.p.). The DHP-sensitive Ca channel activity was measured with depolarizing pulses of various amplitudes and durations as described (Ma et al., 1991). To determine the effect of PKC on channel properties, we routinely first measured channel activity in the absence of PKC, then PKC (purified to homogeneity from chick brain according to the procedure of Woodgett and Hunter, 1987) was introduced to the intracellular solution at a concentration of 0.02 μM. The recording of channel activity was initiated 2–4 min after the addition of PKC, and the experiments usually lasted for 20–40 min without apparent rundown of channel activity.

Biochemical studies of the phosphorylation of Ca channel subunits under the conditions used for the lipid bilayer experiments

T tubule membranes (1 mg protein) were mixed with exogenous phospholipid vesicles (phosphatidylethanolamine:phosphatidylserine:cholesterol in a ratio of 1:1:0.2) in the same buffer (2 ml) used as the *cis* solution in the bilayer experiments, except that the protease inhibitors typically used for biochemical experiments were also added (1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, 1 μM pepstatin, 0.1 μM calpastatin, and 1 μg/ml aprotinin). The mixture was sonicated for 30 min in a bath sonicator at 4°C to form mixed vesicles.

Phosphorylation was performed in aliquots of 1 ml in the presence of 0.02 μM PKC. The reaction was initiated by the addition of 3 mM [γ-^{32}P]ATP-Mg (specific activity 200–300 cpm/pmol). After 10 min, the reaction was stopped by the addition of 1 vol of an ice-cold solution containing 50 mM Na_2KPO_4, 50 mM NaF, and 20 mM ethylenediaminetetraacetate. As a control, the same reactions were performed in the presence of 10 μg/ml of the PKC pseudosubstrate 19–31 (Peninsula Laboratories Inc., Belmont, CA), a specific inhibitor of PKC (House and Kemp, 1987). Solubilization and partial purification of DHP-sensitive Ca^{2+} channels were performed as described (Chang et al., 1991). Phosphorylation of the subunits of the channel was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Calculation of the stoichiometry of phosphorylation was based on the specific activity of the [γ-^{32}P]-ATP, the ^{32}P content of the excised subunit bands, and the amount of DHP-receptor applied to the gels (Chang et al., 1991).

RESULTS

Phosphorylation of the subunits of the DHP-sensitive Ca^{2+} channels from skeletal muscle

Previous studies demonstrated that the α_1 and β subunits of the skeletal muscle DHP-sensitive Ca^{2+} channels are substrates for PKC (Nastainczyk et al., 1987; O'Callahan et al., 1988); however, the conditions of the experiments greatly modify the extent of phosphorylation of the proteins. This is especially true for the α_1 subunit, which is an excellent substrate when phosphorylated in T tubule membranes (O'Callahan et al., 1988; Chang et al., 1991) but a very poor substrate when phosphorylated in detergent solution (Nastainczyk et al., 1987; O'Callahan et al., 1988). Because the conditions of the bilayer experiments were different from those previously used to assess phosphorylation of the channels by PKC, it was important to establish the extent of phosphorylation of the channel subunits by PKC under the conditions used for the electrophysiological measurements. Therefore, a biochemical phosphorylation assay was carried out using vesicles formed with T tubule membranes and the lipid mixture used in the planar bilayer

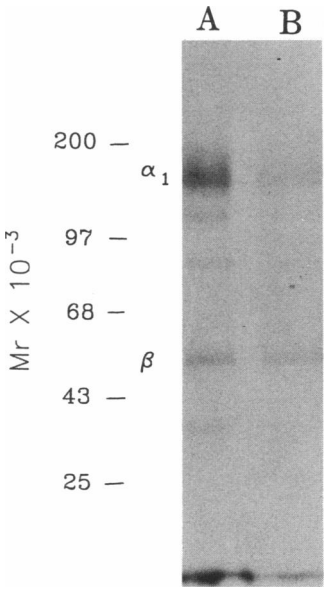

FIGURE 1 Phosphorylation of α_1 and β subunits of the skeletal muscle DHP-sensitive Ca channels by purified PKC. Channels in mixed vesicles, formed by sonication of T tubule membranes and liposomes with the lipid composition used in planar bilayers, were phosphorylated by 0.02 μM PKC in presence of 3 mM [^{32}P]ATP (260 cpm/pmol). The channels were partially purified with WGA-Sepharose and electrophoresed through 5–15% SDS-PAGE. Lane *A* shows an autoradiogram of the phosphorylated and partially purified channels; the position of the bands corresponding to the 165-kD α_1 and the 55-kD β subunits are indicated. Lane *B* shows the autoradiogram obtained when the phosphorylation reaction was performed in presence of 10 μg/ml of the PKC peptide inhibitor (19–31). The position of molecular weight markers is indicated.

experiments. The buffer, ATP, and PKC concentrations were the same as used in the bilayer experiments. The reactions were stopped after 10 min incubation since experiments in the lipid bilayer were performed typically at times between 4 and 20 min after addition of PKC. The phosphorylation of channel subunits was assessed after partial purification of the channels (in the presence of phosphatase inhibitors) and analyzed by SDS-PAGE and autoradiography. The results showed that the α_1 and β subunits of the DHP-sensitive Ca channels were phosphorylated under these conditions (Fig. 1 *A*). The extent of phosphorylation of the α_1 subunit was 0.8 mol ^{32}P/mol of DHP receptor, whereas the level of ^{32}P incorporated into the 55-kD β subunit was threefold lower (0.27 mol ^{32}P/mol protein). In a parallel experiment (Fig. 1 *B*), we tested for the specificity of the PKC-mediated effects by determining the extent of phosphorylation observed in the presence of a specific inhibitor of PKC (House and Kemp, 1987). This agent (10 μg/ml) inhibited the phosphorylation of the 165-kD α_1 subunit and the 55-kD β subunit by 90 and 65%, respectively. These results demonstrated that the phosphorylation of the α_1 subunit that was observed in Fig. 1 *A* was specific for PKC, and that the α_1 subunit is the major target for PKC under the conditions used for this study. They also demonstrated that only part of the phosphorylation of the β subunit could be attributable to PKC. The inhibitor-resistant phosphorylation of the β subunit was most likely due to the endogenous protein kinase in the T tubule membranes that preferentially phosphorylates the β subunit (Imagawa et al., 1987; Chang et al., 1991). The phosphorylation catalyzed by the endogenous kinase was previously shown to be without effect on channel activity (Chang et al., 1991).

Effects of PKC on channel open time

To study the effects of PKC phosphorylation at the single channel level, the DHP-sensitive Ca channels were reconstituted into planar bilayers by incorporating the isolated T tubule membranes from rabbit skeletal muscle as described previously (Ma et al., 1991). When the bilayers were held at either −80 mV or constant 0-mV potentials, no channel activity could be observed. Channel openings became evident when the bilayers were held at −80 mV and depolarizing pulses to −40 mV, or more positive potentials were applied. The channels had a major conductance level of 10 pS, and their current could always be blocked by 20 μM nitrendipine added to the extracellular solution (Ma et al., 1991). To test for effects of PKC, we analyzed the activity of reference channels and channels exposed to PKC in a series of consecutive episodes (acquired at 7-s intervals) of step depolarizations from a holding potential of −80 mV to a test potential of −10 mV (Fig. 2). It appeared from visual inspection of the sweeps that the activity of the channel was changed after phosphorylation by PKC; in particular, the mean open lifetime of the channel appeared to be longer in the presence of PKC.

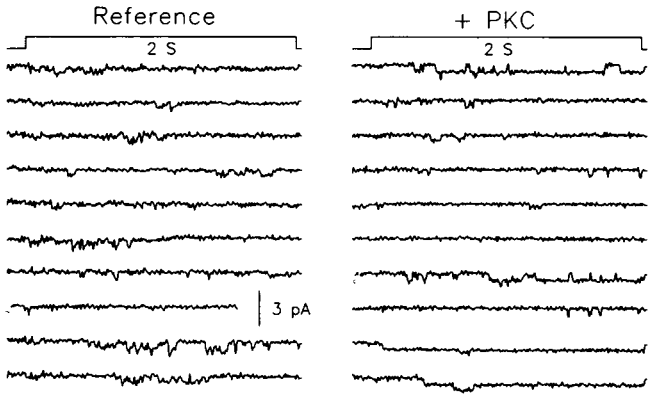

FIGURE 2 Single channel recordings of the skeletal muscle Ca channel in the presence and absence of PKC. DHP-sensitive Ca channels from rabbit skeletal muscle were measured upon fusion of isolated T tubule vesicles with planar lipid bilayers. Channel activity was recorded by depolarizing to −10 mV for 2 s, from an h.p. of −80 mV. Represented are consecutive episodes, acquired at 7-s intervals, for the same channel before (*Reference*) and 7 min after addition of purified PKC to the intracellular solution at a final concentration of 0.02 μM (+ *PKC*). Currents filtered at 100 Hz were digitized at a rate of 2 ms/point.

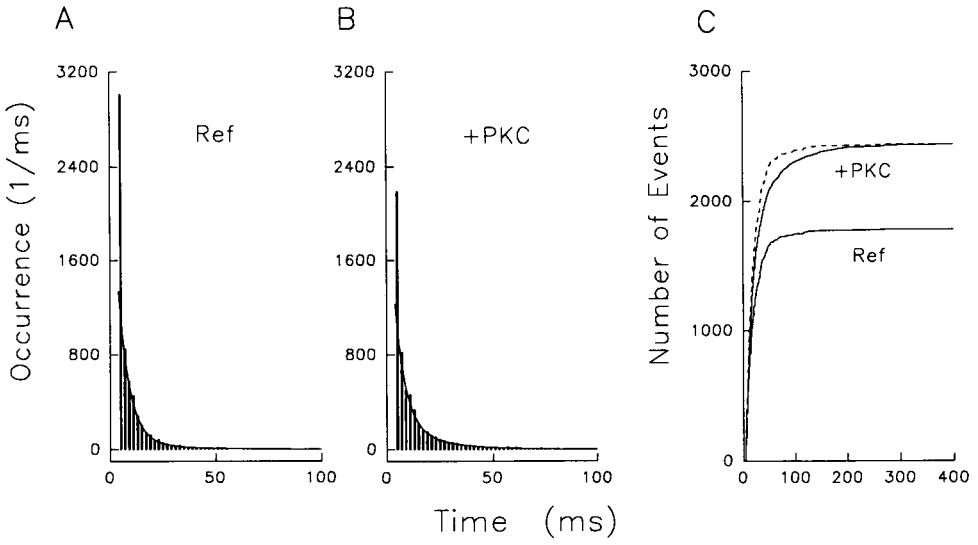

FIGURE 3 Open time histogram at −10 mV. Channel open times at −10 mV (episodes as in Fig. 2) were defined with a 50% threshold criterion using pCLAMP software. Open time histograms were constructed from 176 episodes with the reference channel (*A*) and 496 episodes with the PKC phosphorylated channel (*B*). The histogram of the reference channel was scaled by a factor of 2.82 (496/176) for comparison. Both histograms were fitted as a sum of two exponentials (text, Eq. 1). The best fit parameters in reference were τ_{o1} = 5.3 ms, y_1 = 14,390, τ_{o2} = 25.7 ms, y_2 = 1760. After PKC, τ_{o1} = 5.0, y_1 = 11,601, τ_{o2} = 19.6 ms, y_2 = 4525. (*C*) Cumulative open time histograms constructed from the same set of data in *A* and *B*, after an additional digital filtering at 30 Hz. The best fit parameters with text Eq. 2 in reference were τ_2 = 14.0 ms, N_2 = 1620, τ_3 = 57.8 ms, N_3 = 160. After PKC, τ_2 = 12.3 ms, N_2 = 1608, τ_3 = 52.7 ms, N_3 = 748. The dotted curve corresponds to the reference histogram scaled to the final level after PKC.

Open time histograms of channel events at a test potential of −10 mV were constructed (Fig. 3). When the records were filtered at 100 Hz, the histograms could be fitted as the sum of two exponential terms,

$$y = y_1/\tau_{o1} \exp(-t/\tau_{o1}) + y_2/\tau_{o2} \exp(-t/\tau_{o2}), \quad (1)$$

with time constants of ~5 (τ_{o1}) and 25 ms (τ_{o2}). These values were not changed by the presence of PKC (Fig. 3, *A* and *B*). However, the proportion of long openings relative to short ones (y_2/y_1) was 0.12 for the reference channels and was increased significantly to 0.39 after PKC. This analysis suggests that PKC-mediated phosphorylation increased long openings of the channels.

The filtering at 100 Hz allowed for detection of short openings but was not optimal for analysis of longer openings. As shown in a previous study of the effects of phosphorylation by PKA (Mundiña-Weilenmann et al., 1991*b*), the detection and analysis of long open states became more evident after further filtering (digitally) at 30 Hz. This processing changed substantially the profile of open times. It reduced the baseline noise, increased the average open time of both exponentially distributed terms, reduced the proportion of openings in the short open time term, and yielded a greater frequency of long open states (Ma et al., 1991). As discussed in that paper, these effects of lower frequency filtering probably result from both a reduction in detection of false closings (which improve the detection of long openings) and a loss of detection of brief openings and closings (which results in bursts being counted as single openings).

Within the modified picture provided by filtering at 30 Hz, the effects of PKC were more impressive. Cumulative open time histograms (that is, histograms of the number of events shorter than a certain duration) were constructed using the same records in Fig. 3, *A* and *B*, after further filtering at 30 Hz (Fig. 3 *C*), and fitted as the sum of two exponential terms,

$$N = (N_2 + N_3) - N_2 \exp(-t/\tau_2) - N_3 \exp(-t/\tau_3). \quad (2)$$

The best fit parameters were τ_2 = 14.0 ms, N_2 = 1,620, τ_3 = 57.8 ms, N_3 = 160 (reference); τ_2 = 12.3 ms, N_2 = 1,610, τ_3 = 52.7 ms, N_3 = 748 (+PKC). With this analysis, two features were different for the reference and the phosphorylated channels. First, the total number of openings was ~30% greater for the phosphorylated channels, as judged from the height of the plateau phase of the respective cumulative open time histograms (Fig. 3 *C*). Second, the phosphorylated channels exhibited an almost fivefold greater proportion of long openings than the reference channels. To illustrate this point more clearly, the histogram for the reference channels (Fig. 3 *C*) is also shown after scaling to the same maximum as the phosphorylated channels.

Voltage-dependence of activation

Previous studies revealed that phosphorylation of the skeletal muscle Ca channels with PKA modified the voltage-dependence of activation of the channels (Arreola et al., 1987; Mundiña-Weilenmann et al., 1991*b*). Therefore, we asked if PKC phosphorylation affected this prop-

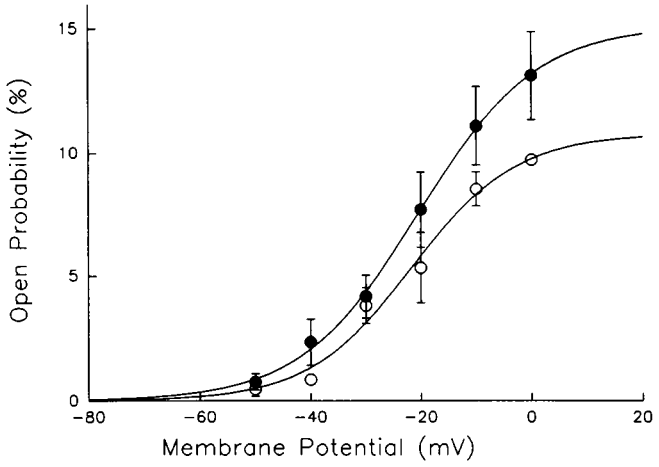

FIGURE 4 Voltage-dependent activation of the Ca channels before and after addition of PKC. Open probability ($\bar{P}_o$) of the channels was calculated as the fraction of the 2-s pulse duration occupied by open states. 15–30 episodes were acquired at each pulse potential, and four to six different voltages (−50–0 mV) were tested for the channels under reference conditions. Thereafter, PKC was introduced to the intracellular solution and the analysis repeated. Values of $\bar{P}_o$ at each potential were averaged over individual experiments (○, reference, n = 5; ●, +PKC, n = 6). Vertical bars span 1 SE. The smooth curves represent the best fit Boltzmann distributions. Parameters are as follows: $\bar{P}_{max}$ = 10.7 (1.2) %, V_t = −21.5 (3.2) mV, K = 9.5 (2.0) mV (reference); $\bar{P}_{max}$ = 15.1 (2.3) %, V_t = −20.5 (1.2) mV, K = 10.6 (0.7) mV (+PKC).

erty. The activation of the DHP-sensitive Ca channels was steeply voltage-dependent (Fig. 4). The mean open probability ($\bar{P}_o$, a nonstationary average) was calculated as the fraction of total time that the channels were open during the 2-s pulses (Ma et al., 1991). The relationship between $\bar{P}_o$ and voltage (V) was described by a Boltzmann distribution function

$$\bar{P}_o(V) = \bar{P}_{max}/[1 + \exp(-(V - V_t)/K)]. \quad (3)$$

For reference channels, the maximum open probability $\bar{P}_{max}$ was 10.7%, the transition voltage V_t was −21.5 mV, and the steepness factor K was 9.5 mV; for the phosphorylated channels, $\bar{P}_{max}$ was 15.1%, V_t was −20.5 mV, and K was 10.5 mV. This analysis indicates that phosphorylation resulted in an increase in the maximum open probability of the channel but did not have a significant effect on the voltage dependence of activation.

Voltage-dependent inactivation

A property of the reconstituted Ca^{2+} channels described previously (Mejía-Alvarez et al., 1991; Ma et al., 1991) is that the channels inactivate with time upon sustained depolarization. This can be seen in the traces for the reference channels in Fig. 2, where channel openings were clustered early in the pulse but became less evident at the end of the 2-s pulse. Previous studies found that inactivation of the channels at −10 mV had a time constant of τ_h = 1.24 s (Ma et al., 1991). To explore the voltage dependence of inactivation, the channels were first subjected to conditioning pulses of 10-s duration to various potentials, and, after a gap of 100 ms, activity was measured during a test pulse to −10 mV (Fig. 5). Inactivation thus measured was dependent on the conditioning voltage and was related to the activation induced by the conditioning pulse. When the reference channels were subjected to conditioning pulses of −50 mV, little inactivation was observed during the test pulse, whereas at conditioning voltages of −30 mV or more, the activity of the reference channels was very low during the test pulse. After a conditioning pulse to −10 mV, inactivation was complete ($\bar{P}_o$ was 0 during the test).

Phosphorylation of the channels with PKC modified slightly the voltage-dependence of inactivation. This was not obvious on visual inspection of the currents (Fig. 5) but became apparent with further analysis. The average open probability during the test pulse was normalized to its value in the absence of conditioning to yield an availability function ($H[V_c]$) that was plotted as a function of the conditioning pulse potential (V_c) (Fig. 6). This is a normalized, or relative availability, that takes the availability at the holding potential, $h(0)$ of Ma et al. (1991), as unity. The data were fitted according to the equation

$$H(V_c) = 1/[1 + \exp(V_c - V_h)/K_h]. \quad (4)$$

The best fitted parameters in reference were V_h = −33.3 mV (standard error of fit = 0.9) and K_h = 8.4 mV (0.8). The corresponding values after PKC were V_h = −29.1 mV (0.9) and K_h = 9.1 mV (0.8). Though small, the difference in V_h was significant ($P < 0.04$).

The availability at the resting potential ($h[0]$) was estimated from the frequency of nulls (sweeps with no openings) as

$$h(0) = \frac{M - M_0}{M},$$

where M is the total number of sweeps and M_0 is the number of nulls. The frequency of nulls increased in PKC from 24.2% (1.7) to 29.4% (1.9), a significant change ($P < 0.01$).

Effect of PKC on kinetics of activation and inactivation

Experiments were performed to determine whether the rates of channel activation and inactivation were modified as a result of PKC mediated phosphorylation (as was the case with PKA) (Mundiña-Weilenmann et al., 1991*b*). Data from 12 experiments, in which depolarizing pulses of 2-s duration to −10 mV were applied, were ensemble averaged (Fig. 7). The amount of current remaining at the end of the test pulse was significantly higher in the records obtained after exposure to PKC, reflecting either a slower or an incomplete inactivation.

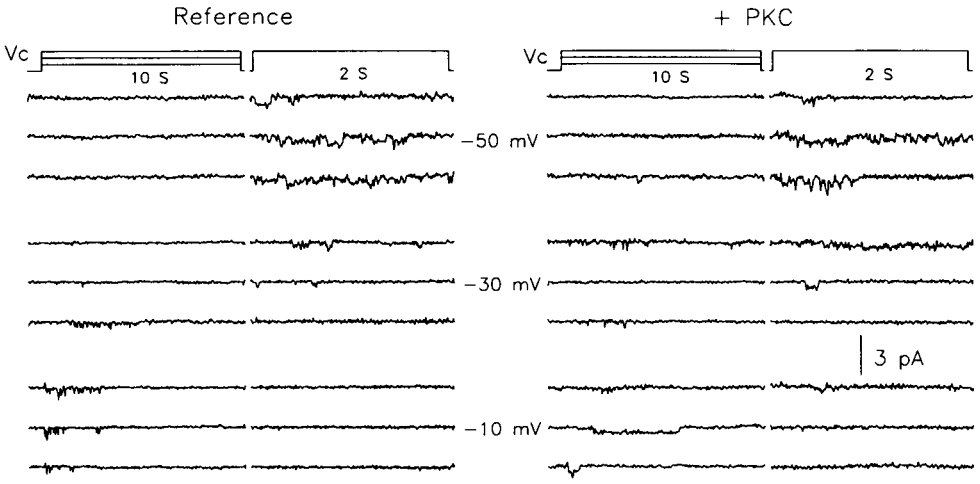

FIGURE 5 Effect of PKC on the voltage-dependent inactivation of the Ca channels. 10-s conditioning pulses to various potentials were applied 100 ms before a test pulse to −10 mV (protocol at top). The figure shows selected episodes at the conditioning voltages indicated (−50, −30, and −10 mV). Records shown in the left and right panels are from the same experiment, performed in the absence and presence of PKC, respectively.

To quantify the effect, we fitted the time dependence of the average current with the function

$$I = I_{max} m^3 h, \tag{5}$$

where $m(t) = m(\infty)[1 - \exp(-t/\tau_m)]$, and

$$h(t) = h(\infty) + [h(0) - h(\infty)] \exp(-t/\tau_h). \tag{6}$$

The activation time constant τ_m was 96 ms (3.0) in reference and 92 ms (2.6) after PKC. The inactivation time constant τ_h was 1.2 s (0.15) in reference and 1.6 s (0.28) in the presence of PKC. The small change in the rate of inactivation is consistent with the shift in the $H(V_c)$ dependency shown in Fig. 6. The steady-state inactivation value at −10 mV, h_∞, also changed, from being not significantly different from 0 in reference to 0.08 after PKC.

Effects of PKC on the rate of channel closing

The changes in the open state properties of the channels upon phosphorylation by PKC were further associated with changes in the kinetics of channel closing (deactiva-

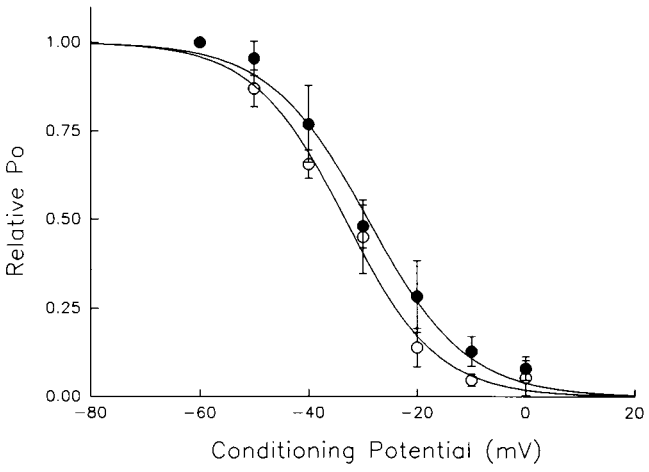

FIGURE 6 Voltage-dependent inactivation of the Ca channels. The $\bar{P}_o$ values during the test pulse of the experiment illustrated in Fig. 5 (reference and +PKC) were plotted as a function of the conditioning voltage. Symbols represent the mean and SE (○, reference, n = 10; ●, +PKC, n = 7). Curves correspond to the best fits according to text, Eq. 4. The reference channels had V_h = −33.3 (0.9) mV, K_h = 8.4 (0.8) mV. The PKC phosphorylated channels had V_h = −29.1 (0.9) mV, K_h = 9.1 (0.8) mV.

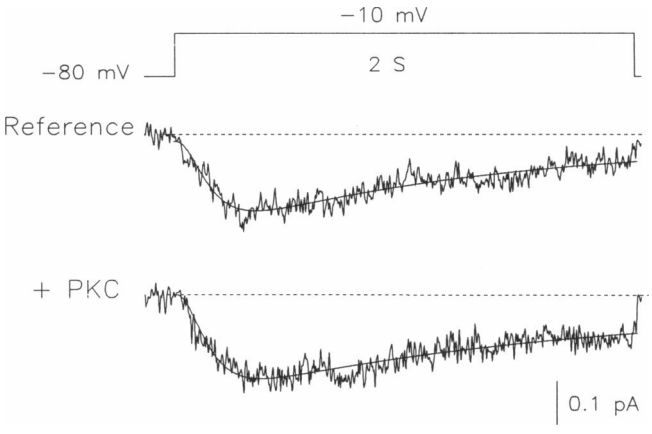

FIGURE 7 Activation and inactivation kinetics of the reference and PKC phosphorylated channels. The ensemble average for reference channels was constructed with records from 32 bilayer experiments (453 sweeps). For the PKC phosphorylated channel, data from 12 experiments were used (253 episodes). Null episodes were excluded from the ensemble averages; they were 24.2 (1.7) % (*Reference*) and 29.4 (1.9) % (*+PKC*) of all the episodes. The smooth curves represent the best fit according to text Eq. 5. The best fit parameters in reference were τ_m = 96.4 (3.0) ms, τ_h = 1.19 (0.15) s, I_{max} = −0.231 (0.008) pA, $h(\infty)$ = 0; in PKC, τ_m = 92.3 (2.6) ms, τ_h = 1.58 (0.28) s, I_{max} = −0.254 (0.007) pA, $h(\infty)$ = 0.076 (0.01).

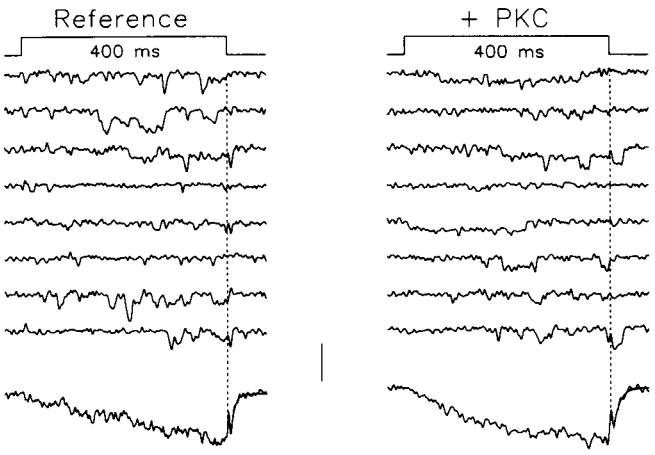

FIGURE 8 Effect of PKC on the deactivation of the DHP-sensitive Ca channel at −80 mV. The figure shows traces obtained after a depolarizing pulse to −20 mV (400 ms) and consecutive episodes of 500-ms duration. Records for the reference channels (*left*) and PKC phosphorylated channels (*right*) were taken from the same bilayer experiment. The ensemble averages (*bottom*) were constructed with 201 episodes for the reference channels and 190 episodes for the PKC phosphorylated channel. Null episodes, which represent ~30% of the total, were excluded from the averages. The vertical bar represents 3 pA for the single channel records and 0.3 pA for the ensemble averages. The OFF portion of the ensemble average was fitted by a single exponential decay, for which the time constants are τ_d = 7.3 (0.3) ms (*Reference*) and 11.8 (0.4) ms (*+PKC*).

tion) observed at the termination of the depolarizing pulse (Fig. 8). To characterize the kinetics of deactivation, single channel episodes of 500-ms duration were analyzed in which 400-ms pulses to −20 mV were applied (the duration was selected to have maximum activation at the end of the pulse). There were differences in the kinetics of deactivation; channels that were open at the time of repolarization appeared to remain open longer in the presence of PKC (Fig. 8). When the single channel records were pooled in ensemble averages, tail currents became apparent and were fitted with a single exponential decay. The time constant was 7.3 ms (0.3) for the reference channels and 11.8 ms (0.4) after exposure to PKC. These results indicate that phosphorylation of the channels by PKC results in a decreased rate of closing.

DISCUSSION

This study demonstrates that PKC modifies the properties of DHP-sensitive Ca^{2+} channels from skeletal muscle under conditions in which the α_1 subunit of the channels is stoichiometrically phosphorylated by the kinase. The results provide direct evidence that PKC can regulate physiologically these channels by phosphorylation.

The biochemical experiments of the present study reinforced our previous observation that the conditions of the phosphorylation experiments can greatly influence the ability of the α_1 subunit to serve as a substrate for PKC. In the present studies, we designed the phosphorylation experiments to imitate the conditions used in the planar bilayer studies, by using vesicles formed by fusion of T tubule membranes and liposomes containing a lipid composition similar to that of the planar bilayer. Under these conditions, the α_1 subunit was the preferred substrate for PKC phosphorylation. The β subunit was also phosphorylated but to a threefold lower extent. These results were consistent with previous studies in which channels were phosphorylated in their native membranes (O'Callahan et al., 1988; Chang et al., 1991), and the α_1 subunit was found to be the preferential substrate. However, the conditions of this experiment did affect the stoichiometry of the phosphorylation of the α_1 subunit, which was only ~1 mol phosphate/mol protein in the present study compared with 2 mol phosphate/mol protein in previous studies. These results are in marked contrast to those obtained when the purified channels are phosphorylated by PKC in solution containing detergent; in that case the α_1 subunit is a very poor substrate for PKC and only the β subunit undergoes significant phosphorylation (Nastainczyk et al., 1987; O'Callahan et al., 1988). Taken together, the results suggest that the lipid environment and/or other factors affect the ability of the α_1 subunit to serve as a substrate for PKC. This finding should be kept in mind for future studies when assessing the ability of PKC to phosphorylate channels or to modulate channel function.

PKC increased the maximum open probability of the available channels

The functional studies at the single channel level showed that the reconstituted Ca channels displayed two open states (τ_{o1} = 5 ms and τ_{o2} = 25 ms) and that the voltage dependence and kinetics of gating (Ma et al., 1991) were close to those observed physiologically (Avila-Sakar et al., 1986). Phosphorylation by PKC resulted in substantial changes in the behavior of the channel. The most notable effects were that PKC caused a threefold increase in the frequency of long openings and increased the maximum ($\bar{P}_{max}$) of the averaged open probability from 0.1 to 0.15.

The effect of PKC to increase $\bar{P}_{max}$ could be due to several factors, because $\bar{P}_o$ is a nonstationary average. Thus, Mundiña-Weilenmann et al. (1991) showed that the increase in $\bar{P}_{max}$ induced by PKA resulted mainly from the slowing of inactivation. Ma et al. (1991) expressed the relationship between changes at the single channel level and the maximum of the average probability in the equation:

$$\bar{P}_{max} = Nh(0)\beta P_{max}. \quad (7)$$

In this equation, N is the number of channels in the membrane, $h(0)$ is the availability of the channel at the

holding potential, P_{max} is the maximum open probability of the available (noninactivated) channels, and

$$\beta = [1 - \exp(-T/\tau_h)]\tau_h/T \quad (8)$$

is a quantity that embodies the effect of inactivation on the average open probability over the pulse duration T. Eq. 7 thus relates changes in $\bar{P}_{max}$ to four possible effects at the single channel level.

Because the time constant of inactivation τ_h increased from 1.2 to 1.6 s, β went from 0.492 in reference to 0.567 in PKC. Also, $h(0)$ decreased from 0.76 in reference to 0.71 in PKC. Substituting these values in Eq. 7, it turns out that NP_{max} went from 0.27 in reference to 0.37 in PKC. Barring a PKC-induced change in the number (N) of functional channels (which is estimated at 2 or 3) (Ma et al., 1991), this change implies a 37% increase in the open probability of the noninactivated channels. This increase was expected as a direct consequence of the increase in the number and proportion of long openings.

The increase in maximum open probability was not accompanied by any change in voltage dependence of channel activation and only by a minimum shift in steady-state inactivation. It thus appears that PKC preferentially affects voltage-independent parameters. As remarked before (Ma et al., 1991), these channels tend to be closed most of the time, even when they are maximally activated by voltage (P_{max} is only ~0.1). This was interpreted (Ma et al., 1991) with the image of two gates or gating processes that can individually close the channel, one controlled by voltage and one voltage independent, with open probability P_{max}. PKC appears to have altered mainly this second gate.

The increase in open probability without a change in voltage dependence is analogous to the enhancement of L-type cardiac Ca channels caused by manipulations that increase intracellular $[Ca^{2+}]_i$, like digitalis-induced increase in $[Na^+]_i$ (Marban and Tsien, 1982) and photorelease from caged Ca (Gurney et al., 1989). The slow onset of the effect in the latter study suggested to Gurney et al. that increased $[Ca^{2+}]_i$ activates a phosphorylation process analogous to the one studied here. Increases in P_o of single L-type Ca channels in neurons in response to stimulation by phorbol esters have been reported (Lipscombe et al., 1988).

The effects of PKC on channel properties have some intriguing parallels with those induced by DHP agonists, such as Bay K 8644. This agent promotes longer channel openings (Hess et al., 1984) and reduces the rate of deactivation of DHP-sensitive channels in cultured skeletal muscle cells without causing significant changes in the voltage-dependence of activation (Cognard et al., 1986). (On the other hand, in cardiac cells, Bay K 8644 alters the voltage dependent properties of L-type Ca channels [Kokubun and Reuter, 1984; Sanguinetti et al., 1986; Bechem and Schramm, 1988].) Since the actions of PKC and Bay K 8644 on the skeletal muscle Ca channel have similarities, it would be interesting to determine whether PKC is enhancing the ability of Bay K 8644 to modulate channel properties or if PKC can produce Bay K-like effects in the absence of the agonist. Unfortunately, it will be difficult to evaluate the effects of PKC in the absence of Bay K 8644 because the open probability becomes extremely low without the agonist.

Comparison with the effects of phosphorylation by PKA

The main effect of PKC-mediated phosphorylation of the skeletal muscle DHP-sensitive Ca channel was to increase the open lifetime of the channel and its maximum open probability. The effects of PKC-mediated phosphorylation on these channels were very different from those of phosphorylation mediated by PKA (Mundiña-Weilenmann et al., 1991*b*). Both kinases increased averaged open probability by ~50% but by different mechanisms. PKA slowed twofold the rate of inactivation, increased the availability at the resting potential, and did not, in the final analysis, modify P_{max}, the open probability of the available channels. In contrast, PKC increased the average open probability even though it decreased the steady-state availability and slowed inactivation only mildly. The activating effect of PKC was almost exclusively due to the increase in long openings, resulting in a sizable increase in P_{max}.

These effects were observed under conditions in which the α_1 subunit was the major phosphorylated channel subunit. Some sites phosphorylated by PKC are different from those phosphorylated by PKA, whereas other sites appear to be common (O'Callahan et al., 1988). Elucidation of the sites responsible for the changes in open time caused by PKC versus those that modify the voltage dependence of activation upon phosphorylation by PKA could provide information about structure-function relationships for these channels.

The differences in the functional consequences of phosphorylation caused by PKA and PKC indicate that the regulation of Ca channel activity by phosphorylation with different kinases does not result in a unique alteration of Ca channel properties. On the contrary, the existence of different mechanisms of channel modulation may be important for the fine tuning of channel activity by neurotransmitter and hormone mediated events.

We thank Judy Ptasienski for the skillful preparation of membranes and Dr. Milos Karhanek for help in data analysis.

This work was supported by grants from National Institutes of Health to Marlene Hosey (HL-23306) and to Eduardo Ríos (AR-32808), and by a grant from the Muscular Dystrophy Association to Eduardo Ríos. Jianjie Ma was a recipient of a Muscular Dystrophy Association postdoctoral fellowship, and Luis M. Gutiérrez was the recipient of a Formación de Personal Investigador postdoctoral fellowship from the Ministerio de Educación y Ciencia of Spain.

Received for publication 16 December 1991 and in final form 26 March 1992.

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