J. Biochem. 120, 170-176 (1996)

Cyclic AMP-Dependent Protein Kinase but Not Protein Kinase C Regulates the Cardiac Ca^{2+} Channel through Phosphorylation of Its α_1 Subunit

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Received for publication, March 1, 1996

The voltage-dependent L-type Ca^{2+} channel in the heart is regulated by cAMP-dependent protein kinase (PKA) and possibly by protein kinase C (PKC). We have investigated the channel modulation through phosphorylation by these protein kinases, using liposomes into which Ca^{2+} channels from bovine heart were reconstituted. Phosphorylation of the proteoliposomes with PKA increased the dihydropyridine-sensitive Ca^{2+} efflux from them by about 70%. PKA rapidly phosphorylated membrane proteins of 210 and 170 kDa. A dihydropyridine-class Ca^{2+} channel blocker, [^{3}H]azidopine, specifically photo-labeled a protein of 210 kDa, suggesting that the 210-kDa phosphoprotein might be the α_1 subunit of the Ca^{2+} channel. In contrast, phosphorylation of the proteoliposomes with PKC failed to modulate the Ca^{2+} efflux. Although PKC catalyzed the phosphorylation of membrane proteins of 150, 130, 95, 67, and 62 kDa, the 210- and 170-kDa proteins were not phosphorylated by this kinase. These results suggest that phosphorylation of the 210-kDa protein in the cardiac sarcolemma by PKA may be responsible for modulation of the channel function, whereas modulation of the channel by PKC, if it occurs, must be the result of an indirect mechanism, *e.g.* phosphorylation of a cytoplasmic protein or an associated channel polypeptide, that cannot function in the reconstituted system.

Key words: Ca^{2+} channel, cAMP-dependent protein kinase, protein kinase C, protein phosphorylation, reconstitution.

In cardiac muscle, the activity of the voltage-dependent L-type Ca^{2+} channel is regulated by neurotransmitters and hormones *via* protein phosphorylation. Several lines of evidence indicate that β-adrenergic modulation of the Ca^{2+} channel is mediated by an increase in the cellular level of cAMP and the consequent activation of PKA (*1-3*). It has also been suggested that in cardiac cells the Ca^{2+} channel is regulated through phosphorylation catalyzed by PKC (*4*). Similar phosphorylation mechanisms involving PKA, PKC, and other protein kinases have been suggested for the regulation of Ca^{2+} channels in other tissues, such as skeletal and smooth muscles, secretory cells, and neuronal cells (*5-7*). In skeletal muscle, the α_1 subunit of the Ca^{2+} channel, which can form a functional ion pore and expresses the receptors for dihydropyridine derivatives, has been shown to be phosphorylated by PKA, in parallel with an increase in the channel activity (*8-10*). Although PKC catalyzes the phosphorylation of skeletal-muscle Ca^{2+} channel polypeptides *in vitro* (*11-14*), the molecular mechanism of modulation of the cardiac Ca^{2+} channel by PKC is not clearly understood. Thus, it remains to be elucidated which protein is phosphorylated as the primary event of modulation of the cardiac Ca^{2+} channel, or whether or not phosphorylation of the channel protein indeed results in modulation of the channel function.

We showed previously that the cardiac L-type Ca^{2+} channel can be functionally reconstituted into liposomes and that phosphorylation of the proteoliposomes with PKA enhances Ca^{2+} efflux from them (*15*). This suggests that phosphorylation of the channel protein or a membrane protein closely associated with the channel is responsible for its modulation by PKA. In this study, we have explored further, using reconstituted proteoliposomes, the substrate protein of PKA responsible for channel regulation, and investigated the possible modulation of the Ca^{2+} channel through phosphorylation by PKC. We report that the α_1 subunit of the Ca^{2+} channel is phosphorylated by PKA, with a resultant increase in Ca^{2+} efflux from the proteoliposomes. On the other hand, PKC does not affect Ca^{2+} efflux nor does it phosphorylate the α_1 subunit of the Ca^{2+} channel.

MATERIALS AND METHODS

Materials—The following materials were purchased from the sources indicated: nifedipine (Bayer); valinomy-

[1] To whom correspondence should be addressed. This work was supported by grants from the Uehara Memorial Scientific Foundation and the Naito Foundation, and Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

Abbreviations: PKA, cAMP-dependent protein kinase; PKC, protein kinase C; WGA, wheat germ agglutinin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PS, α-phosphatidyl-L-serine; PMSF, phenylmethylsulphonyl fluoride; V_1, prepotential; V_2, test potential.

cin (Boehringer Mannheim); fura-2 (Dojin); iodoacetamide (Katayama); aprotinin and soybean trypsin inhibitor (Boehringer Mannheim); TSK 3000G gel (Toyo-Soda); WGA-Sepharose 6MB gel (Pharmacia LKB); Extracti gel D (Pierce); [γ-^{32}P]ATP and [^{3}H]azidopine (Amersham); and Aquasol-2 (Dupont). Soybean phospholipid, TPA, PS, CHAPS, Nonidet P40, benzamidine, PMSF, pepstatin A, leupeptin, and the catalytic subunit of PKA (catalog no. P2645) from bovine heart were obtained from Sigma. Type III (α) PKC was purified from bovine brain according to the method described elsewhere (*16, 17*). Okadaic acid was a generous gift from Dr. A. Takai, Nagoya University, Nagoya.

Reconstitution of Ca^{2+} Channels—Dihydropyridine-sensitive cardiac Ca^{2+} channels were solubilized and reconstituted as reported previously (*15*). Briefly, frozen bovine ventricle tissue was homogenized in 20 mM Tris-HEPES buffer, pH 7.5, containing 250 mM sucrose and a protease inhibitor cocktail (buffer A). The protease inhibitor cocktail consisted of benzamidine (0.1 mM), PMSF (0.1 mM), iodoacetamide (1 mM), pepstatin A (5 μg/ml), leupeptin (5 μg/ml), aprotinin (20 μg/ml), and trypsin inhibitor (20 μg/ml). The sarcolemmal fraction was prepared from the microsomal fraction by affinity chromatography on WGA-Sepharose 6MB gel. Sarcolemmal proteins comprising the Ca^{2+} channel protein were solubilized with 1.2% (w/v) CHAPS in a solution containing 140 mM choline-Cl, 10 mM KCl, and 20 mM Tris-HEPES, pH 7.4 (external solution), and in addition 0.12% (w/v) soybean phospholipid and the protease inhibitor cocktail. The proteoliposomes were formed by removing the detergent with Extracti gel D. Using this method, the Ca^{2+} channels were incorporated in both the outside-out and inside-out conformations.

Phosphorylation of Proteoliposomes—Reconstituted proteoliposomes (0.1 mg/ml protein) were incubated with either the catalytic subunit of PKA (14 U/ml, approximately 10 nM) or type III (type α) PKC (2.4-24 U/ml, approximately 10-100 nM), 0.5-5 mM Ca^{2+}, 1-5 nM TPA, and 40 μg/ml PS, in a reaction mixture comprising 100 μM ATP or [γ-^{32}P]ATP (37 TBq/mol), 3 mM $MgSO_4$, and 20 mM Tris-HEPES, pH 7.5, for 5-20 min at 22-24°C. Then, the proteoliposomes were loaded with Ca^{2+} to measure Ca^{2+} efflux or solubilized to investigate phosphoproteins.

Measurement of Ca^{2+} Efflux—Ca^{2+} efflux from the proteoliposomes was measured, using fura-2, with a spectrofluorophotometer (Shimadzu, RF-5000), at excitation and emission wavelengths of 340/380 and 500 nm, respectively (*15*) (see Fig. 1). Proteoliposomes were loaded with an internal solution comprising 1 mM $CaCl_2$, 10 mM KCl, 140 mM choline-Cl, and 20 mM Tris-HEPES, pH 7.5, by means of a freeze-and-thaw cycle 5-times. To alter the membrane potential of the proteoliposomes, choline-Cl in the external solution was isosmotically replaced with KCl (0.7-140 mM), except that a 350-mM K^+ solution was made by adding an excess of KCl to the 140-mM K^+ solution. Membrane potential V_i was defined as the potential outside minus the potential inside the membrane (*i.e.* the opposite to the conventional definition), considering the inside-out configuration of the reconstituted channels.

An aliquot (5 μl) of the Ca^{2+}-loaded proteoliposomes was incubated in a solution (200 μl) comprising 0.7-350 mM KCl and the K^+ ionophore, valinomycin (0.1 μM), on ice for 1 min, to hold the membrane potential at prepotentials (V_i) ranging from −83 to 63 mV. Then, the mixture was introduced into a cuvette containing 0.5 μM fura-2 and 0.1 μM valinomycin in 2 ml external solution, and the resultant increase in fura-2 fluorescence was measured. The fluorescence intensity was converted to the total calcium concentration using a calibration curve (*15*). Similar experiments were repeated in the presence of 1 μM nifedipine, and the nifedipine-sensitive calcium increase at 1 min after the addition of the proteoliposomes was taken as an integral of the Ca^{2+} efflux through the Ca^{2+} channel.

Photoaffinity Labeling of Proteoliposomes—A dihydropyridine-binding protein was detected with the photoaffinity ligand, azidopine (*18*). Proteoliposomes (6-20 μg protein) were incubated with 50-200 nM [^{3}H]azidopine in the presence or absence of 1 μM nifedipine in a solution (200 μl) comprising 20 mM Tris-HEPES and 2 mM $MgSO_4$, pH 7.4, on ice for 30-60 min, in the dark. Then, the sample was irradiated with a UV lamp (L_{max} 366 nm; Ultra-violet Products, USA, Model UVSL-58) at a distance of 10 cm (1.3 mW/cm^2), again on ice. The proteoliposomes were trapped on a membrane filter and solubilized with SDS. Then the labeled proteins were applied to a gel-filtration column and/or subjected to SDS-PAGE.

Separation of Sarcolemmal Proteins—Reconstituted membrane proteins labeled with either [^{32}P]ATP or [^{3}H]azidopine were solubilized with 1% (w/v) SDS and then applied to a gel-filtration column (TSK 3000G gel; 7.5×600 mm), which had been equilibrated previously with 20 mM Tris-HEPES, 10 mM H_3PO_4, 0.1 mM EGTA, 0.1 mM EDTA, 2 mM DTT, 0.4% Nonidet P40, and the protease inhibitor cocktail, the pH being adjusted with Tris to 7.2. In the experiments on phosphorylation, 1 μM okadaic acid was added to prevent possible dephosphorylation during the separation procedure. The flow rate was 0.4 ml/min. The eluted proteins were detected as the UV absorbance at 280 nm, and the radioactivity in each fraction (10-100 μl) was quantitated by Cerenkov radiation or with a liquid scintillator in Aquasol-2 (a toluene-based scintillation cocktail) using a Beckman LS9000 or Aloka LSC-700 counter.

SDS-PAGE—Fractionated proteins eluted from the gel-filtration column and exhibiting radioactivity were immediately subjected to SDS-PAGE. The gel was calibrated with

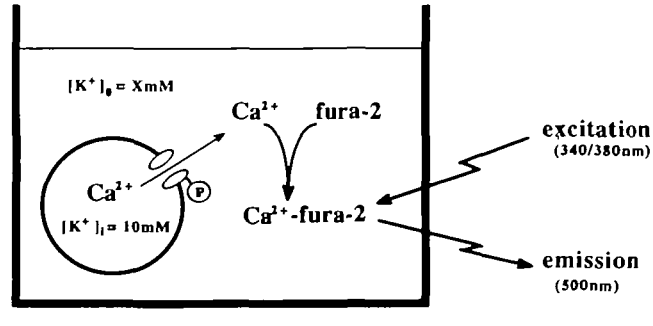

Fig. 1. Schematic illustration of the measurement of Ca^{2+}-flux. The membrane potential of the proteoliposomes was controlled by means of the concentrations of inside and outside K^+, and the K^+ ionophore, valinomycin. Ca^{2+}-efflux induced by membrane depolarization was measured with the fluorescent dye, fura-2, with excitation at 340 and 380 nm, and emission at 500 nm. See the text for further explanation.

a molecular weight standard kit (44-206 kDa; Pharmacia). After electrophoresis, the gel was dried and exposed to film (X-Omat, Kodak or Hyperfilm-^{3}H, Amersham), using an intensifying screen (Cronex Lightning Plus, Dupont), for 1-30 days.

RESULTS

Modulation of Ca^{2+} Efflux through Phosphorylation—In a previous study, we showed that Ca^{2+} efflux from liposomes, into which cardiac sarcolemmal proteins are reconstituted, is enhanced on phosphorylation by PKA (*15*). We examined, therefore, the effects of phosphorylation by PKA and PKC on the voltage-dependent Ca^{2+} efflux and phosphoproteins of proteoliposomes under comparable conditons. The proteoliposomes were incubated first with either the catalytic subunit of PKA (14 U/ml) or activated type III (α) PKC (2.4 U/ml) in the presence of MgATP. Then, Ca^{2+} efflux from the proteoliposomes was measured by changing the membrane potential from the prepotential (V_1; ranging from −83 to +63 mV) to the test potential (V_2; fixed at 0 mV). In the graph shown in Fig. 2, nifedipine-sensitive Ca^{2+} efflux is plotted as a function of V_1. The sign of V_1 was defined as opposite to the conventional electrophysiology, considering that only channels of inside-out orientation were phosphorylated. In the control (non-phosphorylated preparation), Ca^{2+} efflux was maximum (12 pmol/μg protein) at V_1 values of −83 mV and was minimum (1.1 pmol/μg protein) at around 0 mV. The Ca^{2+} efflux was increased by 70% over the control following preincubation of the proteoliposomes with PKA, at negative V_1 values. At positive V_1 values, however, the flux was not different from the control values. Such an increase was not observed for the proteoliposomes preincubated with MgATP alone. Thus, the increase in the Ca^{2+} efflux following preincubation with PKA was considered to be due to phosphorylation of membrane protein(s) in the proteoliposomes. Similar results were obtained in two other experiments. Incubation of the proteoliposomes with PKA for longer than 5 min did not further increase the Ca^{2+} efflux, suggesting that the site is maximally phosphorylated by PKA during the initial preincubation time. The extent of the maximum increase in Ca^{2+} efflux produced on phosphorylation by PKA was 55-80% of the control values for 3 batches of the proteoliposomes. On the other hand, preincubation of the proteoliposomes with type III (α) PKC, the major subspecies present in cardiac tissue (*19*), did not increase Ca^{2+} efflux at any potential. The lack of an effect on Ca^{2+} efflux was not due to an insufficient concentration of PKC used, since a 10-fold higher concentration (24 U/ml) was also ineffective (data not shown).

Phosphoprotein Analysis of the Proteoliposomes—To complement the studies on Ca^{2+} efflux, the phosphoprotein profile of the proteoliposomes was investigated following treatment with protein kinases. The proteoliposomes were incubated with either PKA (14 U/ml) or PKC (2.4 U/ml) for 20 min under the same conditions as those for the Ca^{2+} flux experiments, but this time with radiolabeled ATP. Proteins were then solubilized and separated by SDS-PAGE, and then the phosphoproteins were detected by autoradiography. Figure 3 shows membrane proteins stained with silver (lane a), and autoradiograms for PKA (lane b), and PKC (lane c). Membrane proteins of 210, 170, 150, 100, 83, and 67 kDa were phosphorylated by PKA, whereas phosphoproteins of 150, 130, 95, 67, and 62 kDa were detected for PKC. Phosphorylation of the 210- and 170-kDa proteins, which were substrates for PKA, was not observed, however, with PKC at this concentration or even at 24 U/ml, a 10-fold higher concentration.

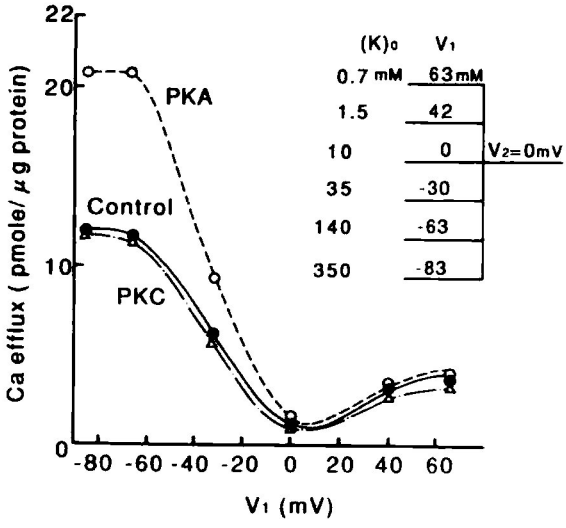

Fig. 2. **Effects of phosphorylation by PKA and PKC on Ca^{2+} efflux from proteoliposomes.** The Ca^{2+} efflux was measured using 0.5 μM fura-2 as described under "MATERIALS AND METHODS." The membrane potential of the proteoliposomes was held at various prepotentials (V_1), and then Ca^{2+} efflux was measured at the fixed test potential (V_2) of 0 mV. Ca^{2+} efflux is plotted against V_1 for the control (filled circles), PKA (open circles, broken line), and PKC (open triangles).

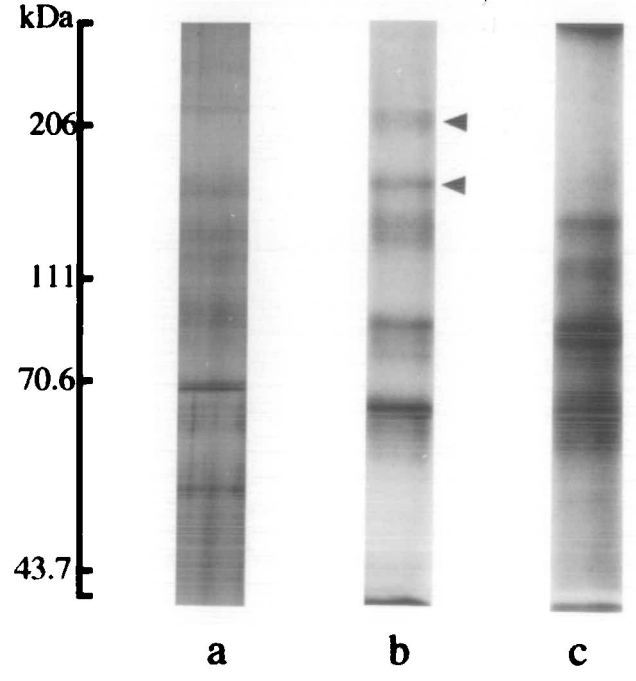

Fig. 3. **Phosphorylation of membrane proteins in the proteoliposomes catalyzed by 14 U/ml PKA (b) and 2.4 U/ml PKC (c).** Membrane proteins stained with silver are shown in lane a. Note that the 210 and 170 kDa proteins were phosphorylated by PKA but not by PKC.

Identification of the Dihydropyridine-Binding Protein— It has been suggested that the cardiac L-type Ca^{2+} channel has a subunit structure similar to that of the Ca^{2+} channel in skeletal muscle. Although the amino acid sequence of the main subunit, α_1, which possesses the ion-conducting pore, has been deduced from cloned cDNA, the molecular mass of the protein in the membrane remains controversial. Therefore, we attempted to identify the α_1 subunit of the cardiac Ca^{2+} channel by means of the specific binding of a dihydropyridine analogue to the α_1 subunit. Phosphoproteins were solubilized with SDS and then separated by gel filtration in the presence of SDS. Figure 4A shows a profile of the eluted proteins and the fractions containing ^{32}P, together with a calibration curve for molecular size. The radioactivity appeared in three major peaks, at fraction numbers 18, 27, and 44, and in minor peaks at 21 and 32–34. The major peaks corresponded to apparent molecular mass of about 200, 140, and 60 kDa, and the minor ones to 170 and 100–110 kDa, respectively. Incorporation of ^{32}P radioactivity in fractions corresponding to molecular mass smaller than 50 kDa was not evident in this experiment. We then subjected the proteins present in fractions numbers 18, 21, and 27 to SDS-PAGE and subsequent autoradiography (Fig. 5, lanes b–d). For fraction number 18, one phosphoprotein of 210 kDa was detected (lane b), whereas for fraction number 21, two bands of 210 and 150 kDa were obtained (lane c). Fraction number 27 gave main bands of 83, 75, and 65 kDa, and minor ones of 100 and 83 kDa (lane d).

Proteins in the proteoliposomes were photoaffinity-labeled with [^3H]azidopine in either the presence or absence of 1 μM nifedipine. Following this, the membrane proteins were solubilized with SDS and then separated by gel filtration under the same conditions as those used for the detection of phosphoproteins. The binding of [^3H]azidopine, which was specifically displaced by nifedipine, peaked at fraction number 18 (Fig. 4B). Thus, the peak of the azidopine binding coincided exactly with that of the first peak of phosphoproteins detected following PKA treatment. The molecular size of the azidopine-binding protein(s) in fraction number 18 was examined by SDS-PAGE and subsequent autoradiography. As shown in Fig. 5, lane e, the azidopine-binding protein was found as a single band at

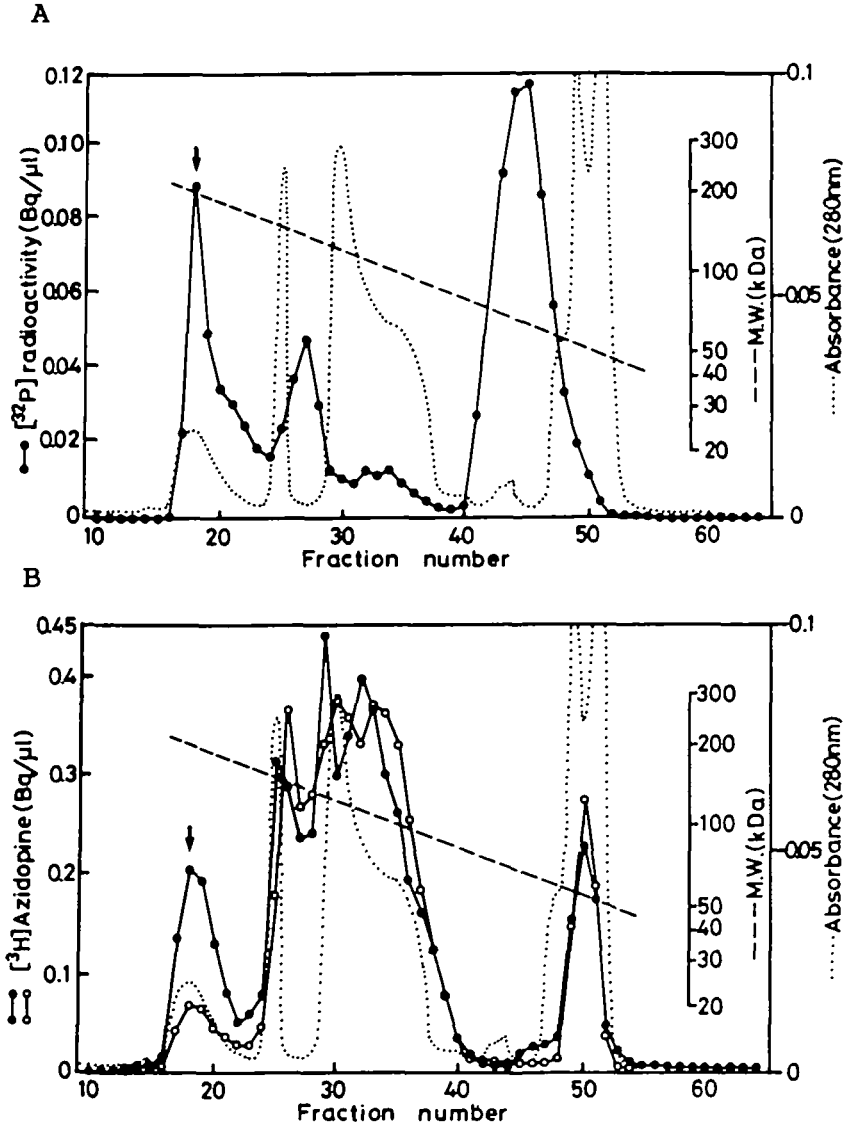

Fig. 4. **Separation of PKA-catalyzed phosphoproteins and [^3H]azidopine binding proteins in the proteoliposomes.** A: Proteoliposomes were incubated with 14 U/ml PKA and 100 μM [γ-^{32}P]MgATP, and then the membrane proteins were solubilized with SDS and separated by gel filtration. Note the major peaks at fractions 18, 27, and 44, and minor ones at 21, 32, and 34. The first peak, fraction 18, corresponding to a molecular mass of 200–210 kDa, is indicated by an arrow. B: Proteoliposomes were incubated with [^3H]azidopine for 1 h in the presence or absence of 1 μM nifedipine and then irradiated with UV light. Filled and open circles indicate [^3H]azidopine binding in the absence and presence of nifedipine, respectively. The nifedipine-sensitive binding peaked at fraction 18, as indicated by an arrow. In A and B, the dotted lines show the protein profile detected as the absorbance at 280 nm, and the broken lines the apparent molecular size determined using a standard kit.

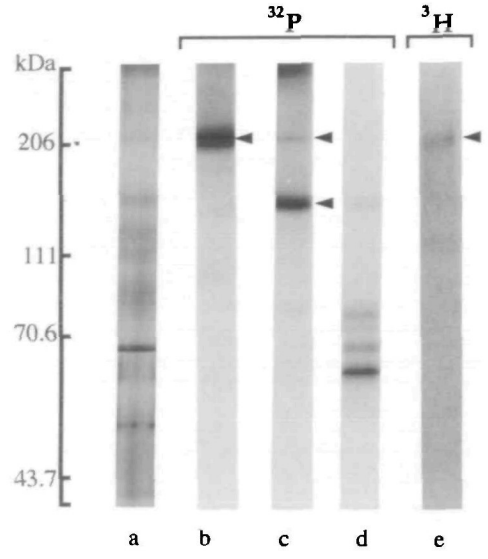

Fig. 5. **Autoradiograms of phosphoproteins and dihydropyridine binding proteins in proteoliposomes.** The phosphoproteins present in fraction 18 in Fig. 4A (lane b), fraction 21 (lane c), and fraction 27 (lane d) were separated by SDS-PAGE and then subjected to autoradiography for 2 days. The radioactivity was incorporated into proteins having molecular mass of 210 kDa (lane b), and 210 and 170 kDa (lane c). Lane e shows the autoradiogram of the [^{3}H]azidopine-binding protein in fraction 18 in Fig. 3B after separation by SDS-PAGE.

210 kDa. This molecular size was again comparable to that of the major phosphorylated band for phosphorylation with PKA (Fig. 5, lane b).

DISCUSSION

The major findings in this study are that: (1) Incubation of proteoliposomes with PKA but not with PKC enhanced the voltage-dependent and dihydropyridine-sensitive Ca^{2+} efflux from the proteoliposomes. (2) PKA but not PKC catalyzed the phosphorylation of 210 and 170 kDa membrane proteins at a concentration which enhances Ca^{2+} efflux from the proteoliposomes. In the present study, we used proteoliposomes reconstituted from bovine sarcolemmal proteins. We showed in a previous study (*15*) that the Ca^{2+} efflux induced by K^+ depolarization is sensitive to Ca^{2+} channel modulators, such as dihydropyridines (nifedipine and Bay K 8644) and a phenylalkylamine derivative, D-600. Therefore, Ca^{2+} efflux from the proteoliposomes is concluded to be that passing through the cardiac L-type Ca^{2+} channels. This finding has been confirmed in the present study.

The Ca^{2+} efflux is dependent on the prepotential (V_l) of the proteoliposomes. It is maximum at negative V_l values and minimum at V_l values around 0 mV. Phosphorylation of proteoliposomes by PKA only enhanced Ca^{2+} efflux at negative V_l values. This voltage-dependent effect of PKA is not due to voltage-dependent phosphorylation of proteoliposomes, because the phosphorylation reaction was performed at the same membrane potential, 0 mV, for all preparations. The potential-dependent increase in the Ca^{2+} efflux is consistent with the idea that Ca^{2+} efflux at positive V_l values represents that through channels of inside-out orientation, and the flux at positive V_l values may be attributed to outside-out channels. Since a flip-flop movement of the channels in the liposomal membrane is unlikely to occur during the Ca^{2+} loading procedure, it may be reasonable to assume that only the inside-out channels are relevant for modulation by phosphorylation. Alternatively, although less likely, the phosphoproteins might be dephosphorylated in a voltage-dependent manner by a membrane-associated intrinsic phosphatase during the incubation at V_l. Nevertheless, it can be safely concluded that Ca^{2+} flux through the reconstituted Ca^{2+} channels is enhanced by phosphorylation by PKA.

PKA catalyzes the phosphorylation of various membrane proteins in proteoliposomes. Among them, 210 and 170 kDa proteins are phosphorylated by PKA but not by PKC, so these phosphoproteins seem to contribute to the different modulation of Ca^{2+} efflux between PKA and PKC. The results of the present study strongly suggest that direct phosphorylation of the 210 and/or 170 kDa proteins is responsible for the modulation of the Ca^{2+} channel by PKA. The photosensitive dihydropyridine-class Ca^{2+} channel antagonist, [^{3}H]azidopine, which is known to bind to the α_1 subunit of the L-type Ca^{2+} channel, specifically labeled a protein of 210 kDa. This finding suggests that the 210 kDa phosphoprotein might be the α_1 subunit of the Ca^{2+} channel, although the possibility that this phosphoprotein is different from the channel subunit but has a similar molecular weight could not be excluded in this study.

The primary sequence of the α_1 subunit of the cardiac L-type Ca^{2+} channel deduced from its cloned cDNA consists of 2171 amino acid residues with a calculated molecular mass of 243 kDa (*20*). Although post-translational modification of this protein is not known, this molecular mass is larger than that of the α_1 subunit of the Ca^{2+} channel in skeletal muscle: which is 212 kDa based on the predicted sequence and 165 kDa for the purified protein. To date, the cardiac α_1 subunit of the Ca^{2+} channel has been reported to have a molecular mass of 165-250 kDa (*18, 21-27*). In intact myocytes, however, the direct phosphorylation of the cardiac α_1 subunit has not been supported (*22-24, 27*). Some studies suggest that, in heterogenous expression systems, the β subunit may be the target of phosphorylation by PKA (*28, 29*), while others support the view that phosphorylation of the α_1 subunit is responsible for modulation of the Ca^{2+} channel by PKA (*22, 23*). Recently, Norman and Leach reported that the rabbit α_1 subunit has a molecular mass of 185-210 kDa and is phosphorylated by PKA (*30*). The results of the present study also suggest that the α_1 subunit is phosphorylated in association with an increase in the Ca^{2+} flux through Ca^{2+} channels. Therefore, we support the view that phosphorylation of the α_1 subunit is responsible for the modulation of the Ca^{2+} channel by PKA.

Some further comparison of Ca^{2+} channel regulation between cardiac and skeletal muscle may be relevant. In skeletal muscle, regulation of the channel activity by PKA is suggested to be mediated through direct phosphorylation of the α_1 subunit. Analysis of the partially proteolyzed peptides revealed that Ser^{687} (between Repeats II and III) and/or Ser^{1854} (near the carboxy terminus) are most rapidly phosphorylated by PKA (*31, 32*). In cardiac muscle, however, corresponding phosphorylation sites are lacking in the predicted primary sequence (*20*). Thus, the finding

regarding the site of phosphorylation in skeletal muscle cannot be directly applied to cardiac muscles. It is interesting to speculate that the cytoplasmic region near the carboxy terminus of the α_1 subunit, which contains several potential phosphorylation sites (*20*), might be involved in the regulation of the Ca^{2+} channel through phosphorylation. Norman and Leach recently suggested that Ser^{1627} and Ser^{1700} may be the phosphorylation sites used by PKA (*30*). It would be of interest to determine whether or not the phosphorylation of these sites in the α_1 subunit leads to functional modulation of the Ca^{2+} channel activity.

In contrast to the prominent modulatory effect of PKA on the Ca^{2+} channel, the effect of PKC is still controversial. The effects reported have been an increase (followed by a decrease) (*33, 34*), no change (*35, 36*), and a decrease (*37, 38*). In this study, preincubation of the proteoliposomes with PKC did not modulate Ca^{2+} efflux through the reconstituted Ca^{2+} channels. This lack of an effect is not due to a lack of PKC activation, as the phosphorylation of 150, 130, 95, 67, and 62 kDa proteins was observed in the proteoliposomes. The failure of PKC to modulate Ca^{2+} efflux might, however, be related to the failure to phosphorylate the 210 and 170 kDa proteins in the proteoliposomes.

Cardiac L-type Ca^{2+} channels consist of α_1, α_2/δ, and β subunits (*24, 25, 30*), and expression of the corresponding mRNAs has been found (*39, 40*). It has also been reported that co-expression of the α_2/δ and/or β subunits significantly modifies the function of the Ca^{2+} channel (*41, 42*). In our experiments, not only the α_1 subunit but also other subunits were probably reconstituted together into liposomes. Therefore, our results suggest that modulation of the channel by PKC, if it occurs, may be mediated through phosphorylation of a protein other than the channel subunits themselves. Alternatively, some subunit(s) of the channel might be missing in our reconstituted proteoliposomes. Further studies are necessary to clarify the molecular mechanism of the modulation of the Ca^{2+} channel through phosphorylation by PKA and PKC.

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