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Functional reconstitution of skeletal muscle Ca^{2+} channels: Separation of regulatory and channel components

(calcium antagonists/ion channels/phosphorylation/signal transduction)

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ABSTRACT **Regulatory properties of a partially purified Ca^{2+}-channel preparation from isolated rabbit skeletal muscle triads were examined in proteoliposomes. These properties included (*i*) inhibition by phenylalkylamine antagonists, such as verapamil, (*ii*) inhibition by the GTP-binding protein G_o in the presence of guanosine 5′-[γ-thio]triphosphate, and (*iii*) regulation of phenylalkylamine inhibition as a result of phosphorylation by a polypeptide-dependent protein kinase (PK-P). By selective reconstitution of protein fractions obtained by wheat germ lectin and ion-exchange chromatography, a separation of Ca^{2+}-channel activity (fraction C) from regulatory component(s) (fraction R) responsible for verapamil sensitivity was achieved. Reconstitution of fraction C alone yielded vesicles that exhibited channel-mediated $^{45}Ca^{2+}$ uptake that could be directly inhibited by coreconstitution of G_o in the presence of guanosine 5′-[γ-thio]triphosphate. However, the $^{45}Ca^{2+}$ uptake obtained with fraction C was not inhibited by verapamil. Coreconstitution of fractions C and R yielded vesicles in which the sensitivity of $^{45}Ca^{2+}$ uptake to verapamil was restored. The verapamil sensitivity of this preparation could be inhibited by PK-P. Fraction C, obtained by wheat germ agglutinin-Sepharose chromatography followed by DEAE-Sephacel chromatography, included a 180-kDa protein that was phosphorylated by cAMP-dependent protein kinase (PK-A) but not by PK-P and a 145-kDa protein (180 kDa under nonreducing conditions) that was not phosphorylated by either kinase. Fraction R contained proteins that did not adsorb to wheat germ lectin and included 165-kDa and 55-kDa proteins that were phosphorylated by PK-P but not by PK-A. These results suggest a complex model for Ca^{2+}-channel regulation in skeletal muscle involving a number of distinct, separable protein components.**

Regulation of the influx of Ca^{2+} through specific channels located in the plasma membrane is important for the control of excitation–contraction coupling, hormone and neurotransmitter release, cell growth, and many other Ca^{2+}-dependent processes (1). Ca^{2+}-channel activity can be regulated by changes in membrane potential, by phosphorylation, and by the binding of specific Ca^{2+}-channel agonists or antagonists (2–4). Several Ca^{2+}-channel types have been identified that differ in their kinetic and conductance properties and in their ability to be regulated by the above mechanisms (5). Although many of the regulatory properties of the Ca^{2+} channel have been well described in whole-cell systems, the molecular aspects of Ca^{2+}-channel function and regulation remain to be determined. Thus far, biochemical characterizations of the Ca^{2+} channel have focused on identification of the polypeptide components of the complex through the use of radioactively labeled Ca^{2+} antagonists that are presumed to bind to proteins directly associated with the channel. The number of dihydropyridine binding sites in skeletal muscle, however, seems to far exceed the number of functional Ca^{2+} channels (6). The dihydropyridine-binding complex from cardiac muscle is a large transmembrane protein having a hydrodynamic molecular mass of ≈370 kDa (7) and consisting of a number of subunits whose reported apparent molecular masses vary depending on the conditions of the gel electrophoresis system used. The dihydropyridine receptor was first identified to be a 170- to 175-kDa glycosylated polypeptide that could be cleaved to 143-kDa and 30-kDa components under reducing conditions (8, 9) and that copurified with an additional 52- to 57-kDa subunit (8). However, antibodies raised to the dihydropyridine-binding protein immunoprecipitate a complex consisting of 175-kDa, 170-kDa, 52-kDa, and 33-kDa subunits, as determined by $NaDodSO_4$/5–15% polyacrylamide gradient gel electrophoresis under nonreducing conditions (10). The 175-kDa subunit was shown to be the polypeptide identified previously, as it was cleaved to 143-kDa and 32-kDa components under reducing conditions. The 170-kDa subunit is nonglycosylated and does not change its mobility upon reduction (10) and can be photoaffinity-labeled with the dihydropyridine [^{3}H]-azidopine (11). This polypeptide has also been shown to be phosphorylated by an unidentified intrinsic skeletal muscle protein kinase (12). Further biochemical characterization of the 175-kDa and 170-kDa components has clearly distinguished them as two distinct polypeptides (13). The amino acid sequence of the 170-kDa polypeptide containing the dihydropyridine binding site has been determined (14). A polypeptide in the range of 143–180 kDa is associated with the Ca^{2+}-channel complex and is phosphorylated by cAMP-dependent protein kinase (PK-A) (12–18). Functional reconstitution of Ca^{2+}-channel activity has been demonstrated by single-channel recording techniques (17, 19) and by $^{45}Ca^{2+}$ uptake (20), but considerable disagreement exists as to the identity of the proteins responsible for the influx of Ca^{2+}.

This report describes the purification and functional reconstitution of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized protein fractions from isolated rabbit skeletal muscle triads. One fraction contained Ca^{2+}-channel activity (fraction C) that was insensitive to the Ca^{2+} antagonist verapamil. Sensitivity to verapamil was restored upon addition of a fraction separated by wheat germ agglutinin (WGA) chromatography (fraction R). This sensitivity was abolished by exposure of fraction R to polypeptide-dependent protein kinase (PK-P). After coreconstitution of a purified preparation of Ca^{2+} channel (fraction C) with a highly purified preparation of the bovine brain protein G_o, Ca^{2+}-channel activity could be inhibited by

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PK-A, cAMP-dependent protein kinase; PK-P, polypeptide-dependent protein kinase; GTP[γ-S], guanosine 5′-[γ-thio]triphosphate; G protein, GTP-binding protein; WGA (WGL), wheat germ agglutinin (lectin).

guanosine 5'-[γ-thio]triphosphate (GTP[γ-S]), providing evidence that skeletal muscle Ca^{2+} channels can be regulated by direct interaction with GTP-binding proteins (G proteins). By identifying protein components present in fraction C and fraction R, it was possible to assign functions to some of the components that constitute the Ca^{2+}-channel complex. Further, these results suggest that Ca^{2+}-channel regulation involves a complex interaction of a number of separable protein components.

MATERIALS AND METHODS

Materials. $^{45}CaCl_2$, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([^{3}H]methoxycarbonyl)pyridine-3-carboxylate ([^{3}H]PN200-110), and [γ-^{32}P]ATP were from New England Nuclear. GTP[γ-S] was from Boehringer Mannheim; Bay K 8644, from Miles Institute of Preclinical Pharmacology (New Haven, CT); and Extracti-Gel D, from Pierce. WGA-Sepharose and DEAE-Sephacel were from Pharmacia. All other chemicals were from Sigma. PK-A was generously supplied by E. Fischer (University of Washington, Seattle). PK-P was purified from yeast (21). Preparations of purified G_o (containing 80% G_o and 20% G_i) and purified G-protein βγ-subunit complex were donated by J. Benovic (Duke University Medical Center).

Ca^{2+}-Channel Purification and Reconstitution. Rabbit skeletal muscle triads (250 mg of protein) were prepared as described (22), incubated with 5 μM Bay K 8644, and solubilized in 60 ml of 1% (wt/vol) CHAPS/10% (vol/vol) glycerol/200 mM KCl/50 mM Tris·HCl, pH 7.4/1 mM phenylmethylsulfonyl fluoride (CHAPS/glycerol/Bay K 8644) at 4°C. Solubilized material was centrifuged at 150,000 × g for 1 hr, and the supernatant was concentrated to 5 ml by ultrafiltration through an Amicon YM100 filter and applied to a 20-ml WGA-Sepharose column equilibrated in CHAPS/glycerol/Bay K 8644. The column was washed with 80 ml of the same buffer. The first 40 ml of the wash was discarded and the final 40 ml was concentrated to 2 ml by ultrafiltration through an Amicon YM100 filter (this concentrated material is referred to as fraction R). Glycosylated proteins were eluted with 200 mM *N*-acetyl-D-glucosamine in CHAPS/glycerol/Bay K 8644, diluted 1:10 into 20 mM Tris·HCl, pH 7.4/1% CHAPS/1% glycerol/1 mM phenylmethylsulfonyl fluoride, and loaded onto a 3-ml DEAE-Sephacel column equilibrated with the same buffer. Channel protein was eluted from the DEAE column with 250 mM potassium oxalate in CHAPS/glycerol (fraction C). Thus, three distinct protein fractions were used in the experiments presented: a crude eluate from the wheat germ lectin column (WGL fraction), the wheat germ lectin wash fractions (fraction R), and the DEAE eluate (fraction C). These protein fractions were reconstituted either individually or together into crude soybean lipid vesicles by Extracti-Gel D chromatography (23).

Electrophysiological Recording. Single-channel recordings were obtained from bilayers by the contact method (17). Ten to 20 μl of vesicles (containing 20 fmol of dihydropyridine receptor as determined by [^{3}H]PN200-110 binding) were added to 500 μl of 90 mM $BaCl_2$/5 mM Hepes/NaOH, pH 7.4, in a Teflon container at 24°C. Ten microliters of a mixture of phosphatidylethanolamine, phosphatidylserine, and cholesterol in a weight ratio of 70:15:15 in *n*-hexane (1 mg of lipid per ml) was applied to the top of the bath to form a monolayer. Bilayers were formed at the tip of a patch-clamp pipette containing 90 mM $BaCl_2$/5 mM Hepes/NaOH, pH 7.4, by two passes of the pipette tip through the monolayer. The currents were filtered at 1 kHz and recorded on video tape (16-bit resolution, Unitrade pulse-code modulator). For analysis, the data were transferred in digital format to a PDP 11/24 computer through the parallel port of the pulse-code modulator.

$^{45}Ca^{2+}$ Uptake Measurements. $^{45}Ca^{2+}$ uptake was measured as described (24). To remove extravesicular potassium oxalate, 300-μl samples of reconstituted vesicles were desalted on 1-ml Sephadex G-50 (fine) columns equilibrated in isosmotic (450 mosM) 200 mM KCl/50 mM Tris·HCl, pH 7.4. $^{45}Ca^{2+}$ uptake was then measured by diluting 200 μl of vesicles into 2 ml of 200 mM KCl/50 mM Tris·HCl, pH 8.5/0.3 mM $CaCl_2$ containing 2 μCi of $^{45}Ca^{2+}$ (1 μCi = 37 kBq). Alternatively, vesicles were diluted into 200 mM NaCl/50 mM Tris·HCl, pH 7.4/3 mM $CaCl_2$ (2 μCi of $^{45}Ca^{2+}$)/2 pM valinomycin, with identical results. After incubation at 22°C the reaction was stopped at the designated times by adding 200-μl aliquots to a stop solution containing assay buffer together with 5 mM $MgSO_4$ and 1 mM $CdCl_2$. The proteoliposomes were then passed through 3-ml Dowex 50 columns equilibrated in 450 mosM sucrose to remove extravesicular $^{45}Ca^{2+}$ (24). Radioactivity determined by liquid scintillation spectroscopy was used to measure accumulated intravesicular $^{45}Ca^{2+}$.

Phosphorylation and Electrophoresis Methods. In a final volume of 50 μl, the phosphorylation reaction mixture contained 20 mM Hepes/NaOH (pH 7.4), 5 mM $MgCl_2$, 10 mM thioglycerol, 25 ng of PK-P, and 25 μl of fraction R (1 mg/ml) or 25 μl of fraction C (0.5 mg/ml), in the presence or absence of 5 μg of histone 1. The reaction was started by adding 5 μl of 100 μM [γ-^{32}P]ATP (4000–5000 cpm/pmol). Phosphorylation conditions for PK-A (1 μg) were identical to those used for PK-P except that histone was omitted. After 1 hr of incubation at 22°C, the reaction was stopped by adding 10 μl of 5× $NaDodSO_4$ sample buffer, and the entire sample was analyzed by $NaDodSO_4$/10% PAGE. The gels were dried and exposed to Kodak XAR-5 film, and autoradiograms were developed as described (25).

RESULTS

Electrophysiological Characterization of Reconstituted Ca^{2+} Channels. Single-channel Ca^{2+} currents were recorded by incorporation of reconstituted channels (WGL fraction) into bilayers formed on the tip of a patch-clamp pipette (17). We observed Ca^{2+}-channel activity only when the channel was solubilized and purified in the presence of Bay K 8644, in agreement with the findings of Curtis and Catterall (20).

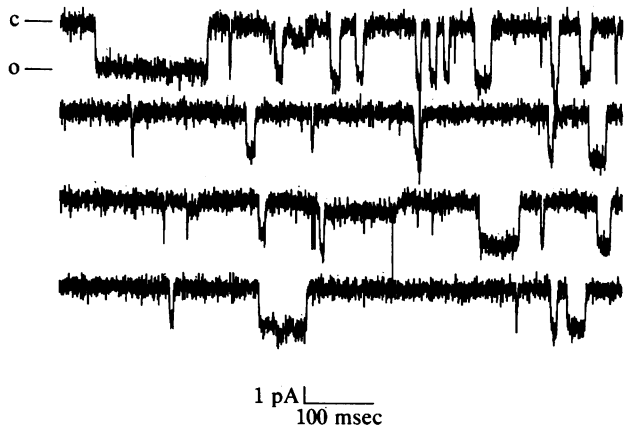

FIG. 1. Single-channel currents recorded from phospholipid bilayers containing reconstituted Ca^{2+} channels. Channels were solubilized from isolated triads and reconstituted as described in *Materials and Methods*. Wheat germ lectin-purified Ca^{2+} channels (WGL fraction) were incorporated into phospholipid bilayers formed at the tip of a patch clamp pipette containing 90 mM $BaCl_2$ in 5 mM Hepes/NaOH, pH 7.4. Steady-state single-channel currents from a representative experiment elicited by holding the membrane potential at −100 mV are shown. Closed and open states of the channel are indicated by c and o, respectively.

Ca^{2+} channels solubilized in CHAPS and reconstituted into phospholipid vesicles (Fig. 1) displayed many of the characteristics of L-type Ca^{2+}-channel currents recorded from cardiac cell-attached patches and from incorporation of crude skeletal muscle membrane vesicles into lipid bilayers (26, 27). The reconstituted channels showed a conductance of 23 pS in symmetrical 90 mM $BaCl_2$ at -100 mV that could be blocked by the phenylalkylamines, such as verapamil. Channel open times varied from milliseconds to more than a second. Also seen in Fig. 1 is an example of a 5- to 8-pS conductance often seen when phospholipids without protein were used.

Inhibition of $^{45}Ca^{2+}$ Uptake by Phenylalkylamines and G Proteins. The ability of phenylalkylamines and G proteins to inhibit $^{45}Ca^{2+}$ uptake into phospholipid vesicles containing reconstituted Ca^{2+} channels was examined at different stages of purification. $^{45}Ca^{2+}$ uptake into vesicles reconstituted with the WGL fraction was inhibited by verapamil (Fig. 2). The order of potency for inhibition by other phenylalkylamines was desmethoxyverapamil > verapamil > gallopamil (data not shown). The concentrations of the inhibitors used (100 μM) in these experiments are consistent with the concentrations required to displace bound [^{3}H]PN200-110 in our reconstituted system and are consistent with the concentrations required to block Ca^{2+} currents in mammalian skeletal muscle fibers (28). When a purified preparation of bovine brain G proteins [80% G_o and 20% G_i (29)] was inserted into lipid vesicles along with the WGL fraction, it had no effect on $^{45}Ca^{2+}$ uptake, either in the presence or in the absence of the nonhydrolyzable GTP analog GTP[γ-S] (Fig. 2).

A reconstituted preparation purified by WGL and subsequent DEAE chromatography (fraction C) exhibited $^{45}Ca^{2+}$ influx comparable to that seen in preparations purified by WGL alone (Fig. 3). However, the additional DEAE purification step resulted in the loss of the inhibitory response of $^{45}Ca^{2+}$ uptake to verapamil. A considerable loss of dihydropyridine binding was also observed (data not shown). However, when the DEAE preparation was coreconstituted with a purified preparation of G_o, GTP[γ-S]-dependent inhibition of $^{45}Ca^{2+}$ uptake was observed (Fig. 3). The G_o-mediated inhibition was dependent on GTP[γ-S], as no effect was observed in the absence of GTP[γ-S] or when GDP was substituted for GTP[γ-S] (data not shown). Similarly, no effect was seen when GTP[γ-S] was added to vesicles containing the WGL fraction or fraction C alone. Coinsertion of purified G-protein $\beta\gamma$ subunits from bovine brain (without α subunit) had no effect on $^{45}Ca^{2+}$ uptake.

Restoration of Reconstituted Verapamil Sensitivity. Extensive washing of solubilized material adsorbed to WGA-Sepharose prior to elution with *N*-acetyl-D-glucosamine also resulted in a loss of verapamil sensitivity upon reconstitution of the WGL fraction. To identify the protein components involved in verapamil-induced inhibition of $^{45}Ca^{2+}$ influx, a protein fraction not retained on the WGL column (fraction R) was examined for its ability to restore verapamil sensitivity upon coreconstitution with fraction C. The insertion of fraction C into proteoliposomes resulted in a verapamil-insensitive $^{45}Ca^{2+}$ influx, as shown above, whereas fraction R did not reconstitute $^{45}Ca^{2+}$ uptake (Fig. 4*A*). However, coreconstitution of fraction R with fraction C restored the ability of verapamil to inhibit $^{45}Ca^{2+}$ uptake (Fig. 4*B*).

Phosphorylation by Protein Kinases. The ability of PK-P and PK-A to phosphorylate the protein components of fractions C and R was examined. Two major polypeptides in fraction R (165 kDa and 55 kDa) were substrates for PK-P but not for PK-A (Fig. 5*A*). Upon phenyl-Sepharose chromatography, the two peptides copurified as a single complex. Several proteins in fraction C, including a 180-kDa polypeptide, served as substrates for PK-A but not for PK-P (Fig.

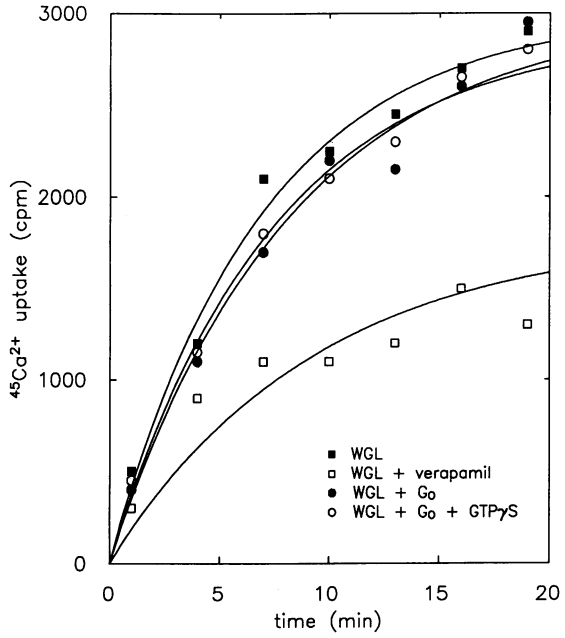

FIG. 2. Verapamil inhibition of $^{45}Ca^{2+}$ uptake into phospholipid vesicles containing the WGL fraction. Four hundred microliters of the WGL fraction (≈1 nmol of [^{3}H]PN200-110 binding sites) was reconstituted with 10 μg of G_o (with or without 50 μM GTP[γ-S]) in 0.8% cholate/50 mM Tris·HCl, pH 7.4 (WGL + G_o) or with 50 μl of G_o buffer alone (WGL). $^{45}Ca^{2+}$ uptake was initiated by dilution of 200 μl of vesicles (without G_o) into 2 ml of 200 mM KCl/50 mM Tris·HCl, pH 8.5/0.3 mM $CaCl_2$ containing 2 μCi of $^{45}Ca^{2+}$ and ethanol (<0.05%) as control (■) or 100 μM verapamil (□) or by dilution of 200 μl of vesicles (with G_o) into the same reaction buffer in the absence (●) or presence (○) of GTP[γ-S]. Nonspecific $^{45}Ca^{2+}$ uptake was determined with protein-free vesicles at each time point (1200 cpm after 20 min) and subtracted from the experimental data in this and all subsequent figures.

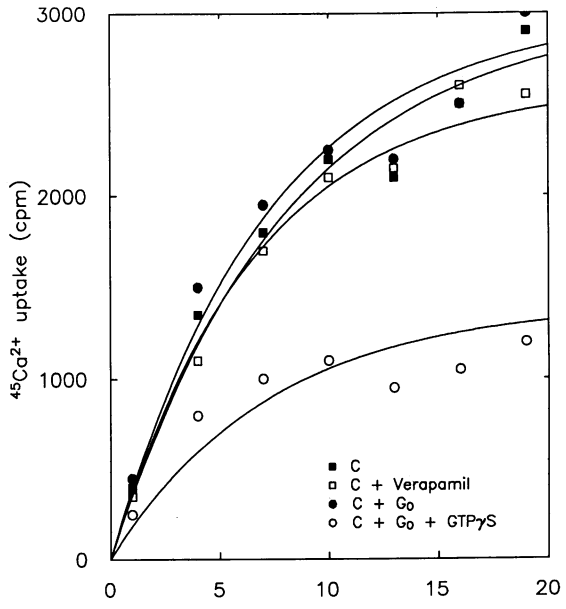

FIG. 3. G-protein inhibition of $^{45}Ca^{2+}$ uptake into phospholipid vesicles containing fraction C. Two hundred microliters of fraction C was reconstituted with 10 μg of G_o (with or without 50 μM GTP[γ-S]) in 0.8% cholate/50 mM Tris·HCl, pH 7.4 (C + G_o) or with 50 μl of G_o buffer alone (C). $^{45}Ca^{2+}$ uptake was initiated by dilution of 200 μl of vesicles (without G_o) into 2 ml of 200 mM KCl/50 mM Tris·HCl, pH 8.5/0.3 mM $CaCl_2$ containing 2 μCi of $^{45}Ca^{2+}$ and ethanol as control (■) or 100 μM verapamil (□) or by dilution of 200 μl of vesicles (with G_o) into the same reaction buffer in the absence (●) or presence (○) of GTP[γ-S].

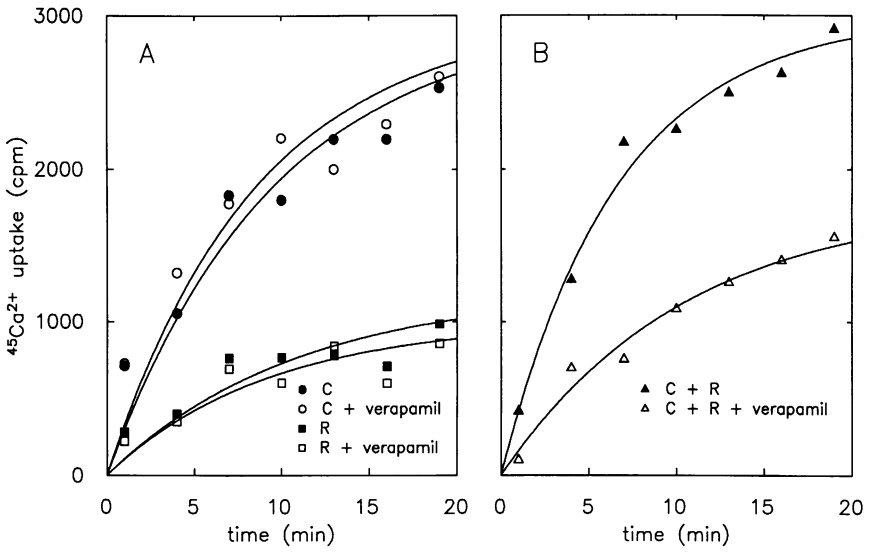

FIG. 4. Restoration of reconstituted verapamil sensitivity. (*A*) Two hundred microliters of either fraction C (plus 200 μl of WGA-Sepharose wash buffer) or fraction R (plus 200 μl of DEAE-Sephacel elution buffer) was reconstituted. $^{45}Ca^{2+}$ uptake was initiated by dilution of 200 μl of vesicles containing fraction C into 2 ml of 200 mM KCl/50 mM Tris·HCl, pH 8.5/0.3 mM $CaCl_2$ containing 2 μCi of $^{45}Ca^{2+}$ and ethanol as control (●) or 100 μM verapamil (○). Vesicles containing fraction R were also diluted into reaction mixture containing ethanol as control (■) or 100 μM verapamil (□) and assayed as described. (*B*) Two hundred microliters of fraction C was combined with 200 μl of fraction R (C + R) and reconstituted. $^{45}Ca^{2+}$ uptake was measured in the presence of ethanol (▲) or 100 μM verapamil (△).

5*A*). The major protein component in fraction C, as determined by silver staining, was a 145-kDa peptide (180-kDa peptide under nonreducing conditions) that was not phosphorylated by either kinase (data not shown). To examine the functional consequences of phosphorylation by PK-P, experiments identical to those illustrated in Fig. 4 were performed. Phosphorylation of fraction R peptides before coreconstitution with fraction C eliminated the ability of verapamil to inhibit $^{45}Ca^{2+}$ uptake (Fig. 5*B*). ATP, histone, or ATP plus histone had no effect on $^{45}Ca^{2+}$ uptake. The dependence of phosphorylation on histone, which serves as an activator of PK-P, can be seen clearly in Fig. 5*A*.

DISCUSSION

The results suggest that the regulation of Ca^{2+}-channel function is mediated by a complex mechanism involving multiple protein components. The isolated channel in fraction C is inhibited by activated G proteins, but this inhibition does not occur in the presence of one or more regulatory components found in fraction R. The components in fraction R, on the other hand, confer verapamil sensitivity, and the interaction of these components with the channel is regulated by phosphorylation. Specific labeling of phosphoproteins in both fractions has identified potential protein components of the Ca^{2+}-channel complex.

Biochemical characterization of the Ca^{2+}-channel complex thus far has focused on proteins that appear to bind radiolabeled Ca^{2+} antagonists, and there is considerable disagreement among different laboratories as to the exact size and subunit composition of these receptor proteins. Because of these difficulties in subunit designation, we chose to identify the proteins by their ability to be phosphorylated by protein kinases. In fraction R we observed 165-kDa and

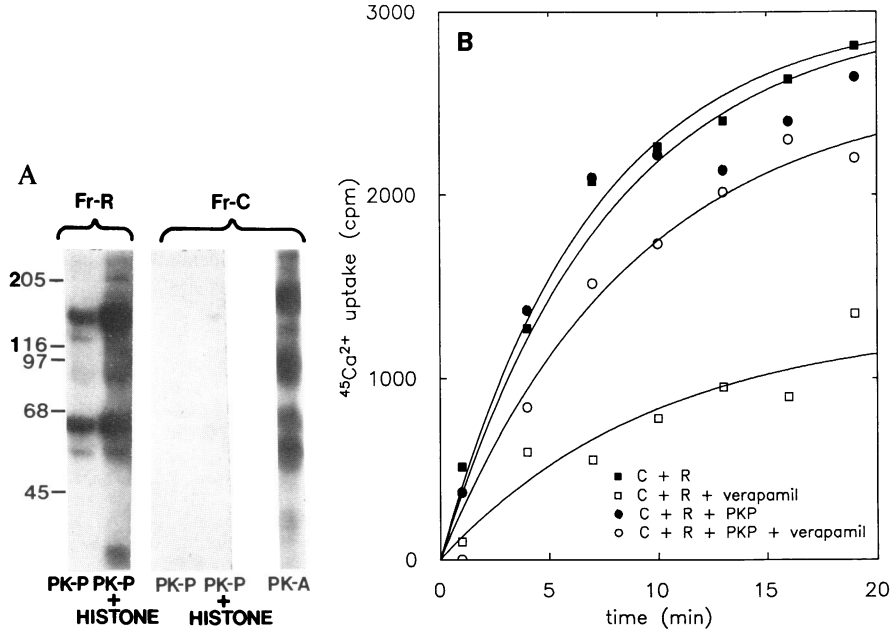

FIG. 5. Phosphorylation of fractions C and R by protein kinases. (*A*) Autoradiograms of phosphorylated substrates of PK-P and PK-A. Fraction R was incubated with PK-P in the absence or presence of histone. Fraction C was incubated with PK-P in the absence or presence of histone or was incubated with PK-A. (*B*) Two hundred microliters of fraction C and of fraction R was reconstituted as described in *Materials and Methods*, except for the inclusion of 0.2 mM ATP and 30 μg of histone 1, either in the absence (C + R) or presence of 100 ng of PK-P (C + R + PK-P). $^{45}Ca^{2+}$ uptake was initiated by diluting 200 μl of C + R vesicles into 2 ml of 200 mM KCl/50 mM Tris·HCl, pH 8.5/0.3 mM $CaCl_2$ containing 2 μCi of $^{45}Ca^{2+}$ and ethanol as control (■) or 100 μM verapamil (□). Two hundred microliters of C + R + PK-P vesicles was diluted into an identical reaction mixture containing ethanol (●) or 100 μM verapamil (○).

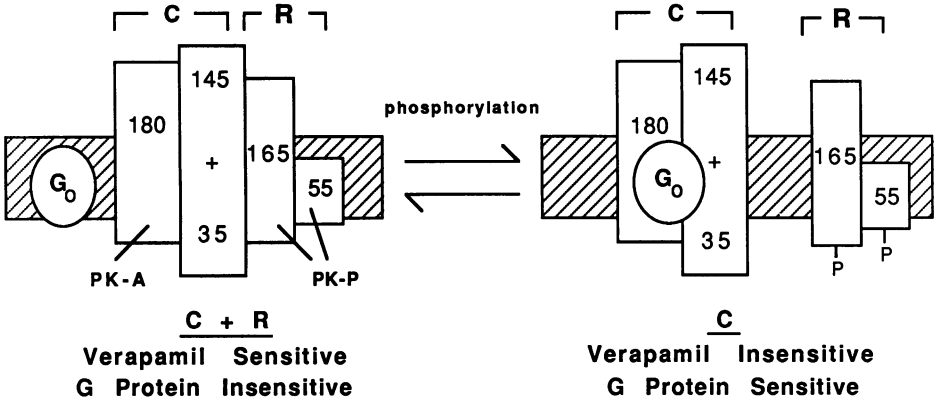

FIG. 6. Proposed model of Ca^{2+}-channel regulation depicting the separation of regulatory (R) and channel (C) components. The Ca^{2+}-channel complex is shown to exist in equilibrium between two states, one in which the channel is sensitive to Ca^{2+} antagonists (e.g., verapamil) and insensitive to inhibition by G proteins and another in which the channel can be blocked by G proteins but is insensitive to Ca^{2+} antagonists. Phosphorylation by PK-P promotes the Ca^{2+}-antagonist-insensitive state. Molecular sizes (kDa) and locations of polypeptide components are indicated.

55-kDa polypeptides that are phosphorylated by PK-P. In fraction C we observed a 180-kDa polypeptide under reducing conditions that is phosphorylated by PK-A, and a 145-kDa polypeptide that is not phosphorylated by either kinase. Under nonreducing conditions, the 145-kDa band shifts to 180 kDa. Upon insertion of fraction C into proteoliposomes, a verapamil-insensitive $^{45}Ca^{2+}$ uptake was observed. Verapamil sensitivity was restored by coreconstitution of fraction C with fraction R. Based on the susceptibility to phosphorylation by PK-A, we suggest that the 180-kDa protein in fraction C may be the same described by Hosey *et al.* (18) as a 165-kDa protein. The 145-kDa protein in fraction C appears to be the same as the 143-kDa subunit of Curtis and Catterall (8), the 150-kDa glycoprotein of Sharp *et al.* (11), and the 140- to 150-kDa glycoprotein of Cooper *et al.* (30), which all show a shift to a higher molecular mass under nonreducing conditions. A discrepancy exists in that Cooper *et al.* (30) refer to this protein (isolated from cardiac muscle) as the dihydropyridine-binding protein, whereas Sharp *et al.* (11) suggest that this glycoprotein is not the receptor for dihydropyridines. Rather, they propose that a 170-kDa skeletal muscle protein, which is not a glycoprotein and does not shift under reducing conditions, binds dihydropyridines. Imagawa *et al.* (12) reported that 170-kDa and 52-kDa proteins in their preparations are phosphorylated by an endogenous unidentified kinase. In our preparation the endogenous kinase phosphorylates the 165-kDa and 55-kDa proteins found in fraction R. It is not clear whether the 165-kDa protein, the 55-kDa protein, or both are required for verapamil sensitivity.

G proteins regulate Ca^{2+}-channel currents in neuronal and cardiac cells (31–33). Our data show that G_o-mediated inhibition of $^{45}Ca^{2+}$ influx in proteoliposomes occurs only with more highly purified channel preparations (fraction C). Crude preparations (WGL fraction) are insensitive to G_o. This observation suggests that a component separated from the complex during purification inhibits the functional interaction of G_o with the channel. This raises the possibility that one or more components of fraction R (possibly the 165-kDa and 55-kDa proteins) may be part of a regulatory mechanism. Since presumably the phosphorylation of the 165-kDa and 55-kDa proteins by PK-P prevents the Ca^{2+}-channel inhibition by verapamil, we propose that the phosphorylation may alter the ability of the regulatory component of fraction R to interact with the channel component of fraction C. Fig. 6 is a schematic representation of our working hypothesis.

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