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Phosphorylation of the calcium antagonist receptor of the voltage-sensitive calcium channel by cAMP-dependent protein kinase

(transverse-tubule membranes/receptor purification/dihydropyridines/ion transport/ion channel)

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ABSTRACT **Physiological studies indicate that voltage-sensitive calcium channels are regulated by cAMP and protein phosphorylation. The calcium antagonist receptor of the voltage-sensitive calcium channel from transverse-tubule membranes consists of three subunits, designated α, β, and γ. The catalytic subunit of cAMP-dependent protein kinase phosphorylates both the α and β subunits of the purified receptor at a rate and extent that suggests they are potential physiological substrates of this enzyme. The phosphorylation of the α and β subunits in transverse-tubule membranes was analyzed by two-dimensional gel electrophoresis. In intact transverse-tubule membranes, the α subunit is not significantly phosphorylated. However, the β subunit, identified by its M_r, pI, and binding to wheat germ agglutinin-Sepharose, was one of the substrates selectively phosphorylated by cAMP-dependent protein kinase in transverse-tubule membranes. These results suggest that cAMP-dependent phosphorylation of the β subunit of the calcium antagonist receptor may be an important regulatory mechanism for calcium channel function.**

Voltage-sensitive calcium channels mediate calcium-dependent depolarization of excitable cells, cause increased cytosolic calcium concentration in response to membrane depolarization, and initiate excitation–contraction and excitation–secretion coupling (1, 2). Several lines of evidence indicate that the activity of calcium channels is modulated directly or indirectly by cAMP-dependent protein phosphorylation. In the heart, the positive inotropic and chronotropic effects of norepinephrine and other β-adrenergic agonists are due to increased inward calcium current during the plateau phase of the cardiac action potential (3, 4). These effects are mimicked by intracellular injection of cAMP (5, 6) or of the catalytic subunit of cAMP-dependent protein kinase (7, 8), in agreement with the proposal that cAMP-dependent protein phosphorylation is the final step in the regulatory pathway. In the nervous system, intracellularly perfused molluscan neurons lose their voltage-sensitive calcium current unless ATP, Mg^{2+}, and cAMP or the catalytic subunit of cAMP-dependent protein kinase are included in the perfusate (9, 10), and similar requirements for maintenance of calcium currents are observed in dorsal root ganglion neurons (11). These results suggest that modulation of calcium channel properties by a regulatory pathway involving cAMP-dependent protein phosphorylation may be a common property of these ionic channels in different tissues. However, it is not known whether the calcium channel itself is the site of this phosphorylation or whether another protein regulator is phosphorylated which in turn modulates calcium channel properties.

Skeletal muscle transverse tubules (T-tubules) have large voltage-sensitive calcium currents that are blocked by dihydropyridine calcium channel antagonists such as nitrendipine (12, 13). Radioligand binding studies with [^{3}H]nitrendipine show that these membranes contain the highest concentration of calcium antagonist receptor sites of any system examined to date (14, 15). We recently have developed methods to solubilize the calcium antagonist receptor of the voltage-sensitive calcium channel with digitonin (16) and to purify it to near homogeneity from skeletal muscle T-tubule membranes (17). The purified calcium antagonist receptor consists of a complex of three subunits: α with M_r of 135,000 ± 5000 (mean ± SD, $n = 11$), β with M_r of 50,000, and γ with M_r of 33,000. The α subunit has a higher apparent M_r (162,000) in the unreduced state, suggesting that it contains an internal disulfide bond that restricts polypeptide unfolding (17). In this report, we describe phosphorylation of these subunits by cAMP-dependent protein kinase in purified preparations of the calcium antagonist receptor and *in situ* in T-tubule membranes.

MATERIALS AND METHODS

Materials. [^{3}H]Nitrendipine and [γ-^{32}P]ATP were obtained from New England Nuclear. The catalytic subunit of type I cAMP-dependent protein kinase, prepared from bovine skeletal muscle according to method 1 of Bechtel *et al.* (18), was provided by B. Flug from the laboratory of E. G. Krebs. The sodium channel from rat brain was prepared as described (19).

Preparation of T-Tubule Membranes. T-tubule membranes (15) and the purified calcium antagonist receptor were prepared from rabbit fast muscle as previously described (17), with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), pepstatin A (1 μM), 1,10-phenanthroline (1 mM), iodoacetamide (1 mM), antipain (1 μg/ml), and leupeptin (1 μg/ml) present in all solutions. Protein concentrations were determined by the dye-binding assay of Bradford (20).

Phosphorylation Assay. The standard reaction mixture containing 0.5–5.0 pmol of calcium antagonist receptor (membrane-bound, solubilized, or purified), 6 mM $MgCl_2$, 6 mM EGTA, 2 μg of the catalytic subunit of cAMP-dependent protein kinase, and 0.06 μM [γ-^{32}P]ATP (2.9×10^3 cpm/fmol) in a final volume of 120 μl. The reaction was initiated by the addition of catalytic subunit, and samples were incubated for 10 min at 37°C, unless otherwise stated. The reaction was stopped by addition of 90 μl of 100 μM EDTA, samples were cooled to 4°C, and unbound ^{32}P was removed by centrifugation through a 2-ml Sephadex G-50 column (21). Samples then were immediately processed for polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Samples for NaDodSO$_4$/PAGE were mixed with concentrated sample buffer to give final concentrations of 3% (wt/vol) NaDodSO$_4$, 12 mM

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Abbreviations: T-tubule, transverse tubule; WGA, wheat germ agglutinin.

EDTA, 12.5% (vol/vol) glycerol, 30 mM Tris Cl (pH 7.6), and 20 mM *N*-ethylmaleimide or dithiothreitol. Samples were placed in boiling water for 3 min and then analyzed in a discontinuous gel system according to Laemmli (22), consisting of a 3% stacking gel and a separation gel containing a linear 5–15% (wt/vol) acrylamide gradient. Gels were stained with either Coomassie brilliant blue R-250 or silver (23). Two-dimensional gel electrophoresis was carried out as described by O'Farrell (24). Solid urea was added to samples (150 μl) to bring the concentration to 9 M, and then concentrated lysis buffer was added to give 2% Nonidet P-40, 1.5% ampholytes, and 2.5% 2-mercaptoethanol. After isoelectric focusing, tube gels were equilibrated in 10% NaDodSO$_4$/10% glycerol/30 mM Tris Cl, pH 6.8/20 mM dithiothreitol. The second dimension was run in the NaDodSO$_4$/polyacrylamide gradient gel system described above. Gels were stained with Coomassie blue, destained, dried, and exposed to Kodak X-Omat film. The amount of ^{32}P incorporated into labeled bands was determined as described (25).

RESULTS

Phosphorylation of the α and β Subunits of the Purified Calcium Antagonist Receptor. The calcium antagonist receptor was purified and incubated with [γ-^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase (see *Materials and Methods*). The labeled receptor then was mixed with a 10-fold excess of unlabeled receptor to allow visualization of the minor subunit bands, denatured with NaDodSO$_4$ without reduction, and analyzed by polyacrylamide gel electrophoresis. Protein was detected by silver stain, and ^{32}P incorporation, by autoradiography. As shown previously (17), the purified calcium antagonist receptor consists of three subunits—α, β, and γ—which comprise nearly all of the protein of the purified preparation (Fig. 1*A*, lane 1). The apparent M_rs of these subunits under nonreducing conditions are 165,000, 53,000, and 32,000, respectively (Fig. 1*A*, lane 1).

Fig. 1*A* (lane 2) presents an autoradiogram showing incorporation of ^{32}P into the subunits of the calcium antagonist receptor. The α and β subunits are labeled but the γ subunit is not. Migration of the phosphorylated β subunit corresponds almost exactly to the migration of the unlabeled subunit. In contrast, the phosphorylated α subunit (lane 2) migrates slightly more rapidly than the majority of the unlabeled α subunit (lane 1). Evidently, phosphorylation alters the binding of NaDodSO$_4$ or the conformation of the denatured polypeptide sufficiently to increase its electrophoretic mobility.

To provide further evidence that the phosphorylated protein bands that comigrate with the α and β subunits are indeed components of the calcium antagonist receptor, we compared the sedimentation of the phosphorylated bands and the receptor in sucrose gradients. The calcium antagonist receptor was labeled with [^{3}H]nitrendipine, solubilized, and purified by chromatography on wheat germ agglutinin (WGA)-Sepharose and DEAE-Sephadex as described previously (17). The partially purified preparation was sedimented through a linear sucrose gradient, and specifically bound [^{3}H]nitrendipine was determined in each fraction. The calcium antagonist receptor migrated in fractions 14 and 15 (Fig. 2*B*). Aliquots of individual sucrose gradient fractions were phosphorylated in the presence of the catalytic subunit of cAMP-dependent protein kinase, reduced with dithiothreitol, and subjected to NaDodSO$_4$/PAGE. Two phosphorylated bands comigrated with the peak of the [^{3}H]nitrendipine–receptor complex (Fig. 2). A labeled band corresponding to an apparent M_r of 50,000, identical to that of the β subunit of the calcium antagonist receptor, was most prominent in fractions 14 and 15 (Fig. 2*A*). Similarly, these two fractions contained a doublet consisting of a prominent labeled band at M_r 145,000 and a fainter band at M_r 165,000 (Fig. 2*A*) which had the same apparent M_rs and electrophoretic behavior as the α subunit of the calcium antagonist receptor after incomplete reduction (17). Phosphorylated bands with apparent M_rs of 40,000 and 28,000 were observed in each sample, representing the catalytic subunit of cAMP-dependent protein kinase and an unidentified protein, respectively.

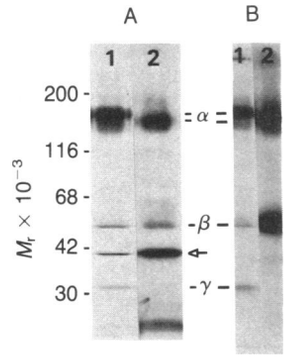

FIG. 1. Phosphorylation of the α and β subunits of the calcium antagonist receptor. (*A*) An aliquot (1.5 pmol) from the peak of the [^{3}H]nitrendipine–receptor complex from the final sucrose gradient purification step was phosphorylated under the standard reaction conditions. One-tenth of the phosphorylated sample (0.15 pmol) was added to an aliquot (1.5 pmol) of unphosphorylated receptor from the same sucrose gradient fraction, boiled in NaDodSO$_4$/PAGE sample buffer containing 20 mM *N*-ethylmaleimide, and subjected to electrophoresis in a 5–15% gradient gel. The wet gel was silver-stained, wrapped in plastic, and exposed to Kodak X-Omat film for 5 days. Photographs of the silver-stained gel (lane 1) and autoradiograph (lane 2) were taken and printed to equal scale. Autophosphorylated and silver-stained catalytic subunit are marked by the arrow. Positions of M_r standards are shown at left. (*B*) An aliquot (2 pmol) of purified calcium antagonist recptor was phosphorylated in the presence of 3 μM [γ-^{32}P]ATP (25 cpm/fmol) and mixed with an equal amount of unphosphorylated receptor labeled with [^{3}H]nitrendipine. This sample was sedimented through a second 5–20% sucrose gradient, and the peak of bound [^{3}H]nitrendipine was analyzed by NaDodSO$_4$/PAGE and silver-staining as described above (lane 1). Lane 2 is an autoradiograph of lane 1. Note that with the higher concentration of ATP (3 μM vs. 0.06 μM), ^{32}P incorporation into the β subunit is greater than that for the α subunit (also see Fig. 3).

When the catalytic subunit was omitted from the phosphorylation reaction, no endogenous phosphorylation activity was associated with the purified calcium antagonist receptor preparation with or without combinations of calcium, calmodulin, or phosphatidylserine (data not shown).

Fig. 2*B* compares quantitatively the concentration of calcium antagonist receptor, as assessed by [^{3}H]nitrendipine binding to the concentration of the phosphorylated protein bands with electrophoretic behavior similar to the α and β subunits of the calcium antagonist receptor, as assessed by liquid scintillation counting of individual protein bands excised from the polyacrylamide gels. The phosphorylated protein bands comigrated quantitatively with the purified calcium antagonist receptor. Furthermore, when the purified calcium antagonist receptor was phosphorylated and sedimented on a second sucrose gradient along with purified receptor labeled with [^{3}H]nitrendipine, the phosphorylated protein bands comigrated with the [^{3}H]nitrendipine-labeled receptor as well as with silver-stained bands for the α, β, and γ subunits (Fig. 1*B*, lanes 1 and 2).

Additional evidence that the α subunit is phosphorylated was derived from comparison of the apparent M_r of the corresponding phosphorylated protein band. Like the α subunit, the phosphorylated protein has an apparent M_r of 163,000 ± 4000 (n = 6) before reduction (Fig. 1, lane 2) and 145,000 ± 4500 (n = 7) after reduction (Fig. 2*A*). We conclude that both the α and β subunits of the purified calcium antagonist re-

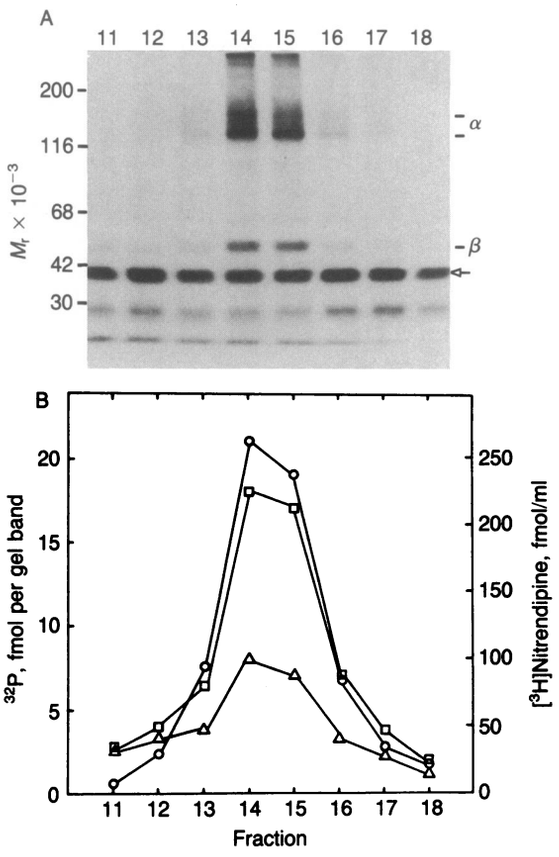

FIG. 2. Comigration of phosphorylated α and β subunits with the [^{3}H]nitrendipine–receptor complex on sucrose gradients. (*A*) Equal aliquots of fractions from the final sucrose gradient step of the calcium antagonist receptor purification (17), containing 0–5 pmol of receptor, were phosphorylated under the standard reaction conditions, boiled in NaDodSO$_4$/PAGE sample buffer containing 20 mM dithiothreitol, and electrophoresed in a 5–15% gradient gel. After drying, an autoradiograph was prepared by exposure to Kodak X-Omat film for 2 hr. Autophosphorylated catalytic subunit is marked by the arrow. (*B*) Bands marked α and β were located and excised from the gel, dissolved in 30% H_2O_2, and the ^{32}P content of α (□) and β (△) bands was determined by liquid scintillation counting. The concentration of the [^{3}H]nitrendipine–receptor complex in each gradient fraction (○) was determined by liquid scintillation counting. The top of the sucrose gradient is to the left.

ceptor are substrates for cAMP-dependent protein kinase *in vitro*.

Time Course of Phosphorylation of the α and β Subunits of the Purified Calcium Antagonist Receptor. In general, physiological substrates for cAMP-dependent protein kinase are phosphorylated more rapidly *in vitro* than proteins that are not natural substrates. For example, the α subunit of the voltage-sensitive sodium channel was found to be phosphorylated *in vitro* as rapidly as known substrates of the enzyme (25) and subsequently was shown to be phosphorylated *in situ* within 15 sec by endogenous cAMP-dependent protein kinase in synaptosomes (26). To evaluate the potential physiological significance of phosphorylation of the calcium antagonist receptor, the initial rates of phosphorylation of the α and β subunits were determined and compared to that for the α subunit of the sodium channel from rat brain under identical reaction conditions (37°C, 0.3 μM catalytic subunit). The initial rates of phosphorylation of the α and β subunits of the calcium antagonist receptor were 10% and 11%, respectively, of the rate of phosphorylation of the α subunit of the sodium channel (Fig. 3*A*). At longer times in the presence of 3 μM catalytic subunit, the extents of phosphorylation of the α and β subunits approached 0.85 and 0.98 mol of ^{32}P per mole of calcium antagonist receptor, respectively (Fig. 3*B*). In the same experiments, phosphorylation of the α subunit of the sodium channel reached 1.9 mol of ^{32}P per mol (data not shown). Thus, the concentration of available phosphorylation sites was 2-fold greater for the sodium channel samples. Comparison of the initial rates of phosphorylation, corrected to equivalent concentrations of phosphorylation sites, indicates that α and β subunits of the calcium antagonist receptor are phosphorylated 22% and 21% as rapidly as the α subunit of the sodium channel. This is within the range of rates observed for accepted physiological substrates of the kinase, suggesting that phosphorylation of these two subunits might occur in intact skeletal muscle.

Selective Phosphorylation of the β Subunit of the Calcium Antagonist Receptor in T-Tubule Membranes. In the presence of low concentrations of ATP, the catalytic subunit of cAMP-dependent protein kinase selectively phosphorylated two major bands in T-tubule membranes, with apparent M_rs of 145,000 and 50,000 under reducing conditions (Fig. 4*A*). As the M_rs of these polypeptides are similar to those of the α and β subunits of the purified calcium antagonist receptor, the M_rs and isoelectric points (pIs) of the polypeptides phosphorylated in T-tubule membranes were compared in two-dimensional gel electrophoresis to the phosphorylated α and β subunits of the purified calcium antagonist receptor. The purified, phosphorylated receptor consists of the phosphorylated α subunit (M_r 145,000, pI 6.4–6.2), which is poorly resolved in the isoelectric focusing gel, and the phosphorylated β subunit (M_r 50,000), which exhibits two forms, spot 3 (pI 5.15) and spot 2 (pI 5.30) (Fig. 4*D*). In contrast, when phosphorylated T-tubule membranes are solubilized with digitonin and analyzed by two-dimensional gel electrophoresis, the M_r 145,000 band (Fig. 4*A*) consists of two widely separated spots (pIs 5.5 and 4.8, Fig. 4*B*). Neither of these corresponds to the α subunit of the calcium antagonist receptor. The phosphorylated band of M_r 50,000 in T-tubule membranes (Fig. 4*A*) consists of a major component equivalent to the β subunit (spot 2) and a tailing component (spot 1, pI 5.45) (Fig. 4*B*). Evidently, the β subunit of the calcium antagonist receptor is a preferred substrate for cAMP-depen-

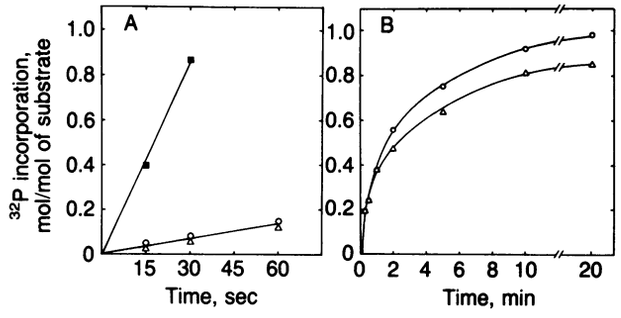

FIG. 3. Time course of phosphorylation of the α and β subunits of the calcium antagonist receptor and the α subunit of the sodium channel. All samples contained 0.5 pmol of receptor in a final reaction volume of 120 μl (4 nM receptor). (*A*) Samples were incubated at 37°C with 3 μM [γ-^{32}P]ATP (25 cpm/fmol) and 0.3 μM catalytic subunit for the indicated times, electrophoresed on a 5–15% gradient gel, and autoradiographed, and the appropriate gel bands were cut out to determine ^{32}P incorporated by liquid scintillation counting. ○, α subunit of the calcium antagonist receptor; △, β subunit of the calcium antagonist receptor; ■, α subunit of the sodium channel incubated with the same sucrose gradient solutions as the calcium antagonist receptor. (*B*) Samples of the α (△) and β (○) subunits of the purified calcium antagonist receptor were phosphorylated with 33 μM [γ-^{32}P]ATP (5 cpm/fmol) and 3 μM catalytic subunit for the indicated times and analyzed as in *A*. All points represent the mean of two determinations from separate calcium antagonist receptor purifications. The experiment was repeated with three additional receptor preparations, with similar results.

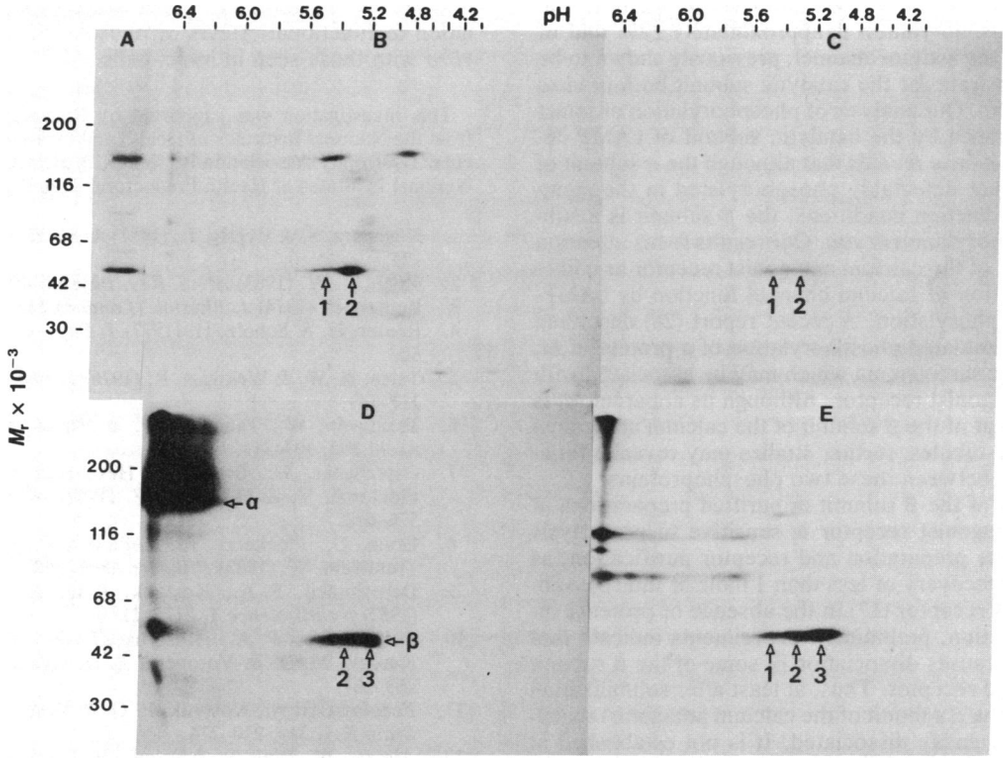

FIG. 4. Selective phosphorylation of the β subunit of the calcium antagonist receptor in T-tubule membranes. (*A*) T-tubule membranes containing 0.5 pmol of calcium antagonist receptor were phosphorylated and subjected to $NaDodSO_4$/PAGE in a 5–15% gel, and an autoradiograph was prepared by exposure to Kodak X-Omat film for 16 hr. (*B*) T-tubule membranes containing 5 pmol of receptor were phosphorylated, solubilized, and dissolved in lysis buffer, and 0.5 pmol was subjected to isoelectric focusing (8800 volt-hr) followed by separation on a $NaDodSO_4$/5–15% polyacrylamide gradient gel in the second dimension. Autoradiographs were prepared after exposure for 1 week. (*C*) An aliquot of the phosphorylated calcium antagonist receptor from *B* was purified by chromatography on WGA-Sepharose (17) in the presence of 10 mM EDTA, 10 mM sodium phosphate (pH 7.4), and protease inhibitors; 0.5 pmol then was subjected to two-dimensional gel electrophoresis and autoradiography for 3 weeks. (*D*) Purified calcium antagonist receptor (5 pmol) was phosphorylated and analyzed by two-dimensional gel electrophoresis and autoradiography (16-hr exposure). (*E*) Digitonin-solubilized T-tubule membranes containing 5 pmol of receptor were phosphorylated and then purified by chromatography on WGA-Sepharose (17). An aliquot (0.5 pmol) of receptor was analyzed by two-dimensional gel electrophoresis and autoradiography (1-week exposure). The three phosphorylated forms of the β subunit are indicated by vertical arrows and the numbers 1, 2, and 3. The pH values shown were obtained from slicing a blank isoelectric focusing gel run at the same time as the gels containing samples.

dent protein kinase in intact T-tubule membranes, but the α subunit is not.

To examine the identity of the phosphorylated proteins of M_r 50,000 more closely, the calcium antagonist receptor was partially purified from phosphorylated T-tubule membranes. Purification of digitonin-solubilized membranes by chromatography on WGA-Sepharose provides 30-fold enrichment in the calcium antagonist receptor and eliminates 97% of the other proteins (17). After WGA-Sepharose chromatography of phosphorylated and solubilized T-tubule membranes, only the M_r 50,000 phosphorylated species from pI 5.3–5.5 remains (Fig. 4*C*). This region could be resolved into the two spots observed in phosphorylated T-tubule membranes, spots 1 and 2. Thus, the M_r 50,000 protein phosphorylated in intact T-tubule membranes and purified by chromatography on WGA-Sepharose corresponds in M_r and, for spot 2, in pI to the β subunit of the calcium antagonist receptor.

The phosphorylated β subunit of the completely purified receptor migrated at a slightly lower pI (spots 2 and 3, Fig. 4*D*) than the phosphorylated β subunit in T-tubule membranes (spots 1 and 2, Fig. 4 *B* and *C*). From spots 1 to 2 to 3, the pI shifts by 0.15 pH unit increments, consistent with the sequential addition of phosphate (27). It seemed possible that the lower pI of the phosphorylated β subunit in the purified calcium antagonist receptor might represent exposure of an additional phosphorylation site upon solubilization. To test this hypothesis, membranes were solubilized, phosphorylated, and purified by chromatography on WGA-Sepharose (Fig. 4*E*). Again, a M_r 50,000 phosphorylated protein was purified. However, with phosphorylation of solubilized membranes, the isoelectric points equivalent to spots 1, 2, and 3 were observed, with spot 3 the major product (Fig. 4*E*). Thus, solubilization appears to expose an additional site for phosphorylation on the β subunit that is not accessible in the membrane (compare Fig. 4 *C* and *E*). This result is consistent with the increase in phosphate incorporation per mol of solubilized β subunit (Fig. 4*E*) as compared to the membrane-associated β subunit (Fig. 4*C*). Phosphorylation of either intact or solubilized membranes selectively labels a polypeptide identified as the β subunit by its apparent M_r, pI, and purification on WGA-Sepharose (Fig. 4 *B–E*). Our experiments with intact T-tubule membranes suggest that spots 1 and 2 are most likely to represent the physiologically significant phosphorylation states of the β subunit (Fig. 4 *B* and *C*).

DISCUSSION

Physiological evidence suggests that phosphorylation by the catalytic subunit of cAMP-dependent protein kinase modulates calcium channel function in a number of cell types (3–11). The most direct mechanism for such regulation would be phosphorylation of a polypeptide component of the channel that can regulate channel activity. In this report, we have demonstrated that cAMP-dependent protein kinase phosphorylates both the α and β subunits of the highly purified

calcium antagonist receptor from T-tubule membranes. The rate of their phosphorylation is approximately 22% that of the α subunit of the sodium channel, previously shown to be an excellent substrate for the catalytic subunit both *in vitro* and *in situ* (25, 26). Our analysis of phosphorylation of intact T-tubule membranes by the catalytic subunit of cAMP-dependent protein kinase reveals that although the α subunit of the receptor is not detectably phosphorylated in the membrane with our reaction conditions, the β subunit is a substrate for phosphorylation *in situ*. Our results focus attention on the β subunit of the calcium antagonist receptor as a likely site of regulation of calcium channel function by cAMP-dependent phosphorylation. A recent report (28) described isoproterenol-stimulated phosphorylation of a protein of M_r 42,000 in cardiac sarcolemma which may be associated with the calcium antagonist receptor. Although its apparent M_r is 20% less than that of the β subunit of the calcium antagonist receptor from T-tubules, further studies may reveal a functional similarity between these two phosphoproteins.

The recovery of the β subunit in purified preparations of the calcium antagonist receptor is sensitive to proteolysis during membrane preparation and receptor purification, as reflected in the recovery of less than 1 mole of intact β subunit per mole of receptor (17) in the absence of protease inhibitors. In addition, preliminary experiments indicate that heating at 37°C causes dissociation of some of the β subunit from the purified receptor. Thus, at least after solubilization with digitonin, the β subunit of the calcium antagonist receptor is labile and easily dissociated. It is not established at present whether the β subunit represents a permanently attached polypeptide component of the receptor or an extrinsic regulatory protein that can reversibly associate with the receptor.

In mammalian fast skeletal muscle, β agonists increase twitch tension and duration over a period of 5 min (reviewed in ref. 29), and these effects are mediated by an increase in cAMP (30–32) after stimulation of β receptor coupled to adenylate cyclase (33, 34). Although the mechanical effects are thought to involve the sarcoplasmic reticulum calcium pump (32), β receptor and adenylate cyclase are preferentially localized in the T-tubule membrane (34), providing the enzymatic machinery required for β-adrenergic modulation of T-tubule calcium channels by cAMP-dependent protein kinase. In view of this possible physiological role for phosphorylation of the calcium antagonist receptor of T-tubules by cAMP-dependent protein kinase, it is of interest to estimate the rate of phosphorylation of T-tubule calcium channels *in vivo*.

Skeletal muscle contains 0.3 μmol of cAMP-dependent protein kinase per kg wet weight (35) and 0.1 μmol of [^{3}H]nitrendipine-binding sites per kg wet weight (15). Assuming a K_m for ATP of 19 μM (18), a homogenous distribution of enzyme and substrate, and saturating concentrations of ATP and cAMP, extrapolation from the initial rate data of Fig. 2 indicates that all the calcium antagonist receptors in skeletal muscle could be phosphorylated in 1.4 min. However, calcium channels are in T-tubules (12–15) at a density of approximately 172 per μm^2 (15) and cAMP-dependent protein kinase is in the cytosol (35), which represents less than 20% of the muscle wet weight (36). If we assume that the relevant microenvironment for interaction of the soluble kinase with the membrane-bound channel is a layer of aqueous phase 0.1 μm thick over the membrane surface, linear extrapolation from our initial rate measurements indicates that all the calcium antagonist receptors could be phosphorylated in 17 sec. Thus, the reaction should certainly occur rapidly enough to play a role in the response to β-agonists. Definitive determination of the role of phosphorylation of the subunits of the calcium antagonist receptor in regulation of calcium channel function in skeletal muscle requires reconstitution of functional calcium channels from purified components and correlation of functional effects of phosphorylation observed *in vitro* with those seen in intact cells.

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