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Multiple Phosphorylation Sites in the 165-Kilodalton Peptide Associated with Dihydropyridine-Sensitive Calcium Channels†

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ABSTRACT: Evidence from electrophysiological and ion flux studies has established that dihydropyridine-sensitive calcium channels are subject to regulation by neurotransmitter-mediated phosphorylation and dephosphorylation reactions. In the present study, we have further characterized the phosphorylation by cAMP-dependent protein kinase and a multifunctional Ca/calmodulin-dependent protein kinase of the membrane-associated form of the 165-kDa polypeptide identified as the skeletal muscle dihydropyridine receptor. The initial rates of phosphorylation of the 165-kDa peptide by both protein kinases were found to be relatively good compared to the rates of phosphorylation of established substrates of the enzymes. Phosphorylation of the 165-kDa peptide by both protein kinases was additive. Prior phosphorylation by either one of the kinases alone did not preclude phosphorylation by the second kinase. The cAMP-dependent protein kinase phosphorylated the 165-kDa peptide preferentially at serine residues, although a small amount of phosphothreonine was also formed. In contrast, after phosphorylation of the 165-kDa peptide by the Ca/calmodulin-dependent protein kinase, slightly more phosphothreonine than phosphoserine was recovered. Phosphopeptide mapping indicated that the two kinases phosphorylated the peptide at distinct as well as similar sites. Notably, one major site phosphorylated by the cAMP-dependent protein kinase was not phosphorylated by the Ca/calmodulin-dependent protein kinase, while other sites were phosphorylated to a high degree by the Ca/calmodulin-dependent protein kinase, but to a much lesser degree by the cAMP-dependent protein kinase. The results show that the 165-kDa dihydropyridine receptor from skeletal muscle can be multiply phosphorylated at distinct sites by the cAMP- and Ca/calmodulin-dependent protein kinases. As the 165-kDa peptide may be the major functional unit of the dihydropyridine-sensitive Ca channel, the results suggest that the phosphorylation-dependent modulation of Ca channel activity by neurotransmitters may involve phosphorylation of the 165-kDa peptide at multiple sites.

Voltage-dependent calcium channels are believed to be regulated by neurotransmitter-mediated phosphorylation and dephosphorylation reactions. Evidence from electrophysiological and ^{45}Ca flux studies has shown that cAMP and cAMP-dependent protein kinase can influence the opening of Ca channels in cardiac and skeletal muscle (Reuter, 1983; Tsien et al., 1986; Schmid et al., 1985; Arreola et al., 1987), as well as in vertebrate (Fedulova et al., 1985) and invertebrate neurons (Chad & Eckert, 1986). Other studies have suggested that certain Ca channels also are subject to regulation by agents which activate protein kinase C (DeRiemer et al., 1985; Rane & Dunlap, 1986; Di Virgilio et al., 1986; Miller, 1987). Ca channels are also known to be regulated by intracellular calcium, which could conceivably involve phosphorylation mediated by Ca/calmodulin (CaM)[1]-dependent protein kinases. As dephosphorylation would be expected to reverse effects of phosphorylation, several studies have shown that Ca channel activity can be regulated by phosphoprotein phosphatases (Kameyama et al., 1986; Chad & Eckert, 1986; Hescheler et al., 1987). Despite the numerous observations concerning effects of protein kinases and phosphatases on Ca channel activity, very little is known about the molecular events underlying the regulation of Ca channels by phosphorylation and dephosphorylation. It remains to be determined if components of the channels, or regulatory proteins, are reversibly phosphorylated in intact cells in response to signaling by different neurotransmitter-activated pathways.

It is now recognized that there are muliple types of Ca channels which differ in their voltage dependencies, kinetics, and pharmacological sensitivities (Nowycky et al., 1985; Cognard et al., 1986; Miller, 1987). Only one type of Ca channel, known as the L type (Nowycky et al., 1985), slow or dihydropyridine (DHP)-sensitive Ca channel, has been well

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[1] Abbreviations: DHP, dihydropyridine; T-tubule, transverse tubule; PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)pyridine-3-carboxylate; WGA, wheat germ agglutinin; $NaDodSO_4$, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CaM, calmodulin.

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characterized biochemically. L-type Ca channels were first purified as high-affinity DHP receptors by several laboratories (Borsotto et al., 1984, 1985; Curtis & Catterall, 1984). Recent studies indicate that a polypeptide of 165 kDa appears to be the major regulatory component of these channels. Results from photoaffinity labeling studies have shown that the 165-kDa polypeptide contains receptors for at least four different chemical classes of Ca channel inhibitors: the dihydropyridines, phenylalkylamines, benzothiazepines, and bepridil (Galizzi et al., 1986; Striessnig et al., 1986, 1987; Hosey et al., 1987; Sharp et al., 1987; Takahashi et al., 1987; Vaghy et al., 1987). With regard to neurotransmitter-mediated regulation, several studies have reported that the 165-kDa peptide can serve as a substrate for cAMP-dependent (Curtis & Catterall, 1985; Hosey et al., 1986, 1987; Takahashi et al., 1987) and Ca/calmodulin-dependent (Hosey et al., 1986) protein kinases. The structure of the 165-kDa peptide has been predicted from cDNA clones (Tanabe et al., 1987) and suggests that this peptide is a membrane protein capable of forming an ion channel. Indeed, the predicted structure of the 165-kDa peptide (Tanabe et al., 1987) has strong homology to the 260-kDa polypeptides which comprise voltage-dependent sodium channels (Noda et al., 1984, 1986). Thus, it appears that the 165-kDa peptide not only is potentially capable of forming an ion channel but also contains sites necessary for regulation by neurotransmitters and Ca channel active drugs. The purpose of the present study was to characterize in detail the phosphorylation of the 165-kDa peptide by cAMP- and Ca/calmodulin-dependent protein kinases. The results establish that the 165-kDa peptide can be multiply phosphorylated at distinct sites by these enzymes.

Experimental Procedures

Materials. [γ-^{32}P]ATP and (+)-[^{3}H]PN200-110 were purchased from Amersham (Arlington Heights, IL). The synthetic peptide substrates for protein kinases were purchased from Peninsula Laboratories (Belmont, CA). Wheat germ agglutinin–Sepharose was purchased from Sigma (St. Louis, MO) or prepared according to methods supplied from Pharmacia (Piscataway, NJ). Calmodulin and protease inhibitors were purchased from Sigma (St. Louis, MO). Chicken gizzard myosin light chains were a generous gift of Dr. Primal de-Lanerolle (University of Illinois). All other reagents were reagent grade or better and obtained from commercial sources.

Membrane Preparation and Protein Purification. Transverse tubule (T-tubule) membranes were prepared from frozen rabbit skeletal muscle according to the procedure of Galizzi et al. (1984). All solutions contained 0.1 mM phenylmethanesulfonyl fluoride, 1 mM iodoacetamide, and 10 μg/mL soybean trypsin inhibitor to prevent proteolysis during membrane isolation. The isolated T-tubule membranes were routinely found to contain 60–100 pmol of DHP receptors/mg of protein using (+)-[^{3}H]PN200-110 as the ligand (Borsotto et al., 1984). Partially purified preparations of DHP receptors were obtained from these membranes by using wheat germ agglutinin (WGA)–Sepharose to purify CHAPS-solubilized receptors as previously described (Hosey et al., 1986). All buffers used for solubilization and purification contained the protease inhibitors listed above as well as 2 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μM pepstatin. The catalytic subunit of cAMP-dependent protein kinase was purified to homogeneity from bovine heart (Sugden et al., 1975). The multifunctional Ca/CaM-dependent protein kinase, or synapsin I kinase (Kennedy et al., 1983), was partially purified from rat brain by using sequential chromatography on DE-23 and CaM–Sepharose (Klee, personal communication). The specific activities of the partially purified preparations ranged from 30 to 100 nmol (mg of protein)$^{-1}$ min^{-1} using chicken gizzard myosin light chain as substrate. As noted by other investigators, the enzyme was highly dependent on CaM for activity and was unstable in that greater than 50% of the activity was lost within 1 week of storage at 4 °C (Fukunaga et al., 1982).

Protein Phosphorylation Reactions. T-Tubule membranes were incubated with the designated protein kinase and [γ-^{32}P]ATP in reaction mixtures which contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 0.05–0.1 mM [γ-^{32}P]ATP (300–3000 cpm/pmol), ±0.1–1.5 μM catalytic subunit of cAMP-dependent protein kinase, ±6–30 μg/mL Ca/CaM-dependent protein kinase, and 0.05–0.6 mg/mL T-tubule membranes. Reactions containing the Ca/CaM-dependent protein kinase also contained 1 μM calmodulin, 0.5 mM $CaCl_2$, and 0.05% 2-mercaptoethanol. The reactions were carried out for 2–10 min at 37 °C. Phosphorylation was terminated by adding 2 volumes of "stop buffer" (50 mM sodium potassium phosphate, pH 7.4, 50 mM NaF, 20 mM EDTA, and all six protease inhibitors) and centrifugation as described (Hosey et al., 1986). The phosphorylated DHP receptors were solubilized and partially purified, and the phosphopeptide content was analyzed by $NaDodSO_4$–polyacrylamide gradient (5–15%) gel electrophoresis and autoradiography. All buffers used for the isolation of the phosphorylated peptides contained 25 mM sodium potassium phosphate, pH 7.4, 20 mM NaF, and 0.1 mM EDTA to prevent dephosphorylation and all six protease inhibitors to prevent proteolysis. Phosphorylation of the synthetic peptide substrates Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide, Peninsula Laboratories 8650) by cAMP-dependent protein kinase and Pro-Leu-Arg-Arg-Thr-Leu-Ser-Val-Ala-Ala-NH_2 (CaM-dependent protein kinase substrate analogue; Peninsula Laboratories 8863) by the Ca/CaM-dependent protein kinase was carried out under conditions similar to those used for the T-tubule membranes (see legend to Figure 1). Phosphorylation of the synthetic peptides was determined by pipeting aliquots of the phosphorylation reactions onto P81 filter paper squares and processing according to Roskoski (1983).

The calculation of stoichiometries of phosphorylation of the 165-kDa peptide was based on the specific activities of [γ-^{32}P]ATP, the ^{32}P content of the 165-kDa band excised from SDS gels, and the concentration of DHP receptors applied to the gel as measured by the binding of the DHP (+)-[^{3}H]-PN200-110. The values of stoichiometry given herein should be considered minimal values because (i) the concentration of the 165-kDa DHP receptor may be overestimated if, as suspected, DHPs bind to the 165-kDa peptide as well as breakdown products of this peptide and (ii) since the amount of ^{32}P incorporated into the 165-kDa peptide was often estimated after partial purification of the peptide, we cannot rule out the possibility that some dephosphorylation of the peptide occurred during its isolation.

Phosphopeptide Mapping and Phosphoamino Acid Determinations. Two-dimensional phosphopeptide mapping of the 165-kDa peptide was performed as in Hunter and Sefton (1980). After electrophoresis of the purified phosphorylated Ca channel preparations on $NaDodSO_4$ gels, the bands containing the 165-kDa peptide were excised and washed first with 10% 2-propanol/10% acetic acid and then with 50% methanol. After lyophilization, the samples were rehydrated and digested in 0.7 mL of 50 mM ammonium bicarbonate, 1 mM dithiothreitol, and 50 μg/mL TPCK-trypsin at 37 °C for 24 h. This was followed by an additional 3–6 h in 0.3 mL of fresh trypsin solution. The digests were pooled and lyophilized. The res-

idues were resuspended in a minimum volume of 1% ammonium carbonate at pH 8.9 and spotted on the lower edge of cellulose thin-layer chromatography plates (EM Science). The peptides were separated in the first dimension at 550 V for 35 min at pH 8.9 and in the second dimension by ascending chromatography with *n*-butyl alcohol/pyridine/acetic acid/water in the ratio 37:25:7.5:30. In other experiments, phosphopeptide mapping was performed in which the peptides were digested with trypsin as described above or with subtilisin (50 μg/mL for 24 h) and separated in the first dimension at 1000 V for 55 min at pH 1.9 followed by separation in the second dimension as described above.

For phosphoamino acid determination, the 165-kDa peptide was processed as described above through the second lyophilization step. The residue was then hydrolyzed with 6 N HCl at 110 °C for 2 h. After removal of the HCl in a Speed-Vac, the hydrolysate was dissolved in a minimum volume of pyridine/acetic acid/water at 1:10:189, pH 3.5, containing a mixture of standard phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine) as markers. The samples were spotted on cellulose thin-layer chromatography plates and separated at pH 3.5 and 390 V for 3 h. The phosphoamino acid markers were detected with a ninhydrin/cadmium stain.

RESULTS

In a previous study, we reported that a 160-kDa peptide in partially purified preparations of DHP receptors could be phosphorylated by cAMP-dependent protein kinase and by a multifunctional Ca/CaM-dependent protein kinase (Hosey et al., 1986). In that study, the mobility of the 160-kDa phosphopeptide on 4–14% $NaDodSO_4$ gels was similar to that of another peptide which migrated at 170 kDa under nonreducing conditions and at 140 kDa under reducing conditions (referred to as the 170/140-kDa peptide) (Hosey et al., 1986). More recently, we have demonstrated that these represent two peptides which can be differentiated by several criteria and that only the peptide of 160–165 kDa, and not the 170/140-kDa peptide, serves as a substrate for cAMP-dependent protein kinase (Hosey et al., 1987). Similar results have been reported by others (Takahashi et al., 1987; Sieber et al., 1987). The 165-kDa peptide, and not the 170/140-kDa peptide, is known to contain the receptors for Ca channel inhibitors (Striessnig et al., 1987; Sharp et al., 1987; Hosey et al., 1987; Sieber et al., 1987; Vaghy et al., 1987). In the present study, we have further analyzed the phosphorylation of the 165-kDa peptide by cAMP-dependent- and a multifunctional Ca/CaM-dependent protein kinase.

We wished to establish whether the *membrane-associated* 165-kDa peptide contained multiple sites for phosphorylation, and if it could be phosphorylated in an additive manner by the cAMP-dependent and the multifunctional Ca/CaM-dependent protein kinases. In order to do so, we first optimized the conditions of phosphorylation by each kinase alone in order to ensure that each kinase rapidly and maximally phosphorylated the peptide under the conditions used. Incorporation of ^{32}P into the 165-kDa peptide by either kinase was found to be linear with T-tubule membrane protein concentrations of up to 0.15 mg/mL. The rates of phosphorylation depended on the concentrations of protein kinases used. For the C subunit of cAMP-dependent protein kinase, routine experiments contained 0.1 μM kinase. However, in some experiments in which we wished to ensure rapid and complete phosphorylation of the peptide by this enzyme, concentrations of 1–1.5 μM were used, as the rate of phosphorylation of the 165-kDa peptide occurred more rapidly at higher kinase

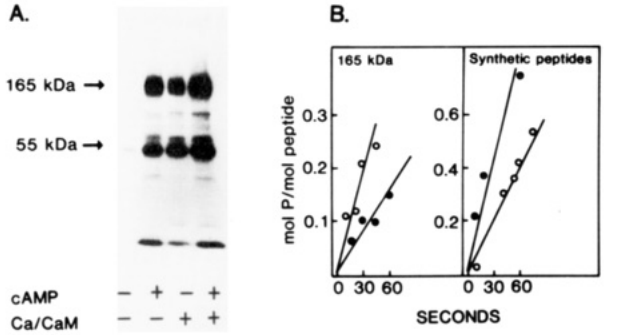

FIGURE 1: Phosphorylation of the 165-kDa DHP receptor in skeletal muscle transverse tubule membranes by cAMP-dependent and Ca/calmodulin-dependent protein kinases. (A) Autoradiogram depicting phosphorylation of the 165-kDa DHP receptor by each protein kinase alone and together. Transverse tubule membranes were phosphorylated with the purified catalytic subunit of cAMP-dependent protein kinase (1 μM) or with partially purified preparations of a Ca/calmodulin-dependent protein kinase (20 μg/mL). The concentrations of protein kinases were high in order to obtain maximal and rapid phosphorylation (see text). DHP receptors were purified from the phosphorylated membranes and electrophoresed on 5–15% gels. Shown is an autoradiogram of the resulting gel. The bands corresponding to the 165-kDa peptide seen in the autoradiogram were excised, and the ^{32}P was quantified by liquid scintillation counting. The amount of ^{32}P incorporated into the 165-kDa peptide under the different conditions was 70 cpm (lane 1, no addition), 410 cpm (lane 2, cAMP-dependent protein kinase alone), 250 cpm (lane 3, Ca/calmodulin-dependent protein kinase alone), and 540 cpm (lane 4, both kinases together). Subtraction of the amount incorporated by the intrinsic kinase from that incorporated by the exogenous kinase indicates that the incorporation achieved by the two exogenous kinases when added together was equivalent to 90% of that expected for additive phosphorylation. (B) Initial rates of phosphorylation of the 165-kDa peptide and two synthetic peptides by cAMP- and Ca/CaM-dependent protein kinases. The left-hand part of the figure shows the rate of incorporation of ^{32}P into the membrane-bound 165-kDa peptide (10 nM) in the presence of either 0.1 μM catalytic subunit of cAMP-dependent protein kinase (●) or 6 μg/mL (approximately 1–2 nM) Ca/CaM-dependent protein kinase (○). The right-hand part of the figure shows the initial rates of phosphorylation of the synthetic peptide "Kemptide" (25 nM) by cAMP-dependent protein kinase (0.1 μM) (●) and the Ca/CaM-dependent protein kinase substrate analogue (400 μM) by the Ca/CaM-dependent protein kinase (6 μg/mL) (○).

concentrations (O'Callahan and Hosey, unpublished observations; Nastainczyk et al., 1987). The amounts of Ca/CaM-dependent protein kinase used depended on the relative purity of the enzyme preparations. As these preparations were usually no greater than 10% pure, the maximum amount of kinase used was ~2–10 nM. After establishing the phosphorylation conditions, we determined if phosphorylation by the two kinases was additive. T-Tubule membranes were incubated with one or both kinases under conditions to ensure maximal phosphorylation by each kinase alone, and the phosphorylation of the 165-kDa peptide was assessed after partial purification (Figure 1A). The autoradiogram of the resulting $NaDodSO_4$ gel confirms our previous observation that both kinases phosphorylate the 165-kDa peptide (Hosey et al., 1986) and additionally shows that phosphorylation by both kinases was greater than achieved by either enzyme alone (Figure 1A). The latter result was verified by direct scintillation counting of the gel pieces. The approximate stoichiometries of phosphorylation achieved in the experiment shown were the following: 0.6 mol of P/mol of 165-kDa peptide by cAMP-dependent protein kinase; 0.4 mol of P/mol of peptide by Ca/CaM-dependent kinase; and 0.8 mol of P/mol of peptide by both kinases together. In other experiments, we estimated that the maximum incorporation of phosphate into the 165-kDa peptide was 0.6–1.5 mol of P/mol

of peptide with the cAMP-dependent protein kinase and 0.4–1.2 mol of P/mol of peptide with the Ca/CaM-dependent protein kinase. As some dephosphorylation may have occurred during the isolation of the 165-kDa phosphoprotein, these values should be considered minimal. The results in Figure 1A also confirmed previous results (Hosey et al., 1986) which showed that a 55-kDa peptide that copurifies with the 165-kDa peptide and has been suggested by some workers to be a subunit of the channel (Curtis & Catterall, 1984; Leung et al., 1988) was also phosphorylated by both protein kinases. The phosphorylation of this peptide was not analyzed in this study. The identities of the other phosphopeptides apparent in Figure 1A are not known.

In order to test whether the 165-kDa peptide might be a "physiological" substrate of the two protein kinases, the initial rates of phosphorylation of the membrane-bound 165-kDa peptide by each protein kinase were compared to the rates of phosphorylation of two peptides that have been established as excellent substrates for the kinases. The synthetic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly ("Kemptide"), which corresponds to the cAMP-dependent phosphorylation site in pyruvate kinase (Kemp et al., 1977), was used as the model substrate for cAMP-dependent protein kinase, while another synthetic peptide, Pro-Leu-Arg-Arg-Thr-Leu-Ser-Val-Ala-NH_2, corresponding to an analogue of a peptide phosphorylated in glycogen synthase by Ca/CaM-dependent protein kinase (Pearson et al., 1985), was used as a model substrate for the Ca/CaM-dependent protein kinase. The results showed that the membrane-bound 165-kDa peptide was phosphorylated at a relatively good rate by both protein kinases under the conditions used (Figure 1B). However, it might be noted that direct comparisons of the "goodness" of the different substrates are difficult to make since the concentration of the 165-kDa peptide was low in order that the rates were linear with protein, and the 165-kDa peptide was phosphorylated in a membrane which contained other substrates (and perhaps inhibitory factors) while the peptide substrates were pure and in solution. On the other hand, the initial rate of phosphorylation of the membrane-associated 165-kDa peptide by cAMP-dependent protein kinase was found to be similar to the rate previously reported for the phosphorylation of the purified 165-kDa peptide under conditions of low ATP and low kinase concentrations (Curtis & Catterall, 1985).

We next determined if prior phosphorylation by one kinase influenced the ability of the other kinase to phosphorylate the 165-kDa peptide. Experiments were performed in which the membrane-bound 165-kDa peptide was maximally phosphorylated for 5 min by one kinase and then for a second 5 min by the same (no further addition) or the other kinase. Incorporation of ^{32}P was followed kinetically (Figure 2). The level of phosphorylation attained in the presence of the two kinases was the same whether the two kinases were added simultaneously (Figure 2, left panel) or after the other had maximally phosphorylated the 165-kDa peptide (Figure 2, right panel). These results established that phosphorylation by cAMP-dependent protein kinase does not preclude subsequent phosphorylation by Ca/CaM-dependent protein kinase, and vice versa (Figure 2), and confirmed the additivity of phosphorylation shown in the autoradiogram in Figure 1A. The stoichiometries of phosphorylation for the experiment shown were 1.3, 0.9, and 1.55 mol of P/mol of peptide for the cAMP-dependent protein kinase alone, Ca/CaM-dependent protein kinase alone, and both kinases together, respectively. As observed in previous studies (Hosey et al., 1986; Imagawa et al., 1987), the results also show that the membranes contained an endogenous protein kinase activity which phosphorylated the 165-kDa peptide at a slow rate (Figure 2, left panel).

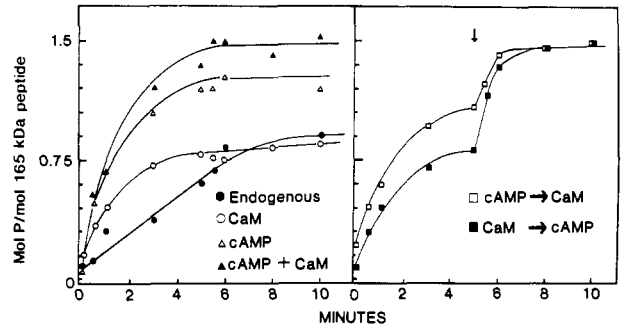

FIGURE 2: Kinetic analysis of the additive phosphorylation of the 165-kDa peptide in transverse tubules by cAMP-dependent and Ca/CaM-dependent protein kinases. Transverse tubule membranes were incubated at 37 °C with no added kinase (●), 30 μg/mL Ca/CaM-dependent protein kinase (○), 1.5 μM cAMP-dependent protein kinase (△), or both kinases simultaneously (▲) for 10 min (left panel), or with either one of the exogenous protein kinases for 5 min before adding (arrow in the right panel) the other exogenous protein kinase for an additional 5 min (□ or ■) (right panel). [γ-^{32}P]ATP (0.1 mM) was added to the reactions at 0, 1.5, 3.0, 5.0, 6.5, and 8.0 min to prevent depletion of ATP by ATPases. After autoradiography, the bands corresponding to the 165-kDa peptide were excised, and the ^{32}P incorporation was quantitated by Cerenkov counting. Similar results were obtained in two separate experiments.

In order to more fully analyze the characteristics of the phosphorylated 165-kDa peptide, we performed phosphoamino acid analysis and phosphopeptide mapping of the 165-kDa peptide phosphorylated by each kinase alone and together. Phosphoamino acid analysis showed that cAMP-dependent protein kinase preferentially phosphorylated the 165-kDa peptide at serine residues although a small amount of phosphothreonine also was recovered; 90% of the ^{32}P was recovered as p-Ser while 10% was found as p-Thr. In contrast, the Ca/CaM-dependent protein kinase phosphorylated at both threonine and serine residues. Although methods used to analyze phosphoamino acids do not result in similar recoveries of the different phosphoamino acids [i.e., the amounts recovered do not necessarily reflect the amounts contained inthe original samples (Bylund & Huang, 1976)], 55–60% of the ^{32}P was recovered as p-Thr and the remainder as p-Ser from samples phosphorylated by the Ca/CaM-dependent protein kinase. The phosphoamino acids recovered from samples phosphorylated by the combination of the two added protein kinases reflected the additivity of the phosphorylations. Phosphorylation catalyzed by the endogenous protein kinase was at both serine and threonine residues.

Two-dimensional phosphopeptide mapping of the 165-kDa peptide revealed that the cAMP- and the Ca/CaM-dependent protein kinases phosphorylated distinct, as well as similar, sites (Figure 3). In terms of distinct sites, a major site phosphorylated by cAMP-dependent protein kinase (indicated by arrow 1 in Figure 3) was not phosphorylated by the Ca/CaM-dependent protein kinase. In addition, two peptide spots (arrows 2 and 3, Figure 3) were phosphorylated to a markedly greater degree by Ca/CaM-dependent protein kinase than by cAMP-dependent protein kinase (this difference was even larger in other experiments). Yet other sites were phosphorylated by each kinase, albeit to different degrees. The similarity of the peptides phosphorylated by both kinases was confirmed by performing phosphopeptide mapping experiments under different conditions (digestion with either trypsin or subtilisin and electrophoresis at pH 1.9 rather than 8.9). From the collective results, it was clear that some of the phospho-

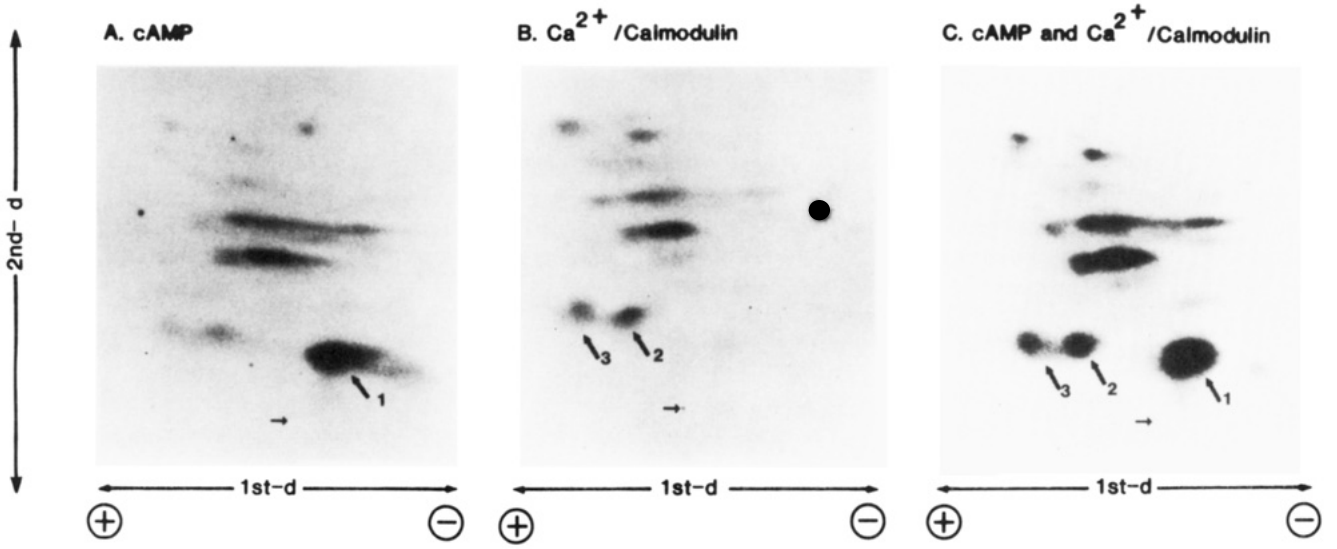

FIGURE 3: Two-dimensional phosphopeptide mapping of the 165-kDa peptide after phosphorylation by cAMP- and/or Ca/calmodulin-dependent protein kinases. The 165-kDa peptide was phosphorylated in T-tubule membranes for 10 min in the presence of the indicated protein kinases (at concentrations stated in the legend to Figure 2) and processed for phosphopeptide mapping as described under Experimental Procedures. The cellulose thin-layer plates were exposed to Kodak X-Omat AR film for 15 days on Cronex screens at −70 °C. The origin is indicated by the small horizontal arrow in each panel. Arrow 1 indicates the peptide preferentially phosphorylated by cAMP-dependent protein kinase, while arrows 2 and 3 indicate the peptides preferentially phosphorylated by Ca/CaM-dependent protein kinase.

peptide spots seen after phosphorylation with cAMP- and/or Ca/CaM-dependent protein kinases were identical (data not shown). While we cannot rule out the possibility that one or more of the similar phosphopeptides contain more than one phosphorylatable residue, the results are consistent with the partial, but not complete, additivity of the phosphorylation shown in Figures 1 and 2. Furthermore, these results, taken together with those of the phosphoamino acid analysis, are consistent with the suggestion that each enzyme phosphorylates several sites in the 165-kDa peptide.

In order to determine whether the use of high concentrations of ATP and cAMP-dependent protein kinase led to phosphorylation of sites not phosphorylated with lower concentrations of these reagents, we performed additional studies with cAMP-dependent protein kinase, in which varied concentrations of ATP (from 0.01 to 0.3 mM) and protein kinase (from 24 nM to 0.6 μM) were utilized. In *each* case, the phosphopeptide maps were similar to those shown in Figure 3. The results showed that there were no particular sites preferentially phosphorylated under different experimental conditions.

Discussion

The results of the present study establish that the 165-kDa component of dihydropyridine-sensitive Ca channels contains multiple sites for phosphorylation by cAMP-dependent and a multifunctional Ca/CaM-dependent protein kinase. These findings suggest that there are several potential regulatory sites in the 165-kDa peptide which conceivably could be involved in the regulation of L-type Ca channels by neurotransmitter-mediated phosphorylation (Reuter, 1983; Tsien et al., 1986; Miller, 1987). As all the phosphorylation reactions analyzed in this report were carried out using the *membrane-bound* form of the DHP receptor as substrate (rather than the purified protein in detergent solution), the sites phosphorylated should correspond to those normally available for phosphorylation in the intact cell.

In the present study, we demonstrated that the membrane-bound 165-kDa peptide was phosphorylated at a good rate by both protein kinases tested. Indeed, the rate of phosphorylation of the membrane-bound substrate by cAMP-dependent protein kinase was similar to that previously reported for the purified 165-kDa peptide by cAMP-dependent protein kinase (Curtis & Catterall, 1985; Nastainczyk et al., 1987). In these previous studies as well as the present study, it was noted that the rate of phosphorylation of the 165-kDa peptide increased considerably at higher concentrations of protein kinases and/or ATP than those used for the initial rate studies. Since the concentrations of ATP and (the 165-kDa peptide) used in the assays to determine the initial rates of phosphorylation of the 165-kDa peptide were lower than those found in intact skeletal muscle, the results are consistent with the possibility that the membrane-associated peptide could serve as a physiological substrate for either protein kinase in vivo. This conclusion agrees with those previously made from studies of the phosphorylation of the purified 165-kDa peptide by cAMP-dependent protein kinase (Curtis & Catterall, 1985; Nastainczyk et al., 1987).

All of the results reported in this study are consistent with the suggestion that the 165-kDa peptide contains multiple sites for phosphorylation by the cAMP- and Ca/CaM-dependent protein kinases. The predicted sequence of the 165-kDa DHP receptor (Tanabe et al., 1987) shows that it contains seven potential sites (Kemp et al., 1977; Krebs & Beavo, 1979) for phosphorylation by cAMP-dependent protein kinase. Six of these seven sites are located in the C-terminal region proposed to constitute a cytoplasmic tail; the seventh site is also found in a predicted cytoplasmic region connecting two hydrophobic domains (Tanabe et al., 1987). Six of the potential cAMP-dependent protein kinase phosphorylation sites contain phosphorylatable serine residues, while one site contains a phosphorylatable threonine. Interestingly, our results show that phosphoserine was the major form of phosphoamino acid recovered after phosphorylation of the membrane-bound 165-kDa peptide by cAMP-dependent protein kinase. However, a small amount of phosphothreonine was also recovered, which could reflect phosphorylation of threonine-1552 by the cAMP-dependent protein kinase. We also found that the 165-kDa peptide was phosphorylated by the Ca/CaM-dependent protein kinase at both serine and threonine residues. While the consensus sequences for substrates of Ca/CaM-dependent protein kinases are not as well established as those for the cAMP-dependent protein kinase, several potential sites

for phosphorylation by this enzyme, predicted according to the substrate requirements observed by Pearson et al. (1985), can also be found in the C-terminal region of the 165-kDa peptide. One possible threonine target for the Ca/CaM-dependent protein kinase is at residue 1661 in the sequence predicted by Tanabe et al. (1987). Some, but not all, of the potential Ca/CaM-dependent protein kinase sites are close to potential cAMP-dependent protein kinases sites (e.g., Ser-1575 may be phosphorylated by Ca/calmodulin-dependent protein kinase while Thr-1552 may be a substrate for cAMP-dependent protein kinase). Such a proximity of potential phosphorylation sites might explain why the results of our phosphopeptide mapping experiments suggest that some of the sites phosphorylated by the cAMP- and the Ca/CaM-dependent protein kinases were common to both kinases. An alternate explanation for the observation that both enzymes appear to phosphorylate some common sites might be that the enzymes phosphorylate adjacent or proximal serines and/or threonines. Such is the case for the phosphorylation of the cardiac protein phospholamban in which cAMP-dependent protein kinase and Ca/calmodulin-dependent protein kinase phosphorylate a pair of adjacent serine and threonine residues (Simmerman et al., 1986). Similarly, cAMP-dependent protein kinase and protein kinase C phosphorylate adjacent serine residues in the nicotinic acetylcholine receptor (Huganir et al., 1984; Safran et al., 1987). In this regard, there are three pairs of adjacent or closely spaced threonine and serine residues (residues 1501 and 1502, 1573 and 1575, and 1756 and 1757) and several pairs of serines in potential phosphorylation sites in the cytoplasmic tail of the 165-kDa peptide. Further studies will be necessary to precisely determine which residues are indeed phosphorylated by the two protein kinases.

We have estimated that cAMP-dependent protein kinase phosphorylated the membrane-bound 165-kDa peptide to the extent of 0.6–1.5 mol of P/mol of peptide, while the Ca/CaM-dependent protein kinase phosphorylated the peptide to the extent of 0.4–1.2 mol of P/mol of peptide. These values for the cAMP-dependent protein kinase catalyzed phosphorylation of the membrane-bound 165-kDa peptide are consistent with the suggested stoichiometries of 0.85–1.0 mol of phosphate/mol of peptide for the cAMP-dependent protein kinase catalyzed phosphorylation of the purified 165-kDa peptide (Curtis & Catterall, 1985; Flockerzi et al., 1986). It might be noted, however, that one factor which may lead to the underestimation of the correct number of phosphorylation sites present in the DHP receptor is that it is possible that we, and others, are not analyzing the intact DHP receptor. The predicted size of the protein from the cDNA is 210 kDa (Tanabe et al., 1987), while the peptide migrates as 155–180 kDa on $NaDodSO_4$ gels (Striessnig et al., 1987; Hosey et al., 1987; Leung et al., 1987; Takahashi et al., 1987). Although the apparent molecular weight varies considerably with the type of gel used (Hosey et al., 1987), it is conceivable that some of the potential cAMP-dependent phosphorylation sites predicted to be contained in the C-terminal segment are absent in the peptide analyzed. Future studies to elucidate the exact nature of the phosphorylated sites will help to resolve these issues. Another factor that could lead to an underestimation of the stoichiometry of phosphorylation is that the 165-kDa peptide is present in the T-tubule membranes in a partially phosphorylated form. We have not attempted to dephosphorylate the T-tubule membranes prior to phosphorylating the membranes with the purified protein kinases.

Recent findings concerning the 165-kDa peptide studied herein strongly suggest that it may be the major functional unit of L-type voltage-dependent Ca channels. The predicted structure of the 165-kDa peptide deduced from molecular cDNA cloning studies suggests it possesses striking homology to the structure of voltage-dependent Na channels, including regions predicted to constitute the voltage sensors (Tanabe et al., 1987; Noda et al., 1984, 1986). From other studies performed with various photoaffinity labeling reagents, it is clear that the 165-kDa polypeptide, which we have found to contain multiple phosphorylation sites, also contains high-affinity receptors for various types of Ca channel blockers, including the dihydropyridines, phenylalkylamines, benzothiazepines, and bepridil (Galizzi et al., 1986; Striessnig et al., 1987; Hosey et al., 1987; Sharp et al., 1987; Takahashi et al., 1987; Vaghy et al., 1987). It has been established that the 165-kDa peptides capable of being photolabeled and phosphorylated are one and the same (Hosey et al., 1987). Therefore, it appears that the 165-kDa peptide structurally resembles an established ion channel protein and contains sites necessary for regulation of its activity by neurotransmitter-mediated phosphorylation events as well as by several classes of established Ca channel inhibitors. Future studies will be directed at elucidating whether this peptide is phosphorylated in intact cells in response to agents known to regulate Ca channel activity.

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