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THE α_1-SUBUNIT OF SKELETAL MUSCLE L-TYPE Ca CHANNELS IS THE KEY TARGET FOR REGULATION BY A-KINASE AND PROTEIN PHOSPHATASE-1C

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Summary: Despite the fact that the phosphorylation-mediated regulation of L-type Ca channels is viewed as a model of ion channel regulation, much remains to be learned about the protein phosphorylation and dephosphorylation reactions that underlie the regulation of the channels. The channel isoform most well studied biochemically is that expressed in skeletal muscle. The α_1- and β-subunits of this channel isoform are substrates for protein kinase A, but it is unknown if phosphorylation or dephosphorylation of both subunits contributes to altered channel properties. Here, we report experiments in which the α_1- and β-subunits were differentially phosphorylated by protein kinase A and dephosphorylated by protein phosphatase 1c under conditions that led to channel regulation. The results suggest that the α_1-subunit plays a key role in the phosphorylation and dephosphorylation-dependent regulation of the L-type Ca channels from skeletal muscle.

Electrophysiological studies have clearly demonstrated that L-type channels in various cell types are regulated by events that appear to involve protein kinase A (PKA)-mediated phosphorylation of the channels or associated regulatory proteins (1,2). However, relatively little is known about the exact nature of the reactions involved. L-type Ca channels from skeletal muscle have been used as a model system to biochemically characterize the channels because the transverse tubule (T-tubule) membranes from skeletal muscle are the richest natural source from which the channels are readily isolated (2). These channels appear to be multi-subunit proteins containing α_1, α_2/δ, β and γ subunits (2,3). From *in vitro* studies, it is known that the α_1- and β-subunits of the skeletal muscle Ca channels can be phosphorylated by PKA (4-6), and that this results in channel activation (6,7). In intact chick and rat skeletal muscle, elevations of intracellular cAMP lead to phosphorylation of the α_1-subunit (8,9) and activation of the channels

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(9). Phosphorylation of the β-subunit was not detected (9). Although several results suggest that the α_1-subunit is the critical target for PKA, the roles of the phosphorylation of each subunit in contributing to channel regulation are not yet clear. In addition, very little is known about the biochemical events that lead to dephosphorylation of the Ca channels and the reversal of the PKA-mediated activation. In the present study, we provide evidence from *in vitro* phosphorylation and dephosphorylation studies to support the hypothesis that phosphorylation of the α_1-subunit of the skeletal muscle L-type channel plays a key role in the regulation of channel activity, while effects of phosphorylation of the β-subunit appear to be of lesser or little consequence.

EXPERIMENTAL PROCEDURES

Materials. Okadaic acid was purchased from GIBCO BRL. Phosphorylase b and phosphorylase kinase were purchased from Sigma. Trypsin-TPCK was from Worthington Biochemicals. The catalytic subunit of PKA was purified to homogeneity from bovine heart (10). The catalytic subunit of protein phosphatase 1c (PP-1c) was partially purified from fresh rabbit skeletal muscle using DEAE-Sepharose and poly(L-lysine)-Sepharose columns according to a modification of published methods (11,12). Phosphatase activity was assayed using [^{32}P]phosphorylase *a* as a substrate which was prepared from phosphorylase *b* (12). Okadaic acid was used to differentiate fractions containing PP1c from PP-2Ac, as PP-2Ac can be inhibited completely by nanomolar concentrations of okadaic acid, while PP-1c is only partially inhibited (13). The specific activity of the partially purified PP-1c was 2.33 U/mg protein. One unit of activity is defined as the amount of enzyme that catalyzes the dephosphorylation of 1.0 μmol of phosphorylase *a*/min at 30°C. The skeletal muscle T-tubule membranes were purified from rabbit skeletal muscle and contained 20-70 pmols dihydropyridine (DHP) receptors/mg protein (14,15). All other materials were from sources previously described (15,16).

Phosphorylation and dephosphorylation of Ca channels in T-tubule membranes. The Ca channels in skeletal muscle T-tubule membranes or the reconstituted Ca channels were phosphorylated with PKA as described (6). The reactions were carried out in the presence of 0.25 μM catalytic subunit of PKA in 0.2-0.3 mg T-tubule membrane protein/ml for 5 min at 30°C and stopped with equal volumes of "stop buffer" (50 mM $NaKPO_4$, 20 mM EDTA, 20 mM NaF) (6). For dephosphorylation studies, the membranes were separated from the phosphorylation reagents by centrifugation at 100,000 x g for 30 min and then washed twice with 5 volumes of buffer A (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol). The dephosphorylation reactions were carried out in buffer A with 0.5-1 mg/ml phosphorylated membrane protein in the absence or presence of 9 mU/ml phosphatase at 30°C for the times indicated and stopped either with SDS sample buffer (18) or with "stop buffer". The samples were separated on 5-15% polyacrylamide gels (18) and stained with Coomassie blue. The ^{32}P content of the α_1-subunit of the Ca channels was determined by excising the relevant bands and counting in a scintillation counter. For dephosphorylation studies, the samples that were treated with phosphatase for 0 min, or not exposed to phosphatase, were defined as the controls, i.e., 100% phosphorylation. Phosphopeptide maps were prepared as previously described (16).

Purification and reconstitution of Ca channels from skeletal muscle. The channels were partially purified using wheat germ agglutinin (WGA)-Sepharose chromatography and, where indicated, sucrose density gradient centrifugation (6,17). The methods used to reconstitute Ca channels and measure Ca influx were described previously (9,15) and involved reconstituting partially purified channels into liposomes containing fluo-3. The measured Ca influx was

sensitive to dihydropyridines and other Ca channel modulators and to the membrane potential established by valinomycin in the presence of a K^+ gradient (9,15).

RESULTS AND DISCUSSION

Phosphorylation of the reconstituted Ca channels by PKA. To investigate the roles of the α_1- and β -subunits in modulation of the activity of L-type Ca channels from skeletal muscle, we performed studies with purified and reconstituted Ca channels and asked if PKA could phosphorylate these channels with the characteristics observed in previous experiments performed directly with channels in T-tubule membranes (6). To do so, the Ca channels were first purified with WGA-Sepharose chromatography and sucrose density gradient centrifugation, reconstituted into artificial liposomes and then subjected to phosphorylation as described under "Experimental Procedures". The time course of phosphorylation of the α_1- and β -subunits by the catalytic subunit of PKA is shown in Fig. 1A and 1B. As can be seen, under these conditions there was a marked selectivity of PKA toward the α_1-subunit of the reconstituted channels. In 3 experiments, the stoichiometry of phosphorylation of the α_1-subunit was calculated to be 0.85 ± 0.12 mol P/mol DHP receptor (Fig 1B). In contrast, the incorporation of ^{32}P into the β -subunit was 5-6 times lower (0.16 ± 0.05 mol P/mol DHP receptor). We have

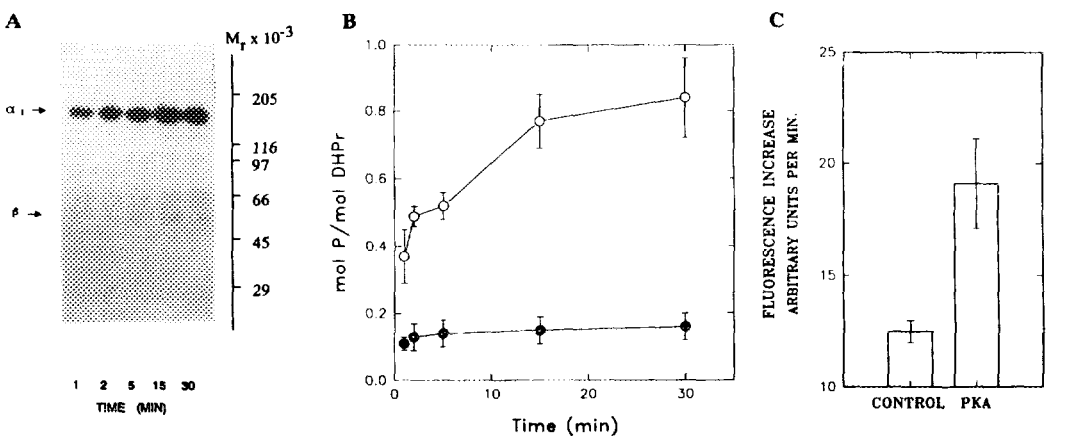

Figure 1. Phosphorylation of the partially purified and reconstituted channels by PKA. Skeletal muscle Ca channels were partially purified using WGA-Sepharose chromatography and sucrose gradients, reconstituted, and phosphorylated in the presence of 50 μM ATP and 0.25 μM PKA. After electrophoresis and autoradiography, stoichiometry of phosphorylation was determined. ***A***: Autoradiogram showing the incorporation of ^{32}P into the α_1 and β peptides at different times of incubation with PKA. ***B***: Time course and extent of phosphorylation of the α_1 (open circles) and β (closed circles) subunits by PKA. The results are means ± SEM from 3 experiments similar to the experiment shown in Fig. 1A. ***C***: Functional consequences of the PKA phosphorylation of skeletal muscle Ca channels after reconstitution. Channels were phosphorylated in the absence (CONTROL) or presence of PKA for 30 min after reconstitution. Results are shown are the rates of initial Ca influx for the different samples; the results are mean ± SEM from 4 experiments.

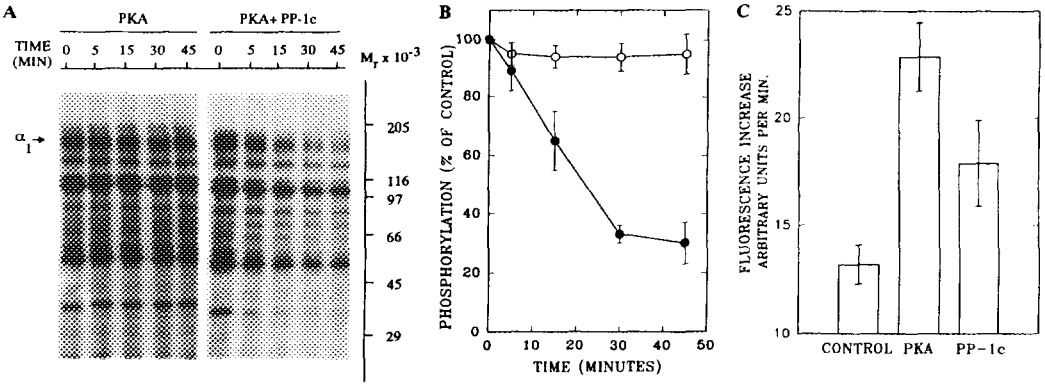

Figure 2. Dephosphorylation of the PKA-phosphorylated channels by PP-1c. T-tubule membranes from skeletal muscle were phosphorylated with PKA and dephosphorylated by PP-1c as described under "Experimental Procedures". ***A***: Autoradiogram from one experiment showing the phosphorylated and dephosphorylated samples at 0 (control), 15, 30, and 45 min after addition of buffer (samples labelled "PKA") or PP-1c (labelled "PKA + PP-1c"). (Slight differences in the separation of low molecular weight peptides in these SDS gels are due to small differences in the acrylamide composition of the two gels). ***B***: The averaged results from four similar experiments measuring the dephosphorylation of the α_1-subunit. The results were calculated after measuring the ^{32}P content of the α_1 band. (Open circles: control phosphorylated channels without phosphatase; filled circles: phosphorylated channels exposed to PP-1c). ***C:*** Functional effect of PP-1c on PKA phosphorylated channels. Unphosphorylated, PKA-phosphorylated and PKA-phosphorylated/PP-1c dephosphorylated channels were purified and reconstituted into fluo-3 containing liposomes. Averaged values of initial rates of Ca influx through control, PKA-phosphorylated or PP-1c dephosphorylated channels as indicated. Data were from four experiments.

calculated that up to 50% of the reconstituted channels are oriented "inside-in" and not accessible to phosphorylation (data not shown), which would explain why the stoichiometries of phosphorylation of both the α_1- and β -subunits of the reconstituted channels were ~2 fold lower than that observed for T-tubule bound channels (6). The effect of PKA-mediated phosphorylation on channel activity was measured by analyzing Ca influx into liposomes using fluo-3 as a Ca indicator (9,15). The initial rate of DHP-sensitive Ca influx was increased from 12.5 $\pm$ 0.5 arbitrary units of fluorescence/min in the liposomes containing the control channels to 19.1 $\pm$ 2.0 after PKA treatment of the reconstituted channels (Figure 1C). This difference was statistically significant ($p<0.05$). Thus, PKA-mediated phosphorylation of the purified and reconstituted channels results in stoichiometric phosphorylation of the α_1-subunit but minimal phosphorylation of the β-subunit. Importantly, these conditions result in activation of channel function.

Dephosphorylation of the Ca channels previously phosphorylated with PKA by PP-1c. We then asked if the PKA-phosphorylated channels could be dephosphorylated by protein phosphatases, and if this would reverse the functional effect of phosphorylation. The study was initiated by analyzing the effects of the catalytic subunit of protein phosphatase type-1 (PP-1c) on PKA-phosphorylated Ca channels in T-tubule membranes. Dephosphorylation was assessed

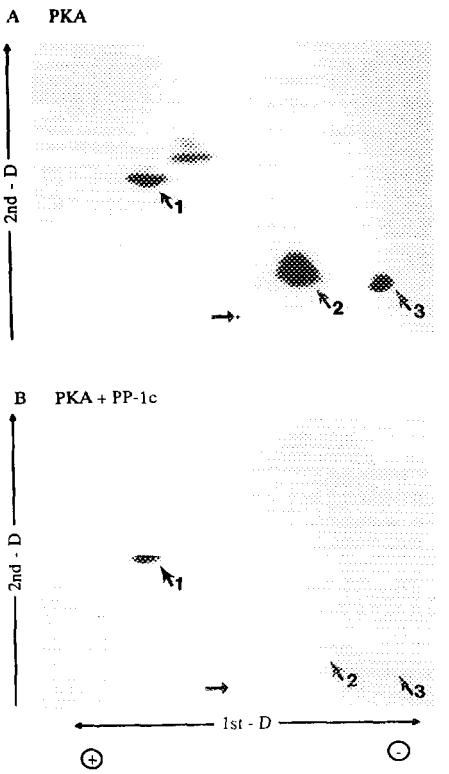

Fig. 3. Two-dimensional phosphopeptide mapping of the α_1 subunit of Ca channels phosphorylated by PKA +/– dephosphorylation by PP-1c. The horizontal arrow indicates the origin, and the numbered arrows indicate the major phosphopeptides. *A:* Two-dimensional phosphopeptide map of the α_1 subunit of the channels phosphorylated by PKA. The starting material contained 1625 cpm (Cerenkov). *B:* Two-dimensional phosphopeptide map of the α_1 subunit of the channels that were phosphorylated by PKA and dephosphorylated by PP-1c. The starting material contained 761 cpm (Cerenkov). Both thin layer chromatography plates were exposed to Kodak X-Omat films at -70°C for 5 days with enhancing screens.

by the decrease in ^{32}P content of the α_1-subunit. As addressed previously, the α_1-subunit in T-tubule membranes can be directly visualized on SDS gels (6,16). PP-1c dephosphorylated the Ca channels which were previously phosphorylated with PKA in a time-dependent manner, while there was very little change in the phosphorylated samples in the absence of added phosphatase (Fig. 2A and 2B). The averaged results of four similar experiments demonstrated that there was ~60% net dephosphorylation of the α_1-subunit by PP-1c which reached a maximal level at 30 min (Fig. 2B).

Previous studies showed that both the α_1- and β -subunits of the Ca channels in the T-tubule membranes are substrates for PKA, however, because the β-subunit co-migrates with other proteins, it cannot be directly visualized in SDS gels of the T-tubule membranes. Therefore to determine if both subunits could be dephosphorylated by PP-1c, the channels were first phosphorylated and dephosphorylated in T-tubule membranes and subsequently purified by WGA-

Sepharose chromatography and sucrose gradient sedimentation (6,17) to allow analysis of the state of phosphorylation of both subunits. Both the α_1- and β -subunits were found to be substrates for PP-1c, but the dephosphorylation occurred to different extents. The α_1-subunit was dephosphorylated by 57%; this decreased the stoichiometry of phosphorylation from ~2 mol P/mol channel to ~1 mol P/mol channel. The β-subunit was dephosphorylated by 34%; this decreased the stoichiometry from 0.5 mol P/mol channel to ~0.3 mol P/mol channel. The results of nine similar experiments from three protein preparations were averaged, and the difference in the dephosphorylated and corresponding phosphorylated subunit was found to be statistically different ($p<0.01$).

The two-dimensional tryptic phosphopeptide maps of the α_1-subunit from PKA-phosphorylated and PKA-phosphorylated/PP-1c-dephosphorylated channels were compared. In the control phosphopeptide map, there were three major phosphopeptides as indicated by the arrows in Fig. 3A. Previous studies indicated that peptides 2 and 3 were uniquely phosphorylated by PKA (16). When subjected to dephosphorylation by PP-1c, phosphopeptides 2 and 3 were almost completely dephosphorylated, while phosphopeptide 1 was dephosphorylated to a lesser extent (Fig. 3B).

In order to determine whether the dephosphorylation catalyzed by PP-1c had any influence on the PKA-mediated activation of the channels, Ca channels were purified from either non-phosphorylated membranes (control), PKA-phosphorylated membranes, and/or PKA-phosphorylated/PP-1c dephosphorylated T-tubule membranes, and reconstituted into liposomes containing fluo-3. Channel activities were measured as described above. Phosphorylation of the channels by PKA increased the initial rate of Ca influx through the channels (Fig. 2C), as observed previously (6). This activation was reversed by treatment with PP-1c (Fig. 2C). The results of 12 similar experiments from four preparations were analyzed by measuring the initial rate of increase in fluorescence due to Ca influx (Fig. 2C); the averaged values indicated that PP-1c significantly reduced activity by 52% compared to the PKA control ($p<0.01$). Taken together, these results demonstrated that PP-1c dephosphorylated the PKA-phosphorylated α_1-subunit *in vitro* by ~60%, and that the dephosphorylation decreased channel activity to a similar extent. Under the same conditions, the phosphorylation of the β-subunit was less than stoichiometric and was minimally changed by PP1-c treatment.

The results of the present studies provide new evidence to suggest that the critical events involved in the regulation of the skeletal muscle L-type channel activity by phosphorylation and dephosphorylation involves the α_1-subunit. First, in the experiments where PKA catalyzed the phosphorylation of the reconstituted channels and enhanced channel activity, only the α_1-subunit was stoichiometrically phosphorylated whereas minimal levels of phosphate were incorporated into the β-subunit (Fig. 1). Second, the extent of

dephosphorylation of the PKA-phosphorylated α_1-subunit by PP-1c correlated well with the extent of reversal of channel activity by PP-1c. Thus, it appears likely that in the case of the concerted regulation by PKA and PP-1c, stoichiometric phosphorylation and dephosphorylation of the α_1-subunit modulate channel activity, while the small and less than stoichiometric changes in the state of phosphorylation of the β subunit are likely to be of lesser consequence.

The phosphatase study reported here provides biochemical evidence for the direct dephosphorylation and regulation of L-type Ca channels by PP-1c, which is known to be one of the major phosphatases present in skeletal muscle (19). It has been shown that Ser 687 of the α_1-subunit of skeletal muscle calcium channels can be rapidly phosphorylated *in vitro* by PKA (20). A comparison of the phosphopeptide maps shown here (Fig. 3A) with those from Rotman *et al.* (21) suggests that phosphorylated Ser 687 might be responsible for the phosphopeptides 2 and 3 in Fig. 3A. Previous studies demonstrated that phosphopeptides 2 and 3 arise uniquely by PKA-mediated phosphorylation (16). The phosphorylation site(s) contained in peptides 2 and 3 appear to be important in the PKA-mediated regulation of the channel activity since dephosphorylation of these sites led to a reversal of the effects of PKA on channel activity.

In addition to the data presented in this paper, previous results from other studies also support the hypothesis that phosphorylation of the α_1-subunit is critical for the regulation of the channels by phosphorylation. In skeletal muscle, Ca currents are increased by β-adrenergic agonists (22). This regulation of channel activity appears to be mediated by the phosphorylation of the α_1-subunit by PKA because when skeletal muscle is exposed to the β-adrenergic agonist isoproterenol or other cAMP-activating agents prior to isolation of the channels, phosphorylation of the α_1-subunit, but not the β-subunit, is increased compared to control, and these conditions lead to increases in channel activity as assessed by reconstitution assays (9). The present studies do not exclude a role of the β-subunit in the regulation of channel activity by PKA-mediated phosphorylation (inducing the right conformation of α_1-subunit in order to be phosphorylated, for example) but suggest that the main event leading to the increase of Ca channel activity is the stoichiometric phosphorylation of the α_1-subunit.

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