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Characterization of the two size forms of the α1 subunit of skeletal muscle L-type calcium channels

(dihydropyridines/electrical excitability/excitation–contraction coupling/ion channel)

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ABSTRACT **The molecular properties of two size forms of the α1 subunit of purified skeletal muscle calcium channels were analyzed. The minor, full-length, form, $\alpha1_{212}$, was found to have an apparent molecular mass of 214 kDa by Ferguson plot analysis, while the major, truncated, form, now designated $\alpha1_{190}$, had an apparent molecular mass of 193 kDa. Antibody mapping of the C-terminal region of $\alpha1_{190}$ with 10 anti-peptide antibodies placed the C terminus between residues 1685 and 1699. Three consensus sites for cAMP-dependent protein phosphorylation are present in the C-terminal region of $\alpha1_{212}$ but not in $\alpha1_{190}$, and they may be important for the regulation of the ion conductance activity of the calcium channel.**

Four classes of voltage-gated calcium channels have been defined on the basis of electrophysiological and pharmacological properties (1). L-type calcium channels mediate long-lasting calcium currents that are sensitive to inhibition by dihydropyridines (DHPs). Most biochemical studies have concentrated on the L-type calcium channel from skeletal muscle transverse tubules (T-tubules), which contain 50- to 100-fold more DHP receptor sites than other sources. The most abundant form of the rabbit skeletal muscle calcium channel is a complex of five subunits: α1 (175 kDa), α2 (143 kDa), β (54 kDa), γ (30 kDa), and δ (24–27 kDa) (reviewed in refs. 2–4). The α1, β, and γ subunits are products of distinct genes (5–7), while the α2 and δ subunits are encoded by the same gene (8), whose protein product is proteolytically processed and disulfide-linked to give the α2δ complex (9). The α1 subunit contains the receptor sites for DHPs and other calcium channel modulating drugs. The sequence of its cDNA predicts a protein of 1873 amino acids (212 kDa) whose structure is homologous to the sodium channel α subunit, with four internally homologous domains each containing six predicted α-helical transmembrane segments (5). This subunit alone is sufficient to form functional calcium channels when expressed in mouse fibroblast cells (10).

The DHP-sensitive calcium channel protein in skeletal muscle has also been proposed to serve as the voltage sensor for excitation–contraction coupling, a process that does not require the entry of extracellular calcium (11). Microinjection of cDNA encoding α1 subunits into myoblasts from mice with the muscular dysgenesis (*mdg*) mutation restores both the DHP-sensitive calcium current and excitation–contraction coupling (12), supporting the hypothesis that the protein product(s) of the α1 gene serve both as active calcium channels and as voltage sensors for excitation–contraction coupling in skeletal muscle. However, only a small fraction of the skeletal muscle DHP receptor sites function simultaneously as active calcium channels *in vivo* or *in vitro* (13, 14). We have previously demonstrated the existence of two size forms of the α1 subunit in purified preparations of skeletal muscle calcium channels, T-tubule membranes, and intact skeletal muscle cells in culture (15, 16). The minor 212-kDa form ($\alpha1_{212}$) contains the complete amino acid sequence encoded by the α1 mRNA, while the major form having an apparent molecular mass of 175 kDa (previously designated $\alpha1_{175}$) is truncated at its C terminus. We have hypothesized that these two forms are specialized to serve as cAMP-modulated ion channels and as voltage sensors for excitation–contraction coupling (15). To examine the functional properties of these two forms directly by expression in recipient cells, it is necessary to determine the C-terminal structure of the truncated form. In this study, we have examined the size of these two forms of the α1 subunit of purified calcium channels in more detail and mapped the C-terminal region with a series of sequence-specific anti-peptide antibodies.

EXPERIMENTAL PROCEDURES

Materials. Calcium channels were purified by chromatography on wheat germ agglutinin (WGA)-Sepharose and DEAE-cellulose (17) from skeletal muscle microsomes that were prepared according to Fernandez *et al.* (18). All buffers contained the following protease inhibitors: phenylmethyl sulfonyl fluoride (0.1 mM), leupeptin (2 μM), pepstatin A (1 μM), antipain (1.6 μM), and *o*-phenanthroline (0.9 mM). Catalytic subunit of cAMP-dependent protein kinase was purified according to Kaczmarek *et al.* (19). The amounts of purified calcium channel were estimated by assuming a molecular mass of 429 kDa for the channel complex.

Synthetic calcium channel peptides (CP) were synthesized by the solid-phase method (20) with N-terminal Lys + Tyr for coupling to carrier protein plus residues 1551–1566 [CP-(1551–1566)], 1573–1587 [CP-(1573–1587)], 1601–1618 [CP-(1601–1618)], 1646–1661 [CP-(1646–1661)], 1662–1676 [CP-(1662–1676)], 1671–1684 [CP-(1671–1684)], 1677–1690 [CP-(1677–1690)], 1685–1699 [CP-(1685–1699)], 1692–1707 [CP-(1692–1707)], or 1738–1752 [CP-(1738–1752)] of the α1 primary sequence. CP-(1670–1676) corresponded to residues 1670–1676 of the α1 primary sequence. Peptides were purified by reverse-phase HPLC on a Waters DeltaPak C_{18} column (25 × 300 mm, 15-μm particles, 300-Å pores) and their identities were confirmed by amino acid analysis.

Antisera against the peptides were prepared as outlined previously (21). The production of antibodies was tested by enzyme-linked immunosorbent assay (ELISA) using microtiter plates with wells coated with 0.5 μg of peptide according to Posnett *et al.* (22). IgG fractions were isolated by affinity chromatography on staphylococcal protein A-Sepharose, with the final volume of IgG being 3 times that of the serum from which it was purified. Antibodies against CP-(1662–1676), CP-(1671–1684), CP-(1677–1690), CP-(1685–1699), CP-

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Abbreviations: DHP, dihydropyridine; ELISA, enzyme-linked immunosorbent assay; T-tubule, transverse tubule; CP, calcium channel peptide; My, myosin; αM, α_2-macroglobulin; βG, β-galactosidase; Ph, phosphorylase b.

(1692–1707), and CP-(1738–1752) were affinity purified as indicated on 1.5-ml columns containing 2 μmol of peptide that had been coupled to CNBr-activated Sepharose. After 2.0 ml of serum had been bound to the columns overnight at 4°C and washed with 20 mM Tris·HCl/0.15 M NaCl, pH 7.4 (TBS), bound IgG was eluted with 5.0 M $MgCl_2$ and dialyzed against TBS in a Centriprep-30 apparatus (Amicon) to a final volume of 1.0–2.0 ml.

Phosphorylation and Immunoprecipitation of Calcium Channels. The standard reaction mixture for phosphorylation of calcium channels contained 50 pmol of purified calcium channels, 50 mM Tris·HCl at pH 7.4, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.56 μM [γ-^{32}P]ATP (1000–3000 Ci/mmol; 1 Ci = 37 GBq), and protease inhibitors as outlined above. The reaction, initiated by the addition of 1 μg of catalytic subunit of cAMP-dependent protein kinase, was carried out at 37°C for 30 min and stopped by the addition of 20 mM EDTA. Bovine serum albumin (5 mg/ml) was added and unbound [γ-^{32}P]ATP was removed by centrifugation through 2-ml Sephadex G-50 columns equilibrated with bRIA (25 mM Tris·HCl, pH 7.4/75 mM NaCl/5 mM EDTA/50 mM KF/20 mM sodium pyrophosphate) containing 0.1% digitonin. Then 0.5 or 5.0 pmol of phosphorylated channel (>80% trichloroacetic acid-precipitable cpm) was incubated with antibody in bRIA containing 1% Triton X-100 and 0.1% bovine serum albumin at 4°C for 4–16 hr. The antigen-antibody complexes were adsorbed to 5 mg protein A-Sepharose and washed four times with bRIA containing 1% Triton X-100 and 1 mg/ml bovine serum albumin, and twice with bRIA. The pellets were incubated at 65°C in SDS/PAGE sample buffer for 15 min prior to electrophoresis.

PAGE and Immunoblotting. SDS/PAGE was carried out according to Laemmli (23) under reducing conditions. Unstained molecular mass markers were used for Ferguson analysis (24). For other experiments, commercial prestained molecular mass markers [myosin (My), 200 kDa; α_2-macroglobulin (αM), 180 kDa; β-galactosidase (βG), 116 kDa; and phosphorylase b (Ph), 97 kDa] were used. Electroblotting to nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes was carried out in a Bio-Rad Mini Protean apparatus for 60 min at 100 V with 25 mM Tris/192 mM glycine/20% (vol/vol) methanol, pH 8.3, as the transfer buffer. Unbound sites were blocked for 2 hr at room temperature with 5% (wt/vol) skim milk powder in TBS. Membranes were then incubated with antibodies in blocking buffer overnight at 4°C, followed by four 5-min washes with TBS/0.05% Tween-20. Immunoreactive bands were visualized by using the Amersham enzyme chemiluminescent immunoblot detection system, with film exposure times indicated in the figures, using horseradish peroxidase-linked protein A as the visualizing reagent. Immunoblotting conditions were adjusted to give bands of comparable intensity.

RESULTS AND DISCUSSION

Molecular Mass of α1. Although the calcium channel α1 subunit is not detectably glycosylated (25), we have observed anomalous migration of $\alpha 1_{175}$ in SDS/PAGE using gels of different acrylamide and crosslinker concentrations. This anomalous electrophoretic migration may be responsible, at least in part, for the large variation in reported apparent molecular mass for $\alpha 1_{175}$ in purified calcium channel preparations [155–175 kDa (25–28)]. To provide a more accurate estimate of the molecular mass of $\alpha 1_{175}$ and $\alpha 1_{212}$, we determined their apparent masses by Ferguson analysis, which compensates for anomalous effects of acrylamide concentration in SDS/PAGE by measurements at multiple acrylamide concentrations and extrapolation to 0% acrylamide (24). The relative mobility of the α1 subunits and the standard proteins varied approximately linearly with acrylamide concentration (Fig. 1*A*). The extrapolated free mobility of the α1 subunits at 0% acrylamide was similar to that of the molecular mass standards (0.45–0.49), with the exception of myosin (205 kDa) whose free mobility was significantly lower (0.32, Fig. 1*A*). The molecular mass of $\alpha 1_{212}$ was determined to be 214 kDa, while that of $\alpha 1_{175}$ was 193 kDa by comparison with the marker proteins (Fig. 1*B*). Since the true molecular mass of $\alpha 1_{175}$ is near 190 kDa (Fig. 1 and see below), we will refer to it as $\alpha 1_{190}$ in the remainder of this paper.

Characterization of Anti-Peptide Antibodies. Estimation of the apparent molecular mass of $\alpha 1_{190}$ by Ferguson plot analysis suggests that it is missing approximately 180 amino acid residues from the C terminus of $\alpha 1_{212}$. To define the C terminus of $\alpha 1_{190}$ more precisely, antisera were prepared against 10 peptides that corresponded to sequences surrounding the expected C terminus of $\alpha 1_{190}$ (Fig. 2*A*) as outlined in *Experimental Procedures*. ELISA indicated each of the antisera contained antibodies that reacted specifically with the corresponding peptide antigens, although the titers of antibodies obtained differed markedly among peptides (Fig. 2 *B* and *C*). For this reason, it was necessary to vary the concentration of antibody used in immunoblotting and immunoprecipitation experiments to give protein bands of comparable intensity. Since the amount of IgG used varied considerably, appropriate controls using equivalent concentrations of nonimmune rabbit IgG were carried out in each case to ensure that the immune recognition of the protein bands was specific.

Mapping of the C Terminus of $\alpha 1_{190}$ by Immunoblotting. The specificity of each antibody for the two forms of α1 was determined in immunoblotting experiments. A 1/200–1/2000 dilution of antibodies directed against CP-(1551–1566), CP-

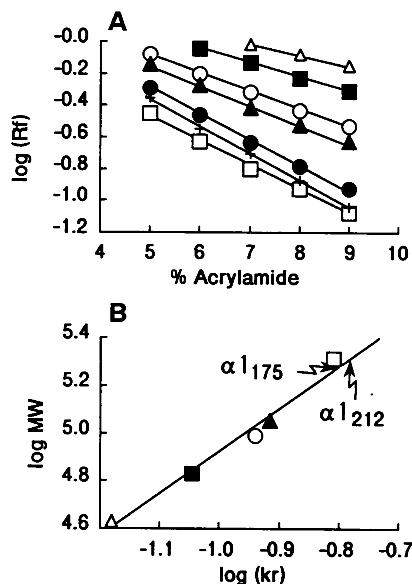

FIG. 1. Molecular mass determination of $\alpha 1_{175}$ and $\alpha 1_{212}$ by Ferguson plot analysis. ^{32}P-labeled calcium channels were immunoprecipitated by antibodies specific for $\alpha 1_{175}$ or $\alpha 1_{212}$ and separated by SDS/PAGE on gels of the indicated acrylamide concentrations under reducing conditions. (*A*) The $\log_{10}$ of the R_f values for $\alpha 1_{175}$ (●), $\alpha 1_{212}$ (+), and molecular mass standards (△, ovalbumin; ■, bovine serum albumin; ○, Ph; ▲, βG; □, My) were plotted against the acrylamide concentration. Data are the mean of two to four determinations. The free mobility (the R_f at 0% acrylamide) is the extrapolated *y*-intercept and was similar for the standards (0.44 ± 0.07) and α1 subunits (0.49 ± 0.01). (*B*) The slopes of the regression lines from *A* (*kr*) were plotted as a function of the logarithm of the molecular mass (Da) for the standard proteins. The masses of the α1 subunits were obtained by fitting their slopes to the calibration line.

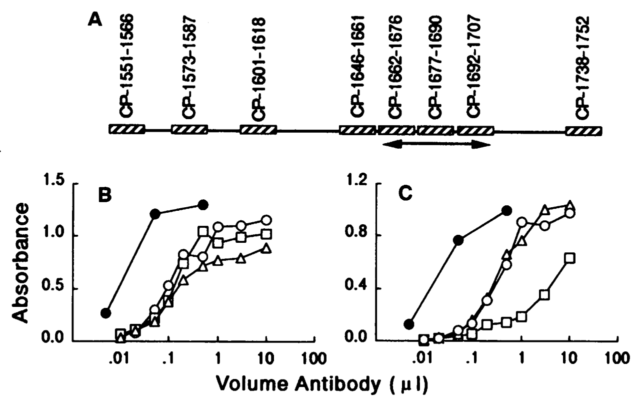

FIG. 2. Location of peptides and immunoreactivity of anti-peptide antibodies. (*A*) Peptides were synthesized corresponding to sequences (hatched boxes) in the C-terminal region of $\alpha 1$. The arrow indicates the region expected to contain the C terminus of $\alpha 1_{190}$. (*B*) The indicated volumes of anti-peptide serum were tested by ELISA in microtiter plates coated with the specific peptides against which each antibody had been prepared. Peptides were △, CP-(1551–1566); □, CP-(1601–1618); ●, CP-(1677–1690); and ○, CP-(1738–1752). (*C*) A similar analysis was carried out for the following peptides: □, CP-(1573–1587); △, CP-(1646–1661); ●, CP-(1662–1676); and ○, CP-(1692–1707).

(1573–1587), CP-(1601–1618), and CP-(1646–1661) recognized $\alpha 1_{190}$ when 0.2–1.0 pmol of purified calcium channel was loaded on the gels (Fig. 3*A*, lanes 1–4). The antibodies against CP-(1662–1676) were less immunoreactive, and 10.0 pmol of calcium channel was required to detect $\alpha 1_{190}$ (Fig. 3*A*, lane 5). After affinity purification and concentration of anti-CP-(1662–1676), $\alpha 1_{190}$ was labeled more strongly and $\alpha 1_{212}$ was also clearly detected in 10-pmol samples of calcium channel protein (Fig. 3*B*, lane 3). Since the amino acid sequence of $\alpha 1_{190}$ is completely contained within the sequence of $\alpha 1_{212}$, it is expected that all antibodies that recognize $\alpha 1_{190}$ will also recognize $\alpha 1_{212}$. However, the small amount of $\alpha 1_{212}$ relative to $\alpha 1_{190}$ makes it difficult to separately detect $\alpha 1_{212}$ under our conditions of SDS/PAGE with antibodies that recognize both, because the broadening of the $\alpha 1_{190}$ band obscures $\alpha 1_{212}$. Anti-CP-(1662–1676) is exceptional among these antibodies in showing relatively strong recognition of $\alpha 1_{212}$ and relatively weak recognition of $\alpha 1_{190}$ in immunoblots.

Immunoblotting of 25.0 pmol of purified calcium channel with the affinity-purified antibodies directed against CP-(1692–1707) and CP-(1738–1752) revealed a single immunoreactive band corresponding in migration position to $\alpha 1_{212}$. The failure of these antibodies to recognize $\alpha 1_{190}$ indicates that the corresponding amino acid sequences are not present in this truncated form and therefore that its C terminus comes before residue 1707. The sensitivity of detection of $\alpha 1_{212}$ with these antibodies is similar to that for anti-CP1, the antibody against the C terminus of $\alpha 1_{212}$ that was originally used to detect the minor 212-kDa form of the $\alpha 1$ subunit (15).

To ensure that the recognition of the two calcium channel forms was specific, antibodies were incubated with the peptides against which they were directed prior to their use in immunoblots. Immunoreactivity of anti-CP-(1601–1618), anti-CP-(1662–1676), and anti-CP-(1692–1707) against the $\alpha 1$ subunits was completely blocked by the corresponding peptides (Fig. 3*B*), indicating that the observed immunoreactivity is specific. These results place the C terminus of $\alpha 1_{190}$ between residues 1662 and 1707.

Mapping of the C Terminus of $\alpha 1_{190}$ by Immunoprecipitation. The specificity of the antibodies was further studied by immunoprecipitation of ^{32}P-labeled calcium channel $\alpha 1$ subunits. As was the case for immunoblotting experiments, protein A-Sepharose-purified antibodies directed against CP-(1551–1566), CP-(1573–1587), CP-(1601–1618), and CP-(1646–1661) immunoprecipitated $\alpha 1_{190}$ (Fig. 4, lanes 1–5), whereas the affinity-purified antibodies directed against CP-(1692–1707) and CP-(1738–1752) immunoprecipitated $\alpha 1_{212}$ (Fig. 4, lanes 7 and 8). With these latter antibodies approximately 10 times as much ^{32}P-labeled calcium channel was needed for visualization of $\alpha 1_{212}$. Equivalent amounts of nonimmune rabbit IgG failed to immunoprecipitate any ^{32}P-labeled proteins in the molecular mass range of interest, as did the antibody directed against CP-(1677–1690). The antibody directed against CP-(1662–1676) immunoprecipitated both $\alpha 1_{190}$ and $\alpha 1_{212}$ (Fig. 4, lane 6). These results confirm the conclusions from immunoblotting experiments and support the location of the C terminus of $\alpha 1_{190}$ between residues 1662 and 1707.

Further Definition of the C Terminus of $\alpha 1_{190}$ with Anti-Peptide Antibodies Purified by Epitope Selection. To more precisely define the C terminus of $\alpha 1_{190}$, two additional antibodies were generated, against CP-(1671–1684) and CP-(1685–1699). In addition, antisera were enriched for anti-peptide antibodies directed against segments of six to eight residues of the $\alpha 1$ primary sequence by purification against

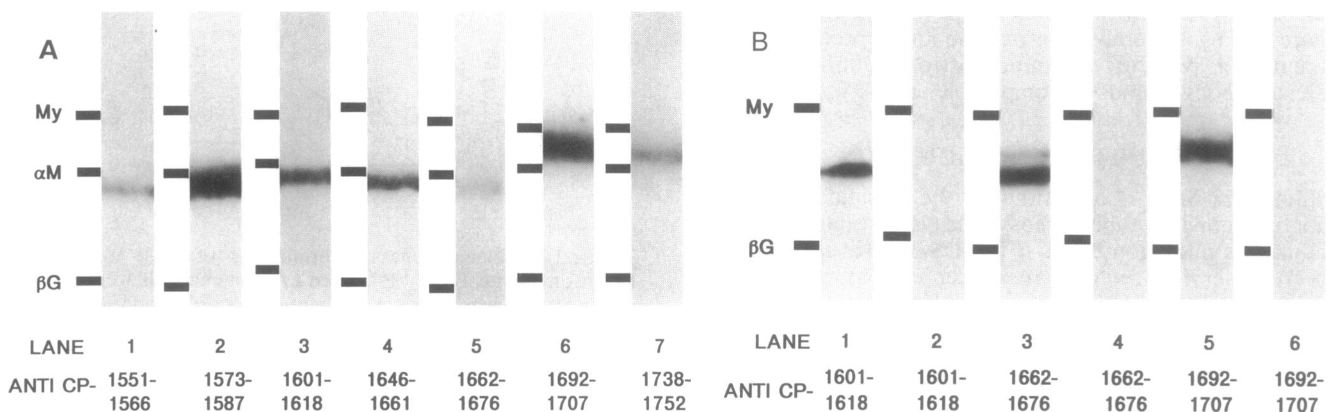

FIG. 3. Immunoblot analysis of calcium channels by using anti-peptide antibodies. Purified calcium channels were analyzed on 6% acrylamide gels, immunoblotted with antibodies directed against the indicated peptides, and visualized on x-ray film. (*A*) The pmol of calcium channel, antibody dilutions (-fold), and film exposure times (sec) were lane 1, 5, 200, 5; lane 2, 1, 200, 1; lane 3, 0.2, 2000, 5; lane 4, 1, 200, 1; lane 5, 10, 200, 5; lane 6, 25, 2, 1; lane 7, 25, 2, 1. (*B*) Anti-peptide antibodies were incubated for 2 hr at room temperature in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of 10 μM peptide against which they were directed before being used in immunoblots. The pmol of calcium channel, antibody dilutions (-fold), and film exposure times (sec) were lanes 1 and 2, 1, 2000, 5; lanes 3 and 4, 20, 3, 2; lanes 5 and 6, 10, 5, 1.

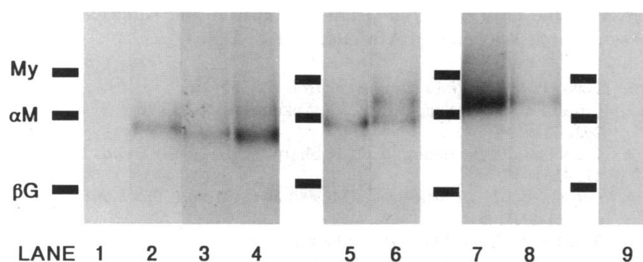

FIG. 4. Immunoprecipitation of phosphorylated calcium channels by anti-peptide antibodies. Either 0.5 (lanes 1–6) or 5.0 (lanes 7–9) pmol of ^{32}P-labeled calcium channels was immunoprecipitated with 9 μg of nonimmune rabbit IgG (lane 1), 25 μg of anti-CP-(1551–1566) (lane 2), 13 μg of anti-CP-(1573–1587) (lane 3), 2 μg of anti-CP-(1601–1618) (lane 4), 12 μg of anti-CP-(1646–1661) (lane 5), 92 μg of anti-CP-(1662–1676) (lane 6), 45 μg of anti-CP-(1692–1707) (lane 7), 29 μg of anti-CP-(1738–1752) (lane 8), or 38 μg of nonimmune rabbit IgG (lane 9), analyzed by SDS/PAGE on 6% acrylamide gels, and exposed to x-ray film for 2 days in the presence (lanes 1, 2, 5, 6) or absence (lanes 3, 4, 7, 8, 9) of an intensifying screen.

overlapping or truncated peptides (Fig. 5*A*). The resulting antibodies were then used in immunoblots.

The antiserum raised against CP-(1671–1684) recognized $\alpha 1_{190}$ when affinity purified against CP-(1670–1676) (Fig. 5*B*, lane 2) or CP-(1677–1690) (data not shown), indicating that the C terminus of $\alpha 1_{190}$ extends beyond residue 1677. Further evidence for this was obtained by purification of anti-CP-(1677–1690) against CP-(1671–1684). The resulting antibodies, directed against residues 1677–1684 of the $\alpha 1$ primary sequence, also recognized $\alpha 1_{190}$ (Fig. 5*B*, lane 3), and immunoreactivity was blocked by preincubation of the antibody with CP-(1677–1690) prior to immunoblotting (Fig. 5*B*, lane 4).

Antibodies directed against residues 1685–1690 of the $\alpha 1$ primary sequence were produced by purifying anti-CP-(1685–1699) against CP-(1677–1690). The antibodies recognized $\alpha 1_{190}$ and $\alpha 1_{212}$ (Fig. 5*B*, lane 5), and preincubation of the antibody with CP-(1677–1690) (data not shown) or CP-(1685–1699) (Fig. 5*B*, lane 6) prior to immunoblotting completely blocked recognition of both forms of $\alpha 1$. Similarly, antibodies recognizing residues 1685–1690 of $\alpha 1$ produced by purification of anti-CP-(1677–1690) against CP-(1685–1699) recognized both forms of $\alpha 1$ (data not shown). These results indicate that the C terminus of $\alpha 1_{190}$ extends beyond residue 1685 of $\alpha 1$.

The antibody against CP-(1685–1699) recognized $\alpha 1_{212}$ (Fig. 5*B*, lane 7), and this recognition was completely blocked by preincubation of the antibody with CP-(1685–1699) (Fig. 5*B*, lane 8). This antibody also labeled $\alpha 1_{190}$ (Fig. 5*B*, lane 7), but this labeling was nonspecific, because it was not blocked by CP-(1685–1699). Antibodies directed against residues 1692–1699 were generated by affinity purification of anti-CP-(1685–1699) against CP-(1692–1707). The resulting antibodies specifically recognized $\alpha 1_{212}$ (Fig. 5*B*, lane 9), and recognition was completely blocked by preincubation of the antibody with CP-(1692–1707) (Fig. 5*B*, lane 10) or CP-(1685–1699) (data not shown). Similarly, antibodies generated by affinity purification of anti-CP-(1692–1707) against CP-(1685–1699) recognized $\alpha 1_{212}$, and this recognition was blocked by preincubation of the antibodies with CP-(1685–1699) or CP-(1692–1707) (data not shown). Failure of these antibodies directed against residues 1692–1699 to recognize $\alpha 1_{190}$ confirms that the C terminus of $\alpha 1_{190}$ does not extend to residue 1699 of the $\alpha 1$ primary sequence.

Molecular Properties of the Two Forms of the $\alpha 1$ Subunit. Ferguson analysis resulted in a more accurate estimate of the molecular mass of the two forms of the calcium channel $\alpha 1$ subunit that we previously described (15). The apparent molecular mass of $\alpha 1_{212}$ (214 kDa) was in close agreement with the mass of 212,018 Da predicted for the full-length $\alpha 1$ subunit from its cDNA sequence (5). The apparent molecular mass (193 kDa) of the protein we previously called $\alpha 1_{175}$, and now refer to as $\alpha 1_{190}$, is considerably larger than the values of 155–175 kDa estimated previously by several groups for purified calcium channels, using 5–15% gradient gels or homogeneous gels at a single concentration of polyacrylamide (15, 25–27, 29), but it is in close agreement with a previous estimate of the size of $\alpha 1$ by Ferguson analysis [194 kDa (30)]. The wide variation in apparent molecular mass of $\alpha 1$ subunits determined from SDS/PAGE likely results from both anomalous migration of the $\alpha 1$ subunits themselves and anomalous migration of the myosin molecular mass marker, which had an unusual value of electrophoretic free mobility in our experiments. The impact of anomalous migration of myosin in standard SDS/PAGE analyses may be substantial, because it is commonly used as the highest molecular mass marker in commercially available kits of molecular mass standards.

A size of 193 kDa predicts that the C terminus of $\alpha 1_{190}$ is near residue 1690 of the predicted $\alpha 1$ sequence. The immunoblotting and immunoprecipitation experiments are consistent with this conclusion. Antibodies against residues 1685–1691 and all anti-peptide antibodies recognizing sequences more toward the N terminus from residue 1685 recognize $\alpha 1_{190}$. Thus, the C terminus of $\alpha 1_{190}$ must be beyond residue 1685 in the $\alpha 1$ sequence. Antibodies against residues 1692–1699 and all antibodies recognizing sequences more toward

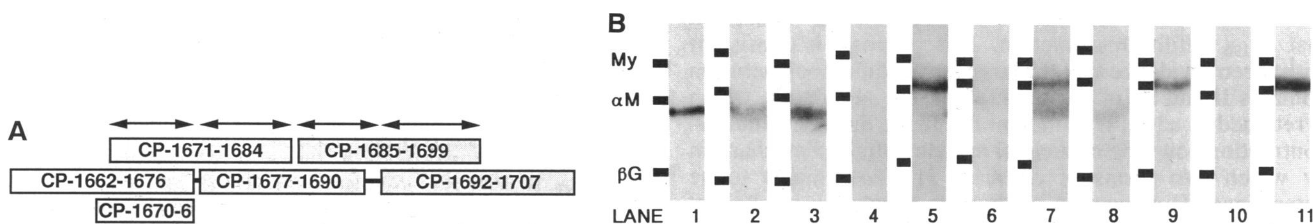

FIG. 5. Immunoblot analysis of calcium channels using anti-peptide antibodies recognizing shorter sequences in $\alpha 1$. (*A*) Peptides corresponding to the indicated sequences (boxes) in the C-terminal region of $\alpha 1$ were synthesized and antibodies were produced against all of these peptides except CP-(1670–1676). Antisera were enriched for antibodies recognizing the sequences indicated by double-headed arrows by purification against overlapping peptides. (*B*) Purified calcium channels were analyzed on 6% acrylamide gels, immunoblotted with antibodies directed against the indicated peptides, and visualized on x-ray film. Antibodies, antibody dilutions (-fold), pmol of calcium channel, and film exposure times (sec) were as follows: lane 1, anti-CP-(1646–1661), 400, 0.1, 30; lane 2, anti-CP-(1671–1684) purified against CP-(1670–1676), 6, 20, 30; lane 3, anti-CP-(1677–1690) purified against CP-(1671–1684), 50, 20, 10; lane 4, anti-CP-(1677–1690) purified against CP-(1671–1684) and preblocked with 1 mM CP-(1677–1690), 50, 20, 10; lane 5, anti-CP-(1685–1699) purified against CP-(1677–1690), 2, 40, 60; lane 6, anti-CP-(1685–1699) purified against CP-(1677–1690) and preblocked with 1 mM CP-(1685–1699), 2, 40, 60; lane 7, anti-CP-(1685–1699) purified against CP-(1685–1699), 50, 20, 30; lane 8, anti-CP-(1685–1699) purified against CP-(1685–1699) and preblocked with 1 mM CP-(1685–1699), 50, 20, 30; lane 9, anti-CP-(1685–1699) purified against CP-(1692–1707), 25, 20, 20; lane 10, anti-CP-(1685–1699) purified against CP-(1692–1707) and preblocked with CP-(1692–1707), 25, 20, 20; and lane 11, anti-CP-(1692–1707) purified against CP-(1692–1707), 5, 20, 3.

the C terminus from residue 1699 recognize only $\alpha 1_{212}$, indicating that the C terminus of $\alpha 1_{190}$ does not extend beyond residue 1699. Thus, we can place the C terminus of $\alpha 1_{190}$ between residues 1685 and 1699 with confidence. The failure of anti-CP-(1692–1699) to recognize $\alpha 1_{190}$ suggests that the C terminus of $\alpha 1_{190}$ does not extend far beyond residue 1692.

It was necessary to use approximately 10-fold higher amounts of purified calcium channels to immunoprecipitate or immunolabel $\alpha 1_{212}$ with similar intensity as $\alpha 1_{190}$. Consistent with our earlier study (15), we conclude that no more than 10% of $\alpha 1$ subunits of purified calcium channels contain the full-length amino acid sequence encoded by the $\alpha 1$ mRNA, while most are present as a smaller form, $\alpha 1_{190}$, whose C terminus is between residues 1685 and 1699.

It is of interest to note that the sequence of residues 1659–1671 (ANTNNANANVAYG) is partially conserved among the L-type calcium channels from rabbit skeletal muscle (5), rabbit heart (31), carp skeletal muscle (32), rat brain (33), and rabbit lung (34) and constitutes the final segment having significant sequence similarity before the amino acid sequences of these channels diverge in the C-terminal region. Because of its high degree of conservation, this segment, which is near the C terminus of $\alpha 1_{190}$, may have an important functional role.

Functional Roles of the Two Forms of the $\alpha 1$ Subunit. We have previously suggested that the two forms of the calcium channel $\alpha 1$ subunit in skeletal muscle are specialized to serve as cAMP-regulated ion channels and as voltage sensors in excitation–contraction coupling (15). The two forms of $\alpha 1$ occur in T-tubule membranes and intact skeletal muscle cells rather than being produced by proteolytic degradation during protein purification (15, 16), and both are phosphorylated in response to physiological stimuli that activate cAMP-dependent protein phosphorylation in intact muscle cells (16). Since it is well established that calcium conductance mediated by the skeletal muscle calcium channel is activated by cAMP-dependent protein phosphorylation (35–38), it is of interest that three phosphopeptides have been identified in $\alpha 1_{212}$ that are absent from $\alpha 1_{190}$ (16). The present study is in agreement with this result in that it predicts that at least residues 1699–1873 of the $\alpha 1$ subunit, which contain three cAMP-dependent protein kinase phosphorylation consensus sequences, are missing in $\alpha 1_{190}$. This result is consistent with our earlier suggestion (15) that phosphorylation of sites in the C terminus of $\alpha 1_{212}$ may be important for regulation of the ion conductance activity of the channel.

Depolarization of the transverse tubule membrane is sufficient to initiate contraction of skeletal muscle without entry of extracellular calcium (39). The $\alpha 1$ subunit of the calcium channel is required for this process (11, 12), and we suggest that $\alpha 1_{190}$ fulfills this function. This proposal is consistent with recent evidence that the large intracellular loop between domains II and III of the skeletal muscle $\alpha 1$ subunit, which is retained in $\alpha 1_{190}$, is sufficient to induce direct excitation–contraction coupling in skeletal muscle (40). The mechanism by which two forms of $\alpha 1$ arise *in vivo* remains to be determined. However, in addition to regulation of skeletal muscle calcium conductance and contraction by phosphorylation, the regulation of the relative amounts of these two forms of the $\alpha 1$ subunit potentially has the ability to control the dual functions of the $\alpha 1$ subunit in this tissue.

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