Immunohistochemical identification and differential phosphorylation of alternatively spliced forms of the α1A subunit of brain calcium channels*

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Biological properties of the α1 subunits of class A brain calcium channels (α1A) were examined in adult rat brain membrane fractions using a site-directed antipeptide antibody (anti-CNA3) specific for α1A. Anti-CNA3 specifically immunoprecipitated high affinity receptor sites for ω-conotoxin MVIIIC (Kd ~ 100 pM), but not receptor sites for the dihydropyridine irsiradine or for ω-conotoxin GVIA. In immunoblotting and immunoprecipitation experiments, anti-CNA3 recognized at least two distinct immunoreactive α1A polypeptides, a major form with an apparent molecular mass of 190 kDa and a minor, full-length form with an apparent molecular mass of 220 kDa. The 220- and 190-kDa α1A polypeptides were also specifically recognized by both anti-BI-NT and anti-BI-1-L-Ct antibodies, which are directed against the NH2- and COOH-terminal ends of α1A predicted from cDNA sequence, respectively. These data indicate that the predicted NH2 and COOH termini are present in both size forms and therefore that these isoforms of α1A are created by alternative RNA splicing rather than post-translational proteolytic processing of the NH2 or COOH termini. The 220-kDa form was phosphorylated preferentially by cAMP-dependent protein kinase, whereas protein kinase C and cGMP-dependent protein kinase preferentially phosphorylated the 190-kDa form. Our results identify at least two distinct α1A subunits with different molecular mass, demonstrate that they may result from alternative mRNA splicing, and suggest that they may be differentially regulated by protein phosphorylation.

In the nervous system, voltage-gated calcium channels are involved in initiation of activity-dependent events such as neurotransmitter release, regulation of action potential duration and frequency, protein phosphorylation, and gene expression (Llinas, 1988; Tsien, 1988; Olivera, et al., 1994). Based on the pharmacological and physiological properties, at least five distinct types of voltage-gated calcium channels, designated L, N, P, Q, and T, have been identified (Bean, 1989; Llinas, 1989; Zhang et al., 1993). Voltage-gated calcium channels are a complex of five subunits: α1, α2, β, γ, and δ (Takahashi et al., 1987; Catterall et al., 1988; Campbell et al., 1988). α1 subunits can function alone as voltage-gated calcium channels when expressed in Xenopus oocytes or mammalian cells (Perez-Reyes et al., 1989; Mikami et al., 1989), whereas coexpression of the other subunits can alter functional properties of α1 subunits (Lacerda et al., 1991; Singer et al., 1991; Varadi et al., 1991; Wei et al., 1991) (reviewed by Isom et al. (1994)). cDNAs encoding five distinct α1 subunits of brain calcium channels have been identified and designated A, B, C, D, and E (α1A–α1E) (Snutch et al., 1990; Snutch and Reiner, 1992: Zhang et al., 1993; Catterall, 1994a). The class C and class D genes encode L-type calcium channel α1 subunits (α1C and α1D), which have a high affinity for dihydropyridines and conduct long lasting Ba2+ currents. In contrast, the class A, B, and E genes encode α1 subunits (α1A, α1B, and α1E) of non-L-type calcium channels, which are more distantly related to L-type calcium channels (23–35% amino acid identity) (Mori et al., 1991; Starr et al., 1991; Dubel et al., 1992; Williams et al., 1992a; Fujita et al., 1993; Niidome et al., 1993; Soong et al., 1993). α1B forms an N-type calcium channel, which is neuro-specific and distinguished by high sensitivity to the cone snail toxin ω-conotoxin GVIA (Dubel et al., 1992; Williams et al., 1992a; Fujita et al., 1993). α1E forms a novel, rapidly inactivating calcium channel, which has some characteristics of a low voltage activated calcium channel (Soong et al., 1993; Williams et al., 1994).

The class A calcium channel (also designated BI) was the first non L-type calcium channel to be cloned, sequenced, and expressed (Starr et al., 1991; Mori et al., 1991). α1A forms high voltage activated calcium channels and Northern blot analysis shows high expression in the cerebellum (Starr et al., 1991; Mori et al., 1991; Sather et al., 1993; Stea et al., 1994a). α1A currents expressed in Xenopus oocytes are insensitive to dihydropyridines and ω-conotoxin GVIA, and therefore α1A subunits may form P-type and/or Q-type channels (Mori et al., 1991; Sather et al., 1993; Stea et al., 1994a). α1A channels expressed in Xenopus oocytes inactivate slowly or rapidly depending on the β subunit expressed with them, and are blocked by ω-agatoxin IVA purified from Agelenopsis aperta venom at high concentration and by ω-conotoxin MVIIIC from Conus ma-

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1 The abbreviations and trivial names used are alphabeta subunits of class A, B, C, D, and E brain calcium channels, respectively; PAS, protein A-Sepharose; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; BSA, bovine serum albumin; WGA, wheat germ agglutinin; PN200-110, isopropyl-4((2,1,3-benzoxadiazol-2-yl)-1,4-di hydriod-2,6-dimethyl-5-methoxy carbonyl)p yridine-3-carboxylate; PAG, polycyramide gel electrophoresis; NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamide) hexanoate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonic acid; TBS, Tris-buffered saline; GABA, γ-aminobutyric acid.
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In contrast, native P-type calcium channels are blocked by low concentrations of ω-agatoxin IVA and by higher concentrations of ω-Conotoxin MVIIIC (Mintz et al., 1992a, 1992b, Hillyard et al., 1992). The pharmacological properties of α1A calcium channel subunits in Xenopus oocytes are distinct from P-type channels, but more closely resemble those of calcium channels in cerebellar granule cells, which have been designated Q-type (Randall et al., 1995; Zhang et al., 1993). In the experiments described in this paper, we used site-directed-antipeptide antibodies against unique sequences in rat brain α1A to identify the corresponding polypeptides and demonstrated that there are multiple isoforms of α1A subunits that may result from alternative RNA splicing and are differentially phosphorylated by second messenger-activated protein kinases.

EXPERIMENTAL PROCEDURES

Materials—[3H]Sidiparine (PN200-110; 80 Ci/mmol), [125I]-ω-Conotoxin GVIA (2200 Ci/mmol), and [1-35S]ATP (3000 Ci/mmol) were purchased from Du Pont NEN. ω-Agatoxin IVA was a generous gift from Drs. N. Saccomano and M. Ahijaliljan, Pfizer. The ECL detection kit for immunoblotting was obtained from Amersham Corp., dotblot from Gallard-Schlesinger (Carle Place, NY), and protein A-Sepharose (PAS) and heparin-agarose from Sigma. cAMP-dependent kinase (PKA) and protein kinase C (PKC) were purified by standard procedures (Kaczmarek et al., 1980; Woobdgett and Hunter, 1987) and kindly provided by Drs. E. I. Rotman and B. J. Murphy, Department of Pharmacology, University of Washington. cGMP-dependent protein kinase (PKG) was obtained commercially from Promega (Madison, WI). Control antibodies (rabbit IgG) were received from Zymed (South San Francisco, CA). All other reagents were of standard biochemical quality from commercial sources.

Production and Purification of Peptides and Antibodies—The peptides CNA3 (KY)SEPQQREHAPPREHV) corresponds to residues 882–896 from commercial sources. The peptide CNA1 ((KY)PSSPERAPGREGREHRHRQ) corresponds to residues 865–881, which are located in a highly variable site in the intracellular loop between domains II and III of the 1A subunit of rat brain calcium channels. The NH2-terminal lysine and tyrosine are not part of the channel sequence and were added for cross-linking and labeling purposes. The CNA1 and CNA3 peptides were synthesized by the solid phase method (Merrifield, 1963) and then purified by reversed phase high pressure liquid chromatography on a Vydac 281TP10 column. The identity of the purified peptides was confirmed by amino acid analysis.

The purified peptides were coupled through amidogroups with glutaraldehyde to bovine serum albumin (BSA), dialyzed against phosphate-buffered saline (10 mM NaH2PO4, pH 7.4, 150 mM NaCl) and emulsified in an equal volume of Freund's complete adjuvant. The coupled peptides were injected into multiple subcutaneous sites on New Zealand White rabbits at 3-week intervals. Antisera were collected after the second injection and tested by enzyme-linked immunosorbent assay using microtiter plates with wells coated with 0.5 μg of peptide (Ponsett et al., 1988). Antibodies were purified by affinity chromatography on the corresponding peptides coupled to CNBr-activated Sepharose. Two ml of the antiserum were bound to the column at 4°C overnight and washed with TBS (10 mM Tris-HCl (pH 7.4), 150 mM NaCl). The bound IgG was eluted with 3 M KCl. The affinity-purified antibodies were then dialyzed against TBS using a Centricon 10 (Amicon).

Anti-β3- and anti-β3-1-3 antibodies were generous gifts from Dr. Masami Takahashi (Mitsubishi-Kasei Life Sciences Institute, Tokyo, Japan), and these antibodies were produced against peptides MARFG-FVYDPYRED (Leveque et al., 1994) and (C)RDQRWSR-PSEGREHTHRQ, which correspond to residues 1–23 and 2254–2273 of the BI-1 cDNA clone encoding a rabbit brain 1A subunit. The NH2-terminally lysine and tyrosine are not part of the channel sequence and were added for cross-linking and labeling and is not part of the α1A subunit sequence.

Radioiodination of ω-Conotoxin MVIIIC—The radiiodinated derivative of ω-Conotoxin MVIIIC was prepared and purified as described before (Kristipati et al., 1994; Wopmann et al., 1994).

Membrane Preparation—Brains were dissected from 15 2-month-old Sprague-Dawley rats, obtained from Bantin and Kingman (Bellevue, WA), and calcium channels were solubilized and partially purified as described previously (Westenbrook et al., 1992). Briefly, samples of rat brain were homogenized and subjected to a brief low speed centrifugation to yield supernatant S1 containing mixed brain membranes. The cell surface membranes were collected by high speed centrifugation. Calcium channels were solubilized with 1.2% digitonin, and insoluble material was removed by high speed centrifugation to yield supernatant S3. The calcium channels were then partially purified by the chromatography on wheat germ agglutinin (WGA)-Sepharose as described previously (Westenbrook et al., 1992).

Radioactive Ligand Binding Studies—For [3H]PN200-110 (isradipine) binding studies, 40 ml of S1 fraction were labeled with 10 μCi of [3H]PN200-110 (85.8 Ci/mmol) at a concentration of 2.9 nM for 1 h on ice. The bound radiogand is stable throughout the subsequent purification. Calcium channels were purified from 250 μl of S3 fraction (6000 cpm) containing 300 μCi KCl, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1.2% digitonin with 0.2% BSA using 15 μg of affinity-purified anti-CNA3, anti-CNC1, or control rabbit nonimmune IgG. After a 1.5-h incubation on ice, 2.5 mg of PAS, prewashed three times with TBS containing 0.1% digitonin and 0.5% BSA, were added to the samples. The samples were mixed on ice for additional 2.5 h, pelleted by centrifugation, and washed three times in TBS, 0.1% digitonin. After the final wash, the antibody-bound PAS complexes were transferred to vials, and the amount of immunoprecipitated [3H]PN200-110 receptors was quantified in a scintillation counter. Total receptor-bound [3H]PN200-110 was determined by filter binding assay. 250 μl of the labeled S3 fraction were precipitated by incubation with 4 ml of ice-cold 10% (v/v) trichloroacetic acid (average molecular weight 8000) in 10 mM MgCl2, and 10 ml Tris-HCl (pH 7.4) for 5 min and poured over Whatman GF/C filters. Samples were washed four times in ice-cold polyethylene glycol solution and quantified in a scintillation counter. The correction factor for ligand-receptor loss in the filter-binding assay was 0.7 (Westenbrook et al., 1992).

Determination of [3H]-ω-Conotoxin MVIIIC binding was done by incubation of 100 μl of S3 fraction containing 0.2% BSA with 0.06 μCi of [3H]-ω-Conotoxin GVIA (2200 Ci/mmol) at a concentration of 0.27 nM for 30 min on ice. Samples were immunoprecipitated with 15 μg of affinity-purified anti-CNA3, anti-CNB2, or control rabbit IgG, and washed four times with TBS, 0.1% digitonin. The matrix was transferred to vials for cDNA binding. Total [3H]-ω-Conotoxin GVIA binding was determined using 100 μl of the labeled S3 fraction in the filter-binding assay described above.
tions, diluted 1:1000 in 100 mM sodium borate (pH 8.5), 0.1% digitonin, were concentrated to a volume of ~500 μl in a Centricon-30 microconcentrator to remove the amine in Tris-HCl buffer. One μmol of sulfosuccinimidyl-6-(biotinamido) hexaneate (NHS-LC-biotin) was used to biotinylate the partially purified membrane fractions. After 2 h of incubation on ice, the reaction was terminated by addition of 0.2 volume of 2M glycine (pH 8.5). Samples were washed twice with TBS, 0.1% digitonin and concentrated to a volume of approximately 300 μl by ultrafiltration.

Biotinylated samples were preabsorbed for 1 h on ice with 300 μl of Sepharose CL-4B and for 2 h on ice with 10 μg of PAS, which was preincubated with 200 μg of control rabbit IgG and washed three times with TBS, 0.1% digitonin in order to remove the nonspecifically binding protein in the sample. After centrifugation, supernatants were incubated for another 2 h on ice with 10 μg of PAS to adsorb the free IgG dissociated from PAS-control rabbit IgG complex. After centrifugation for 1 min on a table top centrifuge, the supernatants were collected and incubated with anti-CNA3 (40 μg), anti-BI-1 (30 μg), anti-BI-1 Ct (30 μg), anti-CNA1 (80 μg), or control antibody (80 μg) for 1.5 h on ice. The immunoprecipitation was performed as described in the section above, and the pellets were extracted for 30 min at 50–60 °C with 20 μl of 1.5% SDS, 50 mM Tris-HCl (pH 7.4), 5 mM dithiothreitol, 1 mM peptatin A, 2 μg/ml leupeptin, and 4 μg/ml aprotonin, and diluted with 250 μl of Triton buffer (1% Triton X-100, 0.5% BSA, 75 mM NaCl, 25 mM Tris-HCl (pH 7.4), 20 mM EDTA). The supernatant was collected and incubated for 1.5 h on ice with the secondary antibodies anti-CNA3 (40 μg) or anti-CNA1 (80-140 μg). Three times of PAS, preincubated as described above, were added, and the samples were incubated on a tilting mixer for 2.5 h on ice. The immunoprecipitated complexes were pelleted by centrifugation, washed three times with Triton buffer and once in 10 mM Tris-HCl (pH 7.4), and extracted for 30 min at 50–60 °C with SDS sample buffer. After a short centrifugation, the supernatants were loaded onto an SDS-PAGE gel. The proteins were blotted, blocked as described above, and nitrocellulose sheets were rinsed with TBS, 5% BSA, 0.2% Nonidet P-40, and 0.05% Tween 20, and incubated for 1 h at room temperature with streptavidin-biotinylated horseradish peroxidase complex, diluted 1:8000 in TBS containing 0.2% Nonidet P-40 and 0.05% Tween 20. After a 3-h wash with 0.2% Nonidet P-40, 0.05% Tween 20 in TBS (6-9 changes), the blots were developed with the ECL reagent.

Immunoprecipitation and Phosphorylation of Calcium Channels— Calcium channels in the WGA extract were concentrated by immunoprecipitation with either affinity-purified anti-CNA3 or control rabbit IgG as described above. Prior to phosphorylation of the immunoprecipitated calcium channels, the resin was washed once in the basic phosphobuffer (50 mM Heps (adjusted to pH 7.4 with NaOH), 10 mM MgCl2, 1 mM EDTA, 0.1% digitonin). Phosphorylation reactions were performed in 50 μl of the reaction mixture containing 0.5–1.0 μg of PKA, PKC, or PKG in the basic phosphobuffer, along with 1 mM dithiothreitol, 1 μM peptatin A, 1 mM EGTA, and 0.2 μM I-125-[G-γ-32P]AT (specific activity 370 Ci/mmol). The mixture was supplemented with 1.5 mM CaCl2, 2 mM MgCl2, and 2.5 mg of phosphodiesterase for PKC and 2 μl of GTP for PKG. Incubations were at 32–34 °C for 30 min with gentle mixing every 2 min. The samples were washed twice with 0.1% digitonin in radiomunnoassay buffer (25 mM Tris-HCl (pH 7.4), 20 mM EDTA, 75 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 50 mM NaF, and 1 mM p-nitrophenyl phosphate), three times with 1% Triton X-100 in radiomunnoassay buffer, and once in 10 mM Tris-HCl (pH 7.4). The pellets were extracted, and the second immunoprecipitations were performed with affinity-purified anti-CP (1382–1400) (20 μg/ml aprotinin, and diluted with 250 μl of 125I-[G-γ-32P]AT and immunoprecipitated labeled brain calcium channels with anti-CNA3 antibodies.

[3H]PN200-110 is a dihydropyridine that specifically binds to L-type calcium channels containing α1C and α1D (Helliwell et al., 1993a). At a concentration of [3H]PN200-110 chosen to saturate all binding sites in a rat brain homogenate, affinity-purified anti-CNC1, an α1D-specific site-directed anti-peptide antibody, immobilized 40% of the total [3H]PN200-110 receptors, whereas anti-CNA3 immunoprecipitated less than 1% of the total [3H]PN200-110 receptors, a value similar to that obtained with control rabbit IgG (Fig. 1A). The binding of 125I-[G-γ-32P]AT-conotoxin GVIA, a selective blocker of the N-type calcium channels containing α1B, was tested similarly. Affinity-purified anti-CNB2, an α1B-specific site-directed anti-peptide antibody, immunoprecipitated over 80% of the total binding sites in S3 fractions. Under the same conditions, anti-CNA3 and control rabbit IgG recognized less than 4% of the total 125I-[G-γ-32P]AT-conotoxin GVIA receptors (Fig. 1B). ω-Conotoxin MVIC blocks Q-type calcium channels containing α1A (Sather et al., 1993; Stea et al., 1994a; Hillyard et al., 1992). A saturating concentration of 125I-[G-γ-32P]AT-conotoxin MVIC was added to the solubilized and partially purified calcium channel preparation. Affinity-purified anti-CNA3 antibodies effectively immunoprecipitated 125I-[G-γ-32P]AT-conotoxin MVIC receptors (Fig. 1C). Anti-CNA3 specifically recognized the ω-conotoxin MVIC receptors, since preincubation of 20 μM CNA3 peptide largely blocked immunoprecipitation of them, whereas the CNA1 peptide at the same concentration did not affect the immunoprecipitation of 125I-[G-γ-32P]AT-conotoxin MVIC receptors with anti-CNA3. In contrast, anti-CNC1 and control rabbit IgG immunoprecipitated only small amounts of 125I-[G-γ-32P]AT-conotoxin MVIC. ω-Conotoxin MVIC has a significant affinity for ω-conotoxin GVIA binding sites on α1A (Hillyard et al., 1992). However, anti-CNB2 did not immunoprecipitate detectable 125I-[G-γ-32P]AT-conotoxin MVIC-labeled α1A at 0.15 μM (Fig. 2A).

Displacement of specific binding of 125I-[G-γ-32P]AT-conotoxin MVIC by unlabeled ω-conotoxin MVIC was observed between 10 pM and 1 nM, with half-maximal inhibition at approximately 100 pM (Fig. 2). The Kd value of approximately 100 pM is in agreement with that determined in rat synaptosomal membranes (10–300 pM) (Hillyard et al., 1992; Kristipati et al., 1994; Wopmann et al., 1994), ω-Agatoxin IVA, a 48-amino acid peptide toxin from funnel web spider venom with no obvious similarity in sequence to ω-conotoxin MVIC, blocks P-type calcium currents with IC50 of 1–2 nM (Mintz et al., 1992a) and α1δ calcium currents at 100–300 μM (Sather et al., 1993, Stea et al., 1994a). To assess whether ω-conotoxin MVIC and ω-agatoxin IVA might bind to the same high affinity sites, the ability of ω-agatoxin IVA to displace high affinity binding of ω-conotoxin MVIC was tested. Displacement of specific binding of 125I-[G-γ-32P]AT-conotoxin MVIC to the ω-conotoxin MVIC receptor site with unlabeled ω-agatoxin IVA occurred only at high concentration with half-maximal inhibition at approximately 1–5 μM (Fig. 2). This result indicated that ω-agatoxin IVA can displace ω-conotoxin MVIC from its high affinity binding site nonspecifically at high concentration, but not at concentrations at which it inhibits calcium channels containing α1A. Evidently, ω-agatoxin IVA does not bind to the same receptor site as ω-conotoxin MVIC in inhibiting class A calcium channels.

Identification of α1A in Rat Brain Membranes—To identify α1A polypeptides, rat brain glycoproteins were isolated by affinity chromatography on WGA-Sepharose, and calcium channels were enriched by adsorption to heparin-agarose and analyzed by immunoblotting (see “Experimental Procedures”). Affinity-purified anti-CNA3 antibody revealed at least three
immunoreactive bands of $\alpha_{1A}$ subunits with apparent molecular masses of 210–230, 180–195, and 160 kDa (Fig. 3). The molecular mass of the largest polypeptide varies between 210 and 230 kDa depending on the concentration of acrylamide used for SDS-PAGE (Fig. 3, lanes 1 and 4). In a 5% acrylamide gel, the polypeptide migrated to a position just above myosin, the 205-kDa marker, whereas in a 7% acrylamide gel its position was between the longer and shorter forms of spectrin, the 240- and 220-kDa markers, respectively. We refer to this band as the 220-kDa form of $\alpha_{1A}$ polypeptide. In a higher resolution autoradiogram, two distinct but closely spaced bands were often separately visualized within this 220-kDa band (Fig. 3, lane 1). The intermediate size form of $\alpha_{1A}$, which we have designated the 190-kDa form, migrated with an apparent molecular mass of 180 kDa in a 5% acrylamide gel, and 195 kDa in a 7% acrylamide gel when compared with the marker proteins. The smallest polypeptide migrated with an apparent molecular mass of 160 kDa in both gels. Anomalous migration was also observed during the determination of molecular masses of the two size forms of the skeletal muscle L-type calcium channel $\alpha_1$ subunit (De Jongh et al., 1991).

The specificity of the interaction of anti-CNA3 antibodies with these polypeptides was tested with the CNA3 peptide. After preincubation with CNA3 peptide at a concentration of 2 $\mu$M, no signal could be detected with anti-CNA3 antibody (Fig. 3, lane 2). The migration positions of $\alpha$- and $\beta$-spectrin, myosin heavy chain, $\alpha_7$-macroglobulin, $\beta$-galactosidase, and fructose-6-phosphate kinase are indicated at the left side of the gel together with their molecular masses in kDa.

**Fig. 2.** Competitive inhibition of $^{125}$I-$\omega$-agatoxin MVIIC binding by unlabeled $\omega$-agatoxin MVIIC or $\omega$-agatoxin IVA. The indicated concentrations of $\omega$-agatoxin MVIIC (closed circles) or $\omega$-agatoxin IVA (open circles) were added to WGA samples with $^{125}$I-$\omega$-agatoxin MVIIC (0.15 nM) and immunoprecipitated with affinity-purified anti-CNA3 antibodies. Bound $^{125}$I-$\omega$-agatoxin MVIIC was determined, and the values were normalized to the specific binding observed in the absence of unlabeled toxins (100%).

**Fig. 3.** Detection of $\alpha_{1A}$ with affinity-purified anti-CNA3 antibodies by immunoblotting. Membrane glycoprotein fractions were isolated from solubilized brain membranes by WGA affinity chromatography, and calcium channels were concentrated by adsorption to heparin agarose, extracted, and analyzed by SDS-PAGE using a 5% acrylamide gel (lanes 1–3) or a 7% acrylamide gel (lane 4). Proteins were transferred onto a nitrocellulose membrane, blocked, incubated with anti-CNA3 (lanes 1, 2, and 4) or anti-CNC1 (lane 3), incubated with horseradish peroxidase-protein A, washed, and visualized with ECL reagent, as described under “Experimental Procedures.” Anti-CNA3 antibodies were preincubated overnight on ice with 2 $\mu$M CNA3 peptide (lane 2). The migration positions of $\alpha$- and $\beta$-spectrin, myosin heavy chain, $\alpha_7$-macroglobulin, $\beta$-galactosidase, and fructose-6-phosphate kinase are indicated at the left side of the gel together with their molecular masses in kDa.
Calcium channels are multisubunit complexes and may interact with other cellular components such as cytoskeletal proteins and synaptic vesicle proteins in immunoprecipitation. Therefore, it is possible that the proteins immunoprecipitated by anti-CNA3 antibodies under native conditions might be associated proteins of similar size to the \( \alpha_1 \) subunit rather than the \( \alpha_2 \) subunit itself. To exclude other proteins from the immunoprecipitates, double immunoprecipitation experiments were performed under conditions that should completely dissociate the calcium channel subunits and associated proteins. Anti-CP(1382–1400), which recognizes a segment of the \( \alpha_2 \) subunit whose sequence is conserved in all calcium channel \( \alpha_1 \) subunits so far characterized, was used as a probe in the second immunoprecipitation. The CP(1382–1400) sequence is accessible to anti-CP(1382–1400) only after solubilization in Triton X-100, which removes the \( \alpha_2 \) and \( \delta \) subunits (Ahlijianian et al., 1991).

Following the double immunoprecipitation with anti-CNA3 and anti-CP(1382–1400) antibodies, two immunoreactive bands corresponding in size to the 220- and 190-kDa polypeptides were visualized (Fig. 4, lane 1), indicating that these immunoreactive polypeptides are \( \alpha_{1A} \) subunits. Two forms of \( \alpha_{1A} \) polypeptides with apparent molecular masses of approximately 220 and 190 kDa were also recognized by the affinity-purified anti-CNA1 antibody, which is directed against a unique amino acid sequence in the intracellular loop between domains II and III of \( \alpha_{1A} \) (residues 865–881) immediately on the NH\(_2\)-terminal side of the CNA3 sequence. Double immunoprecipitation with anti-CNA1 and anti-CP(1382–1400) antibodies revealed two immunoreactive bands with molecular masses of 220 and 190 kDa, and the 190-kDa polypeptide was the major form of \( \alpha_{1A} \) as detected with anti-CNA3 antibody (Fig. 4, lane 5). These observations in immunoblotting and immunoprecipitation experiments demonstrate that \( \alpha_{1A} \) subunits consist of at least two distinct polypeptides that are specifically recognized by anti-CNA3 antibody, and that the 190-kDa polypeptide is a major form of \( \alpha_{1A} \) whereas the 220-kDa polypeptide is a minor form of this subunit.

Identification of the Predicted NH\(_2\)- and COOH-terminal Sequences of the \( \alpha_{1A} \) Polypeptides—\( \alpha_1 \) subunits of skeletal muscle calcium channels and class B, C, and D brain calcium channels each have multiple size forms that are truncated at the NH\(_2\) or COOH termini (De Jongh et al., 1989, 1991; Westenbroek et al., 1992; Hell et al., 1993a, 1993b, 1994; Leveque et al., 1994). To test if this is true for \( \alpha_{1A} \) we used antibodies that recognize the NH\(_2\)-terminal (anti-BI-Nt) or COOH-terminal (anti-BI-1-Ct) of \( \alpha_{1A} \) subunits deduced from cDNA sequence. Two distinct cDNAs encoding \( \alpha_{1A} \) subunits have been cloned: rBA from rat brain and B1 (isoforms B1-1 and B1-2) from rabbit brain (Starr et al., 1991; Mori et al., 1991). The anti-BI-Nt antibody is directed against the NH\(_2\) terminus (residues 1–23) of B1 clone (Mori et al., 1991), which is conserved in all known isoforms of \( \alpha_{1A} \) subunits. Two distinct B1 cDNA clones, designated B1-1 and B1-2, differ from each other in COOH-terminal sequence (Mori et al., 1991). Anti-BI-1-Ct was raised against the COOH terminus (residues 2237–2254) of the B1-1 clone (Mori et al., 1991).
shows that the 190-kDa form of insitu possibly because of insufficient quantity of these polypeptides Anti-BI-1-Ct antibodies did not detect immunoreactive bands with molecular weights or may recognize different immunoreactive polypeptides with equivalent molecular weights (Fig. 6, lane 1). Anti-BI-1-Ct antibody revealed an immunoreactive band with an apparent molecular mass of 190 kDa, which was blocked by preincubation with 2 μM BI-1-Ct peptide (lane 4). Molecular markers are given in Fig. 3. 1901), which is conserved in the rbA clone (Starr et al., 1991). We examined the immunoreactive α1A polypeptides for the presence of the predicted NH2- and COOH-terminal sequences using anti-BI-Nt and anti-BI-1-Ct.

Immunoblotting with anti-BI-Nt antibodies revealed four immunoreactive bands with apparent molecular masses of 220, 190, 160, and 95 kDa in a 5% acrylamide gel (Fig. 5, lane 1). These immunoreactive polypeptides were specifically detected with anti-BI-Nt antibodies, since 0.2 μM of the BI-Nt peptide blocked the interaction of anti-BI-Nt with the immunoreactive polypeptides (Fig. 5, lane 2). The immunoreactive band of 190 kDa was the major form, and the band at 220 kDa was a doublet as observed with anti-CNA3. In other blots, we stripped the membrane used for immunoblotting with anti-BI-Nt antibodies by incubation at 50 °C for 30 min in Tris-HCl buffer (pH 6.7) containing 2% SDS and 20 mM dithiothreitol, and re-probed with anti-CNA3 antibodies. Immunoreactive bands with molecular masses of 220, 190, and 160 kDa detected with anti-BI-Nt or anti-CNA3 were identical (data not shown). Anti-BI-1-Ct antibodies revealed an immunoreactive band with an apparent molecular mass of 190 kDa, which was blocked by preincubation with 2 μM BI-1-Ct peptide (Fig. 5, lanes 3 and 4). Anti-BI-1-Ct antibodies did not detect immunoreactive bands with molecular mass values of 220 or 160 kDa in immunoblots, possibly because of insufficient quantity of these polypeptides in situ. Thus, immunoblotting with anti-BI-Nt and anti-BI-1-Ct shows that the 190-kDa form of α1A polypeptide contains both the predicted NH2- and COOH-terminal ends of the α1A subunits.

It is possible that anti-BI-Nt or anti-BI-1-Ct may recognize different immunoreactive polypeptides with equivalent molecular weights or may recognize α1B since the BI-Nt sequence is 54% identical to the corresponding α1B sequence. To exclude this possibility, we performed double immunoprecipitation with anti-CNA3 antibodies and either anti-BI-Nt or anti-BI-1-Ct antibodies (Fig. 6). We used anti-BI-Nt or anti-BI-1-Ct antibodies for the first immunoprecipitation and followed with anti-CNA3 or anti-CP(1382–1400) in the second immunoprecipitation. Double immunoprecipitation with anti-BI-Nt and anti-CP(1382–1400) revealed two distinct immunoreactive polypeptides with molecular masses of 220 and 190 kDa (Fig. 6, lane 1), and immunoprecipitation with anti-BI-1-Ct and anti-CP(1382–1400) antibodies detected two forms of α1A polypeptide with equivalent molecular weights (Fig. 6, lane 4). In double immunoprecipitation with anti-BI-Nt and anti-CNA3 antibodies, α1A polypeptides recognized by anti-BI-Nt were specifically immunoprecipitated with anti-CNA3 antibody, since preincubation of 50 μM CNA3 peptide blocked the interaction of anti-CNA3 with α1A polypeptides (Fig. 6, lane 2 and 3). Similarly, immunoprecipitation with anti-BI-1-Ct and anti-CNA3 detected immunoreactive polypeptides of 220 and 190 kDa and was blocked by 50 μM CNA3 peptide (Fig. 6, lanes 5 and 6). These results demonstrated that anti-CNA3, anti-BI-Nt, and anti-BI-1-Ct antibodies recognized the same immunoreactive α1A polypeptides with molecular mass values of 220 and 190 kDa, and that 220- and 190-kDa forms of α1A have both the predicted NH2- and COOH-terminal ends of α1A. These results indicate that these isoforms of α1A do not result from post-translational proteolytic processing, but may instead be products of alternative RNA splicing.

Phosphorylation of α1A Subunits by Second Messenger-activated Protein Kinases—Calcium channels are regulated by phosphorylation by multiple protein kinases (Tsien et al., 1986; Levanit, 1988; Miller, 1990; Catterall, 1994b). To examine the phosphorylation of α1A subunits by second messenger-activated protein kinases, α1A was purified by immunoprecipitation with affinity-purified anti-CNA3 antibodies. The resulting immune complexes were incubated with different kinases in the presence of [γ-32P]ATP. After washing, the PAS-antibody-channel complexes were dissociated with 1.5% SDS-sample buffer and diluted with 1% Triton, and α1A subunits were re-immunoprecipitated with anti-CP(1382–1400) (see “Experimental Procedures”). Following phosphorylation with PKA, two labeled α1A polypeptides with molecular masses of 220 and 190 kDa were observed. No incorporation of radiolabel was detected with a non-immune rabbit IgG in the first immunoprecipitation (Fig. 7, lane 2), or if 50 μM CNA3 peptide was preincubated prior to the first immunoprecipitation (data not shown). The 220-kDa band was observed as a doublet in a high resolution autoradiogram, as demonstrated in immunoblotting and immunoprecipitation experiments (Fig. 3, lane 1, and Fig. 4, lane 1). Immunoblotting and immunoprecipitation experiments showed that the 190-kDa band is a major form of α1A and the 220-kDa band is minor in quantity. In contrast, PKA phosphorylated the
molecular masses of 220, 170, 150, and 140 kDa were observed. Although one or more of these bands may represent \( \alpha_1A \) subunits of class A channels or other associated proteins, it seems most likely that the bands of 220 and 170 kDa correspond to the 220- and 190-kDa isoforms of \( \alpha_1A \).

\( \alpha_1A \) subunits have been suggested to be components of both P-type and Q-type calcium channels. \( \alpha_1A \) is localized in high density in the cell bodies and dendrites of cerebellar Purkinje cells where P-type calcium currents are recorded, as well as in the cell bodies and nerve terminals of cerebellar granule cells where Q-type calcium currents are recorded (Westenbroek et al., 1995). Coexpression of \( \alpha_1A \) with various calcium channel \( \beta \) subunits results in modulation of the amplitude, time course, and the voltage-dependent properties of the \( \alpha_1A \) calcium currents (Mori et al., 1991; Sather et al., 1993; Stea et al., 1994a; Soong et al., 1994; De Waard et al., 1994). \( \alpha_1A \) calcium currents expressed in Xenopus oocytes inactivate more rapidly than native P-type calcium channels, but coexpression of the rbA-I or rbA-II isoforms of the \( \alpha_1A \) subunit with a \( \beta \) subunit (rbB-I with \( \beta_3 \), or rbA-II with \( \beta_3 \)) in Xenopus oocytes gives currents with much slower inactivation like a P-type calcium channel (Stea et al., 1994a; Soong et al., 1994). However, the sensitivity of \( \alpha_1A \) to \( \omega \)-conotoxin MVIIC and \( \omega \)-agatoxin IVA is not significantly affected in these coexpression studies. These findings suggest that pharmacological and physiological differences between Q-type and P-type calcium channels may be due to an unidentified isoform of \( \alpha_1A \), which may result from alternative RNA splicing or post-translational modifications or may result from assembly with other auxiliary subunits of calcium channels.

Identification of Multiple Alternatively Spliced Forms of \( \alpha_1A \) Subunits—In our immunoblotting, immunoprecipitation, and phosphorylation experiments, affinity-purified anti-CNA3 antibodies identified at least two distinct \( \alpha_1A \) polypeptides: a minor doublet of polypeptides with an apparent molecular mass of approximately 220 kDa and a major polypeptide with an apparent molecular mass of 190 kDa. These polypeptides were specifically recognized by anti-CNA3 antibody, since the CNA3 peptide blocked binding of anti-CNA3 antibody to these immunoreactive polypeptides. Multiple size forms of calcium channel \( \alpha_1 \) subunits were first described for the skeletal muscle calcium channel (De Jongh et al., 1989, 1991), and found for neuronal L-type (Snutch et al., 1991; Hui et al., 1991; Williams et al., 1992b; Hell et al., 1993a) and non-L-type calcium channels (Mori et al., 1991; Starr et al., 1991; Westenbroek et al., 1992; Coppola et al., 1994; Leveque et al., 1994; Williams et al., 1994). In skeletal muscle, the two size forms of \( \alpha_1 \) subunits may arise from post-translational processing because only a single mRNA has been characterized. In contrast, sequencing of cDNA clones encoding the neuronal calcium channels has revealed multiple isoforms in each case which vary in the cytoplasmic loops and COOH-terminal regions (Mori et al., 1991; Soong et al., 1994; Coppola et al., 1994; Williams et al., 1992a, 1992b, 1994; Snutch et al., 1991; Hui et al., 1991; Niidome et al., 1992; Soong et al., 1993). For \( \alpha_1A \) subunits in rat central nervous system, four distinct transcripts were identified by Northern blot analysis (Starr et al., 1991), and alternative RNA splicing of a single rat class A gene has been shown to generate isoforms (rbA-I and rbA-II) that have similar molecular size (approximately 250 kDa; Soong et al., 1994). No alternatively spliced mRNAs encoding \( \alpha_1A \) subunits of substantially different size have been characterized previously in rat brain. However, our immunochromatographic experiments demonstrate that class A calcium channels are composed of multiple isoforms of \( \alpha_1A \) with different molecular size, and that these multiple isoforms of \( \alpha_1A \) may be produced by alternative RNA splicing rather than

**Fig. 7. Phosphorylation of the class A calcium channel \( \alpha_1A \) subunits by PKA, PKC, and PKG.** Class A calcium channel \( \alpha_1A \) subunits were purified from WGA glycoprotein fractions by immunoprecipitation with anti-CNA3 (lanes 1, 3, and 5), or with rabbit control antibodies (lanes 2, 4, and 6). Immunoprecipitated \( \alpha_1A \) was phosphorylated with PKA (lanes 1 and 2), PKC (lanes 3 and 4), or PKG (lanes 5 and 6) and reprecipitated with anti-CP (1382-1400) antibodies as described under “Experimental Procedures.” Molecular mass markers are described in Fig. 3.

220-kDa polypeptide much more extensively than the 190-kDa polypeptide. A plausible explanation for this result is that the 220-kDa form contains PKA phosphorylation sites that are not present in the 190-kDa form. PKA phosphorylated these two \( \alpha_1A \) forms similarly when disulfide bonds were not reduced, when disulfide bonds were reduced with 20 mM dithiothreitol in SDS-sample buffer, or when samples were solubilized with 2% CHAPS or 0.1% digitonin (data not shown).

The 190-kDa form of \( \alpha_1A \) was a substrate for phosphorylation by PKC and PKG (Fig. 7, lanes 3 and 5). Control rabbit IgG was ineffectiv in precipitating the 190-kDa polypeptides phosphorylated by these enzymes confirming the identification of \( \alpha_1A \) (Fig. 7, lanes 4 and 6). The 220-kDa form of \( \alpha_1A \) could not be visualized as a phosphorylated polypeptide by PKC or PKG. However, we cannot exclude the possibility that the 220-kDa form of \( \alpha_1A \) is also a substrate for these kinases, since 220-kDa polypeptide could be present in insufficient quantity for detection of phosphorylation by these enzymes. Nevertheless, the results show that PKA preferentially phosphorylates the 220-kDa form of \( \alpha_1A \) whereas PKC and PKG preferentially phosphorylate the 190-kDa form.
by post-translational proteolytic processing. These results suggest that additional uncharacterized mRNAs encoding different size forms of $\alpha_{1A}$ must be present in rat central nervous system.

The $B1 \ alpha$ subunit cDNA clones from rabbit brain (B1-1 and B1-2) encode Q-type calcium channels when expressed in Xenopus oocytes (Mori et al., 1991; Sather et al., 1993). Analysis of B1 clones revealed multiple isoforms differing by insertion/deletion of 349 amino acids in the loop between domains II and III (residues 772-1,120) and 195 amino acids in COOH-terminal region and by alternative expression of a 28-amino acid substitution in COOH-terminal region (Mori et al., 1991). These differences, which apparently result from alternative RNA splicing, can give rise to at least eight distinct mRNAs encoding multiple size forms of $\alpha_{1A}$, in rabbit brain. The differences in size caused by either of these large deletions would be sufficient to reduce the apparent size of the $\alpha_{1A}$ subunit from 220 to 190 kDa. These findings suggest the possibility that $\alpha_{1A}$ subunits in both rat and rabbit contain multiple splicing cassettes in the loop between domains II and III and in the COOH-terminal region and that the two size forms that we have observed in our biochemical experiments are derived from these alternative splicing events. Because the known cDNAs could encode multiple $\alpha_{1}$ subunits with a size of approximately 190 kDa, it is possible that multiple $\alpha_{1}$ isoforms are contained within the protein bands present in this region of the gel. In addition, because the immunostaining with anti-B1-Ct was weaker than with anti-B1-Nt, it is possible that this band also contains $\alpha_{1A}$ subunits that have been truncated at the COOH terminus by proteolytic processing.

In immunoblotting experiments, affinity-purified anti-CNA3 and anti-B1-Nt antibodies specifically identified an additional immunoreactive band of $\alpha_{1A}$ with an apparent molecular mass of 160 kDa (Figs. 3 and 5). The biochemical properties of the 160-kDa form of $\alpha_{1A}$ polypeptide could not be extensively characterized, since this form was not consistently detected in double immunoprecipitation experiments. However, our results show that the 160-kDa polypeptide contains both the CNA3 sequence and the B1-Nt sequence. It may be an additional spliced isoform of $\alpha_{1A}$ or a proteolytic product of the longer forms of $\alpha_{1A}$ polypeptide, which has a cleaved COOH terminus.

Possible Physiological Significance of Differential Phosphorylation of Class A Calcium Channels—Since P-type calcium currents were first described in the cerebellar Purkinje neurons and the presynaptic terminal of the squid giant synapse (Llinas et al., 1989), intensive studies of P-type and/or Q-type channels have demonstrated their broad distribution in the central and peripheral nervous systems and in the endocrine system (Regan et al., 1991a, 1991b; Hillman et al., 1991; Mintz et al., 1992a, 1992b; Uchitel et al., 1992; Usohizc et al., 1992; Swartz et al., 1993; Regher and Mintz, 1994; Wheeler et al., 1994; Castillo et al., 1994; Artalejo et al., 1994; Stea et al., 1994a, Brown et al., 1994; Westenbroek et al., 1995). On the basis of electrophysiological findings, P-type and/or Q-type calcium channels are involved in excitatory and inhibitory synaptic transmission at central synapses (Takahashi and Moriyama, 1993, Luebek et al., 1993, Mintz and Bean, 1993; Castillo et al., 1994, Wu and Saggau, 1994, Wheeler et al., 1994, Regher and Mintz, 1994) and at the mammalian neuromuscular junction (Uchitel et al., 1992; Bowersox et al., 1995). $\omega$-Aga-toxin IVA-sensitive and $\omega$-conotoxin GVIÐ-resistant calcium channels regulate glutamate release in rat brain synaptosomes (Turner et al., 1992) or in hippocampal slices (Gaur et al., 1994) after potassium-induced depolarization. Immunocytochemical experiments using anti-peptide antibodies clearly demonstrate the subcellular localization of class A calcium channels in the presynaptic terminals of many central neurons (Westenbroek et al., 1995) and in presynaptic terminals at the neuromuscular junction (Oslely and Froehner, 1994; Sugira et al., 1995). These findings indicate that at least one isoform of P-type and/or Q-type calcium channels is localized in presynaptic terminals, controls the neurotransmitter release at central synapses, and may contribute to synaptic plasticity (Wheeler et al., 1994). However, most P-type and/or Q-type calcium currents identified in the electrophysiological experiments are recorded in the somata or dendrites of neurons such as Purkinje neurons, cerebellar granule cells, neocortical pyramidal cells, and dorsal root ganglia (Llinas et al., 1989; Usohizc et al., 1992; Regan et al., 1991a, 1991b; Mintz et al., 1992a, 1992b; Randall and Tsien, 1995), and $\alpha_{1A}$ subunits are also observed in these locations by immunocytochemistry (Westenbroek et al., 1995). These results indicate that class A calcium channels are also localized in the postsynaptic membrane. Since class A calcium channels are present in different subcellular locations and participate in different physiological events, the distinct isoforms we have observed in these experiments may be specialized for localization in specific subcellular compartments and for function in different neuronal processes.

P-type and/or Q-type calcium channels are modulated by GTP-binding proteins (G protein) and protein phosphorylation. P-type channels in Purkinje neurons and spinal cord interneurons are inhibited by $\gamma$-aminobutyric acid (GABA) through GABAB receptor activation and this inhibition is mediated through G proteins (Mintz and Bean, 1993). On the other hand, in hippocampal CA3 pyramidal neurons, P-type calcium channels are potentiated by adenosine through $A_2$ receptor activation (Mogul et al., 1993). This potentiation involves a PKA-dependent process (Mogul et al., 1993). Phosphorylation by second messenger-activated protein kinases is a well-known pathway for functional modulation of neuronal calcium channels. Injection of cerebellar mRNA into Xenopus oocytes leads to the expression of a single type of voltage-gated calcium channels similar to P-type channels, and this calcium current is enhanced by activators of PKA and PKC (Fournier et al., 1993a, 1993b). In contrast, $\lambda_{2A}$ of $\alpha_{1A}$ channels coexpressed with $\beta$ subunit in Xenopus oocyte is not affected by the activation of PKC (Stea et al., 1994b). Whereas the functional effects of phosphorylation of P-type and Q-type calcium channel are still incompletely described, our results provide the first evidence that class A calcium channel $\alpha_{1A}$ subunits are substrates for phosphorylation by PKA, PKC, and PKG, and indicate that the different $\alpha_{1A}$ subunit size forms may be differentially phosphorylated and differentially regulated. Further work is required to determine whether different isoforms of the class A calcium channels are differentially regulated by PKA, PKC, and PKG in vivo and to evaluate the physiological effect of phosphorylation on $\alpha_{1A}$ channel function.

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