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Immunochemical Identification and Differential Phosphorylation of Alternatively Spliced Forms of the α_{1A} Subunit of Brain Calcium Channels*

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Biochemical properties of the α_1 subunits of class A brain calcium channels (α_{1A}) were examined in adult rat brain membrane fractions using a site-directed anti-peptide antibody (anti-CNA3) specific for α_{1A}. Anti-CNA3 specifically immunoprecipitated high affinity receptor sites for ω-conotoxin MVIIC (K_d ~100 pM), but not receptor sites for the dihydropyridine isradipine or for ω-conotoxin GVIA. In immunoblotting and immunoprecipitation experiments, anti-CNA3 recognized at least two distinct immunoreactive α_{1A} 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 α_{1A} polypeptides were also specifically recognized by both anti-BI-Nt and anti-BI-1-Ct antibodies, which are directed against the NH_2- and COOH-terminal ends of α_{1A} predicted from cDNA sequence, respectively. These data indicate that the predicted NH_2 and COOH termini are present in both size forms and therefore that these isoforms of α_{1A} are created by alternative RNA splicing rather than post-translational proteolytic processing of the NH_2 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 α_{1A} 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 *et al.*, 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 *et al.*, 1989; Zhang *et al.*, 1993). Voltage-gated calcium channels are a complex of five subunits: α_1, α_2, β, γ, and δ (Takahashi *et al.*, 1987; Catterall *et al.*, 1988; Campbell *et al.*, 1988). α_1 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 α_1 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 α_1 subunits of brain calcium channels have been identified and designated A, B, C, D, and E (α_{1A}–α_{1E})[1] (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 α_1 subunits (α_{1C} and α_{1D}), which have a high affinity for dihydropyridines and conduct long lasting Ba^{2+} currents. In contrast, the class A, B, and E genes encode α_1 subunits (α_{1A}, α_{1B}, and α_{1E}) 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). α_{1B} 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). α_{1E} 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). α_{1A} 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). α_{1A} currents expressed in *Xenopus* oocytes are insensitive to dihydropyridines and ω-conotoxin GVIA, and therefore α_{1A} subunits may form P-type and/or Q-type channels (Mori *et al.*, 1991; Sather *et al.*, 1993; Stea *et al.*, 1994a). α_{1A} 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 MVIIC from *Conus ma-*

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[1] The abbreviations and trivial names used are: α_{1A}, α_{1B}, α_{1C}, α_{1D}, and α_{1E}, α_1 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-benzoxadialzol-4-yl)-1.4-dihydro-2.6-dimethyl-5-methoxycarbonyl) pyridine-3-carboxylate; PAGE, polyacrylamide gel electrophoresis; NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamide) hexanoate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TBS, Tris-buffered saline; GABA, γ-aminobutyric acid.

gus (Hillyard *et al.*, 1992; Sather *et al.*, 1993; Stea *et al.*, 1994a). In contrast, native P-type calcium channels are blocked by low concentrations of ω-agatoxin IVA and by higher concentrations of ω-conotoxin MVIIC (Mintz *et al.*, 1992a, 1992b, Hillyard *et al.*, 1992). The pharmacological properties of α_{1A} calcium channels 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 anti-peptide antibodies against unique sequences in rat brain α_{1A} to identify the corresponding polypeptides and demonstrated that there are multiple isoforms of α_{1A} subunits that may result from alternative RNA splicing and are differentially phosphorylated by second messenger-activated protein kinases.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Isradipine (PN200-110; 80 Ci/mmol), ^{125}I-ω-conotoxin GVIA (2200 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from DuPont NEN. ω-Agatoxin IVA was a generous gift from Drs. N. Saccomano and M. Ahlijanian, Pfizer. The ECL detection kit for immunoblotting was obtained from Amersham Corp., digitonin 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; Woodgett 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 peptide CNA3 ((KY)SEPQQREHAPPREHV) corresponds to residues 882- 896 (Starr *et al.*, 1991) and the peptide CNA1 ((KY)PSSPERAPGREGPYGRE) corresponds to residues 865–881, which are located in a highly variable site in the intracellular loop between domains II and III of the α_{1A} subunit of rat brain calcium channels. The NH_2-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 amino groups with glutaraldehyde to bovine serum albumin (BSA), dialyzed against phosphate-buffered saline (10 mM NaH_2PO_4 (pH 7.4), 150 mM NaCl) and emulsified in an equal volume of Freund's complete (initial injection) or incomplete 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 (Posnett *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 3.0 M $MgCl_2$. The affinity-purified antibodies were then dialyzed against TBS using a Centriprep 30 (Amicon).

Anti-BI-Nt and anti-BI-1-Ct antibodies were generous gifts from Dr. Masami Takahashi (Mitsubishi-Kasei Life Sciences Institute, Tokyo, Japan), and these antibodies were produced against peptides MARFGDEMPARYGGGGAGAA(C) (Leveque *et al.*, 1994) and (C)RDQRWSRSPSEGREHTTHRQ, which correspond to residues 1–23 and 2254–2273 of the BI-1 cDNA clone encoding a rabbit brain α_{1A} subunit, respectively (Mori *et al.*, 1991). The cysteine residue in each peptide was added to facilitate cross-linking and radiolabeling and is not part of the α_1 subunit sequence.

Radioiodination of ω-Conotoxin MVIIC—The radioiodinated derivative of ω-conotoxin MVIIC was prepared and purified as described before (Kristipati *et al.*, 1994; Woppmann *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 (Westenbroek *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 (Westenbroek *et al.*, 1992).

Radioactive Ligand Binding Studies—For [^{3}H]PN200-110 (isradipine) binding studies, 40 ml of S1 fraction were labeled with 10 μCi of [^{3}H]PN200-110 (85.8 Ci/mmol) at a concentration of 2.9 nM for 1 h on ice. The bound radioligand is stable throughout the subsequent purification steps. Calcium channels were purified from 250 μl of the S3 fraction (~6000 cpm) containing 300 mM 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 [^{3}H]PN200-110 receptors was quantified in a scintillation counter. Total receptor-bound [^{3}H]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% polyethylene glycol (average molecular weight 8000) in 10 mM $MgCl_2$ and 10 mM 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 (Westenbroek *et al.*, 1992).

Determination of ^{125}I-ω-conotoxin GVIA binding was done by incubation of 100 μl of S3 fraction containing 0.2% BSA with 0.06 μCi of ^{125}I-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 γ counting. Total ^{125}I-ω-conotoxin GVIA binding was determined using 100 μl of the labeled S3 fraction in the filter-binding assay described above for [^{3}H]PN200-110.

^{125}I-ω-Conotoxin MVIIC binding was determined by incubation of 400 μl of samples containing 140 μl of WGA extract, 10 mM Tris-HCl (pH 7.4), and 0.2% BSA with 0.1 μCi of labeled toxin (1300 Ci/mmol) at a concentration of 0.15 nM on ice for 30 min. This was added to 15 μg of affinity-purified anti-CNA3, anti-CNC1, or control rabbit IgG, coupled to 2 mg of PAS, and incubated for 4 h on ice in a tilting mixer. Samples were washed quickly three times in 10 mM Tris-HCl (pH 7.4), 75 mM NaCl, 0.1% digitonin, 0.2% BSA. ^{125}I-ω-Conotoxin MVIIC binding in the pellet was counted in a γ counter. For the competitive displacement studies, the unlabeled ligands ω-conotoxin MVIIC or ω-agatoxin IVA at concentrations ranging from 10^{-14} to 10^{-5} M were added to samples with ^{125}I-ω-conotoxin MVIIC and incubated with affinity-purified anti-CNA3, and the bound ^{125}I-ω-conotoxin MVIIC was measured as described above. For peptide block, 20 μM test peptide was added to the affinity-purified antibodies and incubated overnight on ice prior to incubation with samples containing WGA extract.

Immunoblotting of Calcium Channels—To concentrate the calcium channels, WGA column fractions containing 0.1 mg of total protein were incubated for 4 h on ice with 40 μl of heparin-agarose (Sakamoto and Campbell, 1991). The resin was washed three times with 10 mM Tris-HCl (pH 7.4), 0.1% digitonin and once with 10 mM Tris-HCl (pH 7.4). Calcium channels were extracted for 30 min at 50–60 °C with 30 μl of SDS-sample buffer (200 mM Tris-HCl (pH 6.8), 10 mM dithiothreitol, 4 M urea, 8% SDS, 10% glycerol). After separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto a nitrocellulose membrane (0.2 μm) in a buffer containing 12.5 mM Tris (pH 8.3), 96 mM glycine, 0.1% SDS, 15% (v/v) methanol. Unbound sites on the nitrocellulose were blocked for 2 h at room temperature with TBS containing 10% skim milk powder and incubated with affinity-purified anti-CNA3 (1–10 μg/ml), and with protein A-purified anti-BI-Nt, or anti-BI-1-Ct antibodies (50–250 μg/ml) in TBS for 2 h at room temperature. After five 5-min washes at 4 °C with TBS, blots were incubated for 1 h with horseradish peroxidase-protein A, diluted 1:2000 in TBS. After another eight 10-min washes at 4 °C, the blots were developed with the ECL reagent. For peptide block, the corresponding peptide at 0.2–2 μM was added to the affinity-purified antibodies and incubated overnight on ice prior to incubation with the samples.

Biotinylation and Purification by Immunoprecipitation—WGA frac-

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-(biotinamide) hexanoate (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 2 M glycine (pH 8.5). Samples were diluted in 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 mg 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 proteins in the sample. After centrifugation, supernatants were incubated for another 2 h on ice with 10 mg 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-Nt (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 μM pepstatin A, 2 μg/ml leupeptin, and 4 μg/ml aprotinin, 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-CP(1382–1400) (20 μg). Three mg of PAS, pretreated 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 (8–9 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 phosphorylation buffer (50 mM Hepes (adjusted to pH 7.4 with NaOH), 10 mM $MgCl_2$, 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 phosphorylation buffer, along with 1 mM dithiothreitol, 1 μM pepstatin A, 1 mM EGTA, and 0.2 μM [γ-^{32}P]ATP. This buffer was supplemented with 1.5 mM $CaCl_2$, 50 μg of diolein, and 2.5 mg of phosphatidylserine for PKC and 2 μM cGMP 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 radioimmunoassay buffer (25 mM Tris-HCl (pH 7.4), 20 mM EDTA, 75 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerol phosphate, 50 mM NaF, and 1 mM *p*-nitrophenyl phosphate), three times with 1% Triton X-100 in radioimmunoassay 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) as described in the previous section. Samples were analyzed by SDS-PAGE, and autoradiography was performed.

RESULTS

Immunoprecipitation of High Affinity Receptor Sites for Dihydropyridines, ω-Conotoxin GVIA, or ω-Conotoxin MVIIC—The amino acid sequences of the large intracellular loops connecting domains II and III of the neuronal calcium channel α_1 subunits are highly variable and characteristic for each class of channels. For production of specific antibodies against α_{1A}, a unique sequence in this intracellular loop was selected, and the corresponding peptide (CNA3) was synthesized, coupled to BSA as a carrier, and used as an antigen for antigen production as described under "Experimental Procedures." To determine the pharmacological properties of the α_{1A} polypeptides recognized by anti-CNA3, we labeled brain calcium channels with [^{3}H]PN200-110, ^{125}I-ω-conotoxin GVIA, or ^{125}I-ω-conotoxin MVIIC, and immunoprecipitated labeled brain calcium channels with anti-CNA3 antibodies.

[^{3}H]PN200-110 is a dihydropyridine that specifically binds to L-type calcium channels containing α_{1C} and α_{1D} (Hell *et al.*, 1993a). At a concentration of [^{3}H]PN200-110 chosen to saturate all binding sites in a rat brain homogenate, affinity-purified anti-CNC1, an α_{1C}-specific site-directed anti-peptide antibody, immunoprecipitated 40% of the total [^{3}H]PN200-110 receptors, whereas anti-CNA3 immunoprecipitated less than 1% of the total [^{3}H]PN200-110 receptors, a value similar to that obtained with control rabbit IgG (Fig. 1*A*). The binding of ^{125}I-ω-conotoxin GVIA, a selective blocker of the N-type calcium channels containing α_{1B}, was tested similarly. Affinity-purified anti-CNB2, an α_{1B}-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 ^{125}I-ω-conotoxin GVIA receptors (Fig. 1*B*). ω-Conotoxin MVIIC blocks Q-type calcium channels containing α_{1A} (Sather *et al.*, 1993; Stea *et al.*, 1994a; Hillyard *et al.*, 1992). A saturating concentration of ^{125}I-ω-conotoxin MVIIC was added to the solubilized and partially purified calcium channel preparation. Affinity-purified anti-CNA3 antibodies effectively immunoprecipitated ^{125}I-ω-conotoxin MVIIC receptors (Fig. 1*C*). Anti-CNA3 specifically recognized the ω-conotoxin MVIIC receptors, since preincubation of 20 μM CNA3 peptide largely blocked immunoprecipitation of them, whereas the CNC1 peptide at the same concentration did not affect the immunoprecipitation of ^{125}I-ω-conotoxin MVIIC receptors with anti-CNA3. In contrast, anti-CNC1 and control rabbit IgG immunoprecipitated only small amounts of ^{125}I-ω-conotoxin MVIIC. ω-Conotoxin MVIIC has a significant affinity for ω-conotoxin GVIA binding sites on α_{1B} (Hillyard *et al.*, 1992). However, anti-CNB2 did not immunoprecipitate detectable ^{125}I-ω-conotoxin MVIIC-labeled α_{1B} when 0.15 nM ^{125}I-ω-conotoxin MVIIC was used in this experiment.

Displacement of specific binding of ^{125}I-ω-conotoxin MVIIC by unlabeled ω-conotoxin MVIIC was observed between 10 pM and 1 nM, with half-maximal inhibition at approximately 100 pM (Fig. 2). The K_d 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; Woppmann *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 MVIIC, blocks P-type calcium currents with IC_{50} of 1–2 nM (Mintz *et al.*, 1992a) and α_{1A} calcium currents at 100–300 nM (Sather *et al.*, 1993, Stea *et al.*, 1994a). To assess whether ω-conotoxin MVIIC 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 MVIIC was tested. Displacement of specific binding of ^{125}I-ω-conotoxin MVIIC to the ω-conotoxin MVIIC 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 MVIIC from its high affinity binding site nonspecifically at high concentration, but not at concentrations at which it inhibits calcium channels containing α_{1A}. Evidently, ω-agatoxin IVA does not bind to the same receptor site as ω-conotoxin MVIIC in inhibiting class A calcium channels.

Identification of α_{1A} in Rat Brain Membranes—To identify α_{1A} 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

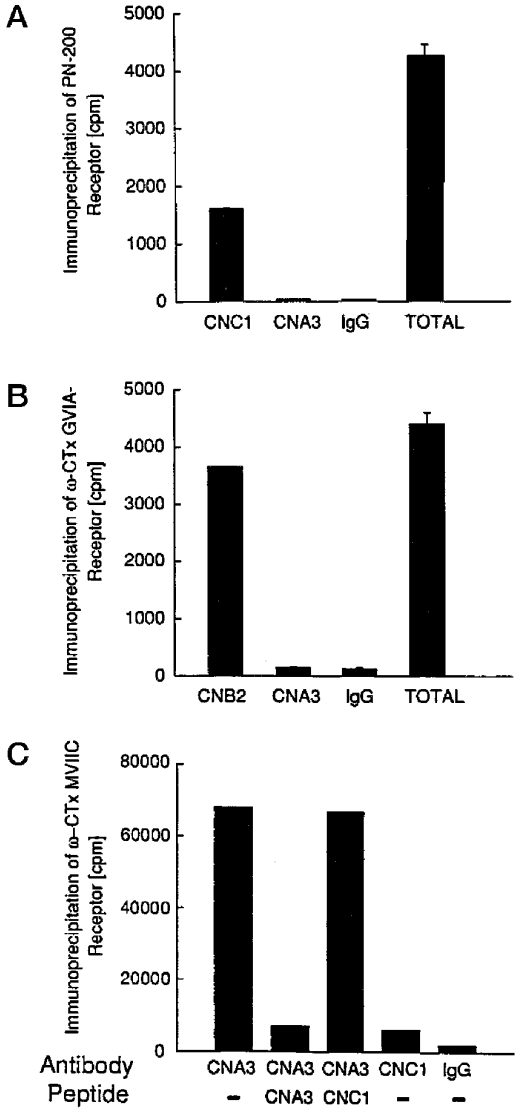

FIG. 1. **Immunoprecipitation of brain calcium channels labeled with [^{3}H]PN200-110, ^{125}I-ω-conotoxin GVIA, or ^{125}I-ω-conotoxin MVIIC.** *A*, rat brain membrane fraction (S1) was incubated with [^{3}H]PN200-110 at the concentration of 2.9 nM, solubilized, immunoprecipitated with anti-CNC1, anti-CNA3, or rabbit control antibodies, and the bound [^{3}H]PN200-110 was counted (see "Experimental Procedures"). Anti-CNC1 was raised against a highly variable site of the class C L-type calcium channel α_1 subunit (Hell *et al.*, 1993a). Total [^{3}H]PN200-110 receptor sites were estimated by filter binding assay. *B*, S3 fractions were incubated with ^{125}I-ω-conotoxin GVIA (0.27 nM) and immunoprecipitated with anti-CNB2, anti-CNA3, or control IgG. Anti-CNB2 is specific for class B N-type calcium channel α_1 subunit (Hell *et al.*, 1994). *C*, calcium channels were purified from the S3 fraction by chromatography on WGA-Sepharose, incubated with ^{125}I-ω-conotoxin MVIIC (0.15 nM), and immunoprecipitated with anti-CNA3, anti-CNC1, or control IgG. The specificity of immunoprecipitation of ^{125}I-ω-conotoxin MVIIC receptors with anti-CNA3 was tested by peptide block. Anti-CNA3 was preincubated with 20 μM CNA3 peptide or with 20 μM CNC1 peptide, and immunoprecipitation was carried out as described under "Experimental Procedures."

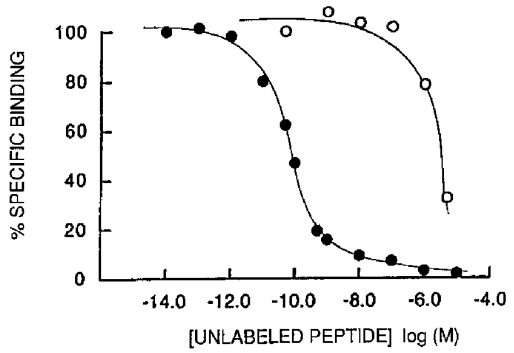

FIG. 2. **Competitive inhibition of ^{125}I-ω-conotoxin MVIIC binding by unlabeled ω-conotoxin MVIIC or ω-agatoxin IVA.** The indicated concentrations of ω-contoxin MVIIC (*closed circles*) or ω-agatoxin IVA (*open circles*) were added to WGA samples with ^{125}I-ω-conotoxin MVIIC (0.15 nM) and immunoprecipitated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

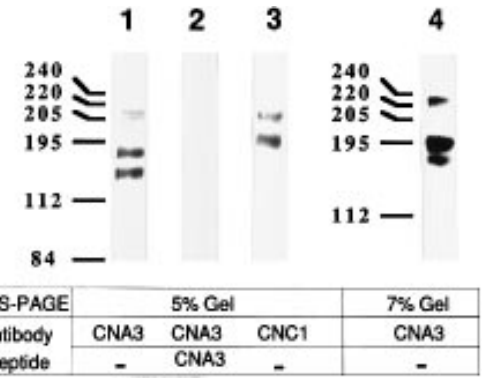

FIG. 3. **Detection of α_{1A} with affinity-purified anti-CNA3 antibodies by immunoblotting.** Membrane glycoprotein fractions were isolated from solubilized brain membranes by WGA affinity chromatography, and calcium channels were concentrated by adsorption to heparin agarose, extracted, and analyzed 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 μM CNA3 peptide (*lane 2*). The migration positions of α- and β-spectrin, myosin heavy chain, α_2-macroglobulin, β-galactosidase, and fructose-6-phosphate kinase are indicated at the left side of the gel together with their molecular masses in kDa.

immunoreactive bands of α_{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 α_{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 α_{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 α_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 μM, no signal could be detected with anti-CNA3 antibody (Fig. 3, *lane 2*). These specific bands were only observed with affinity-purified anti-CNA3 antibodies; affinity-purified anti-CNC1 antibodies against α_{1C} revealed distinct bands with apparent

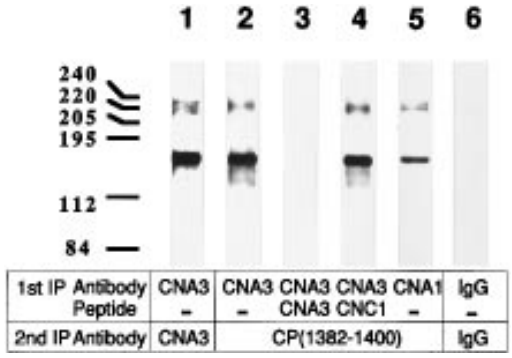

FIG. 4. **Detection of α_{1A} with anti-CNA3 antibodies by double immunoprecipitation.** WGA glycoprotein fractions were biotinylated with NHS-LC-biotin, and α_{1A}polypeptides were purified by double immunoprecipitation. Samples were extracted with SDS-sample buffer, separated by SDS-PAGE, blotted, and incubated with streptavidin-biotinylated horseradish peroxidase complex as described under "Experimental Procedures." Calcium channels were purified by double immunoprecipitation with affinity-purified anti-CNA3 (*lane 1*), with anti-CNA3 and anti-CP(1382–1400) (*lanes 2–4*), with anti-CNA1 and anti-CP(1382–1400) (*lane 5*), or with rabbit control IgG (*lane 6*), and visualized by ECL. Anti-CNA3 antibodies were preincubated overnight on ice with 50 μM CNA3 peptide (*lane 3*) or 50 μM CNC1 peptide as a control of the specificity of the peptide block (*lane 4*). Molecular marker proteins are indicated as in Fig. 3.

molecular mass values of 210 and 190 kDa in the parallel gel (Fig. 3, *lane 3*), and antibodies directed against the other brain calcium channels also recognized distinct polypeptides (data not shown). Thus, the 220-, 190-, and 160-kDa polypeptides are distinct size forms of α_{1A} subunits. From these results, we cannot exclude that one or more of the multiple size forms of α_{1A} is created by *in vitro* proteolysis. However, it seems unlikely because inclusion of high concentrations of several protease inhibitors and careful handling of all instruments including rotors to keep them at 0 °C did not alter the appearance of multiple forms of α_{1A}. In addition, results presented below indicate that the 220- and 190-kDa forms contain the NH_2- and COOH-terminal sequences predicted from cDNA sequence, indicating they have not been proteolytically cleaved. The doublet band with an apparent molecular mass of 220 kDa appears smaller than the deduced molecular mass of α_{1A} from cDNA sequence (250 kDa; Starr *et al.*, 1991), but it may represent the full-length form of α_{1A} because α_1 subunits migrate anomalously in SDS-PAGE (De Jongh *et al.*, 1991). For example, an apparently full-length form of α_{1C} (predicted molecular mass of 250 kDa; Snutch *et al.*, 1991) migrated to a similar position in the same gel (Fig. 3, *lanes 1* and *3*) (Hell *et al.*, 1993a).

We also examined the α_{1A} polypeptides in immunoprecipitation experiments (Fig. 4). To isolate and detect α_{1A} in immunoprecipitation, we biotinylated the WGA-purified glycoprotein fraction, isolated α_{1A} by double immunoprecipitation with affinity-purified anti-CNA3 antibodies, and visualized α_{1A} by the streptavidin-biotin detection method (see "Experimental Procedures"). When samples were analyzed in 5% acrylamide SDS-PAGE gels, anti-CNA3 antibody revealed at least two distinct immunoreactive polypeptides, which correspond to 220- and 190-kDa forms detected in immunoblotting (Fig. 4, *lane 1*). The 190-kDa polypeptide was the major form, whereas 220-kDa polypeptide was a minor form and was often visualized as a doublet band as in immunoblotting. Occasionally, an additional band with a molecular mass of 160 kDa was weakly stained in immunoprecipitation experiments. The 160-kDa polypeptide may not be sufficient in quantity to be detected consistently in double immunoprecipitation.

The immunoreactive polypeptides of 220 and 190 kDa were specifically recognized by affinity-purified anti-CNA3 antibodies in immunoprecipitation, since preincubation with CNA3 peptide at 50 μM eliminated the immunoprecipitation of α_{1A} polypeptides with anti-CNA3, but the CNC1 peptide at the same concentration had no effect (Fig. 4, *lanes 3* and *4*). In addition, neither band was recognized when immunoprecipitated with nonspecific antibodies (Fig. 4, *lane 6*). These specific bands of α_{1A} polypeptide were only observed with affinity-purified anti-CNA3 antibodies, whereas affinity-purified antibodies against α_{1B} revealed distinct bands with apparent molecular masses of 230 and 210 kDa in parallel experiments (data not shown).

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 α_1 subunit rather than the α_1 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 α_1 subunit whose sequence is conserved in all calcium channel α_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 α_2 and δ subunits (Ahlijanian *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 α_{1A} subunits. Two forms of α_{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 α_{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 α_{1A} as detected with anti-CNA3 antibody (Fig. 4, *lane 5*). These observations in immunoblotting and immunoprecipitation experiments demonstrate that α_{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 α_{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 α_{1A} Polypeptides—α_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 α_{1A}, we used antibodies that recognize the NH_2-terminal (anti-BI-Nt) or COOH-terminal (anti-BI-1-Ct) of α_{1A} subunits deduced from cDNA sequence. Two distinct cDNAs encoding α_{1A} subunits have been cloned: rbA from rat brain and BI (isoforms BI-1 and BI-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 BI clone (Mori *et al.*, 1991), which is conserved in all known isoforms of α_{1A} subunits. Two distinct BI cDNA clones, designated BI-1 and BI-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 BI-1 clone (Mori *et al.*,

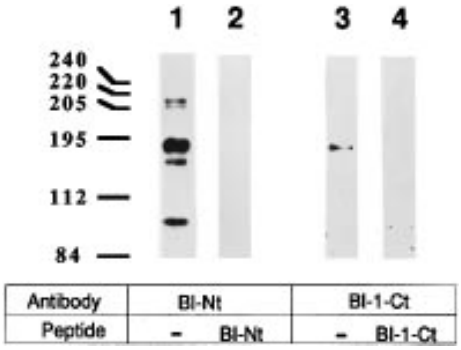

FIG. 5. **Detection of α_{1A} with anti-BI-Nt and anti-BI-1-Ct antibodies by immunoblotting.** Membrane glycoprotein fractions were isolated from solubilized brain membranes by WGA affinity chromatography, and calcium channels were concentrated by adsorption to heparin agarose, extracted, and analyzed by SDS-PAGE. Immunoblotting was performed with anti-BI-Nt (*lanes 1* and *2*) or with anti-BI-1-Ct antibodies (*lanes 3* and *4*) as described before (Fig. 3). To test for nonspecific labeling, anti-BI-Nt antibody was preincubated overnight on ice with 0.2 μM BI-Nt peptide (*lane 2*), and anti-BI-1-Ct was preincubated with 2 μM BI-1-Ct peptide (*lane 4*). Molecular mass markers are indicated in Fig. 3.

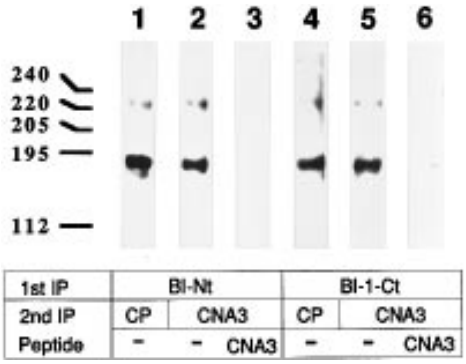

FIG. 6. **Recognition of α_{1A} polypeptide in double immunoprecipitation by anti-CNA3, anti-BI-Nt, and anti-BI-1-Ct antibodies.** WGA glycoprotein fractions were biotinylated with NHS-LC-biotin and purified by double immunoprecipitation with anti-CNA3 antibodies. Samples were extracted with SDS-sample buffer, separated by SDS-PAGE, blotted, and incubated with streptavidin-biotinylated horseradish peroxidase complex as described under "Experimental Procedures." Double immunoprecipitations were performed with anti-BI-Nt and anti-CP(1382–1400) (*lane 1*), with anti-BI-Nt and anti-CNA3 (*lanes 2* and *3*), with anti-BI-1-Ct and anti-CP(1382–1400) (*lane 4*), or with anti-BI-1-Ct and anti-CNA3 antibodies (*lanes 5* and *6*). Anti-CNA3 antibodies were preincubated overnight on ice with 50 μM CNA3 peptide (*lanes 3* and *6*) in the second immunoprecipitation. Note that the blot was overexposed to demonstrate the immunoreactive band at 220 kDa (*lane 4*). Molecular markers are given in Fig. 3.

1991), which is conserved in the rbA clone (Starr *et al.*, 1991). We examined the immunoreactive α_{1A} polypeptides for the presence of the predicted NH_2- 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 α_{1A} polypeptide contains both the predicted NH_2- and COOH-terminal ends of the α_{1A} 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 α_{1B} since the BI-Nt sequence is 54% identical to the corresponding α_{1B} 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 α_{1A} polypeptide with equivalent molecular weights (Fig. 6, *lane 4*). In double immunoprecipitation with anti-BI-Nt and anti-CNA3 antibodies, α_{1A} 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 α_{1A} polypeptides (Fig. 6, *lanes 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 α_{1A} polypeptides with molecular mass values of 220 and 190 kDa, and that 220- and 190-kDa forms of α_{1A} have both the predicted NH_2- and COOH-terminal ends of α_{1A}. These results indicate that these isoforms of α_{1A} do not result from post-translational proteolytic processing, but may instead be products of alternative RNA splicing.

Phosphorylation of α_{1A} Subunits by Second Messenger-activated Protein Kinases—Calcium channels are regulated by phosphorylation by multiple protein kinases (Tsien *et al.*, 1986; Levitan, 1988; Miller, 1990; Catterall, 1994b). To examine the phosphorylation of α_{1A} subunits by second messenger-activated protein kinases, α_{1A} was purified by immunoprecipitation with affinity-purified anti-CNA3 antibodies. The resulting immune complexes were incubated with different kinases in the presence of [γ-^{32}P]ATP. After washing, the PAS-antibody-channel complexes were dissociated with 1.5% SDS-sample buffer and diluted with 1% Triton, and α_{1A} subunits were re-immunoprecipitated with anti-CP(1382–1400) (see "Experimental Procedures"). Following phosphorylation with PKA, two labeled α_{1A} 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 α_{1A} and the 220-kDa band is minor in quantity. In contrast, PKA phosphorylated the

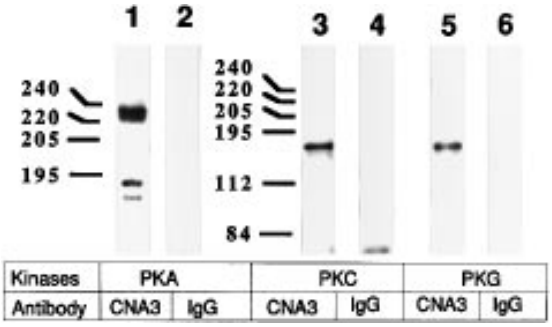

FIG. 7. **Phosphorylation of the class A calcium channel α_1 subunits by PKA, PKC, and PKG.** Class A calcium channel α_1 subunits were purified from WGA glycoprotein fractions by immunoprecipitation with anti-CNA3 (*lanes 1*, *3*, and *5*), or with rabbit control antibodies (*lanes 2*, *4*, and *6*). Immunoprecipitated α_{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 α_{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 α_{1A} was a substrate for phosphorylation by PKC and PKG (Fig. 7, *lanes 3* and *5*). Control rabbit IgG was ineffective in precipitating the 190-kDa polypeptides phosphorylated by these enzymes confirming the identification of α_{1A} (Fig. 7, *lanes 4* and *6*). The 220-kDa form of α_{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 α_{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 α_{1A} whereas PKC and PKG preferentially phosphorylate the 190-kDa form.

DISCUSSION

High Affinity ω-Conotoxin MVIIC Binding Site on α_{1A}—Our pharmacological experiments using radioactive ligands show that anti-CNA3 antibody does not immunoprecipitate either dihydropyridine or ω-conotoxin GVIA binding sites, but specifically immunoprecipitates the high affinity ω-conotoxin MVIIC receptor site (K_d ~100 pM). These results are consistent with the conclusion that α_{1A} forms Q-type calcium channels because ω-conotoxin MVIIC blocks α_{1A} currents expressed in *Xenopus* oocytes and Q-type currents in the cerebellar granule neurons with IC_{50} less than 150 nM (Sather *et al.*, 1993; Stea *et al.*, 1994a; Zhang *et al.*, 1993; Randall and Tsien, 1995). In contrast, native P-type calcium channels are blocked by higher concentrations of ω-conotoxin MVIIC (IC_{50} of 1–10 μM; Hillyard *et al.*, 1992). The K_d in our biochemical experiments is substantially lower than the IC_{50} obtained in electrophysiological experiments with α_{1A} channels ($IC_{50} < 150$ nM). This discrepancy in IC_{50} may result from differences in the ionic strength in the various experimental conditions. Na^+, Ba^{2+}, and Ca^{2+} all inhibit binding of ω-conotoxin MVIIC at physiological concentrations (Kristipati *et al.*, 1994), but are absent from our binding assay solutions.

Our results are closely correlated with covalent cross-linking experiments identifying the polypeptides that bind ω-conotoxin MVIIC (Woppmann *et al.*, 1994). Polypeptides with apparent molecular masses of 220, 170, 150, and 140 kDa were observed. Although one or more of these bands may represent α_2 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 α_{1A}.

α_{1A} subunits have been suggested to be components of both P-type and Q-type calcium channels. α_{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 α_{1A} with various calcium channel β subunits results in modulation of the amplitude, time course, and the voltage-dependent properties of the α_{1A} calcium currents (Mori *et al.*, 1991; Sather *et al.*, 1993; Stea *et al.*, 1994a; Soong *et al.*, 1994; De Waard *et al.*, 1994). α_{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 α_{1A} subunit with a β subunit (rbA-I with $\beta_{2a,}$ or rbA-II with β_{1b}) 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 α_{1A} to ω-conotoxin MVIIC and ω-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 α_{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 α_{1A} Subunits—In our immunoblotting, immunoprecipitation, and phosphorylation experiments, affinity-purified anti-CNA3 antibodies identified at least two distinct α_{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 α_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 α_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 α_{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 α_{1A} subunits of substantially different size have been characterized previously in rat brain. However, our immunochemical experiments demonstrate that class A calcium channels are composed of multiple isoforms of α_{1A} with different molecular size, and that these multiple isoforms of α_{1A} may be produced by alternative RNA splicing rather than

by post-translational proteolytic processing. These results suggest that additional uncharacterized mRNAs encoding different size forms of α_{1A} must be present in rat central nervous system.

The BI α_1 subunit cDNA clones from rabbit brain (BI-1 and BI-2) encode Q-type calcium channels when expressed in *Xenopus* oocytes (Mori *et al.*, 1991; Sather *et al.*, 1993). Analysis of BI 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 α_{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 α_{1A} subunit from 220 to 190 kDa. These findings suggest the possibility that α_{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 α_1 subunits with a size of approximately 190 kDa, it is possible that multiple α_1 isoforms are contained within the protein bands present in this region of the gel. In addition, because the immunostaining with anti-BI-Ct was weaker than with anti-BI-Nt, it is possible that this band also contains α_{1A} subunits that have been truncated at the COOH terminus by proteolytic processing.

In immunoblotting experiments, affinity-purified anti-CNA3 and anti-BI-Nt antibodies specifically identified an additional immunoreactive band of α_{1A} with an apparent molecular mass of 160 kDa (Figs. 3 and 5). The biochemical properties of the 160-kDa form of α_{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 BI-Nt sequence. It may be an additional spliced isoform of α_{1A}, or a proteolytic product of the longer forms of α_{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, 1993; Uchitel *et al.*, 1992; Usowicz *et al.*, 1992; Swartz *et al.*, 1993; Regehr 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 Momiyama, 1993, Luebke *et al.*, 1993, Mintz and Bean, 1993; Castillo *et al.*, 1994, Wu and Saggau, 1994, Wheeler *et al.*, 1994, Regehr and Mintz, 1994) and at the mammalian neuromuscular junction (Uchitel *et al.*, 1992; Bowersox *et al.*, 1995). ω-Agatoxin IVA-sensitive and ω-conotoxin GVIA-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 (Ousley and Froehner, 1994; Sugiura *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; Usowicz *et al.*, 1992; Regan *et al.*, 1991a, 1991b; Mintz *et al.*, 1992a, 1992b; Randall and Tsien, 1995), and α_{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 γ-aminobutyric acid (GABA) through $GABA_B$ 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, I_{Ba} of α_{1A} channels coexpressed with β 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 α_1 subunits are substrates for phosphorylation by PKA, PKC, and PKG, and indicate that the different α_1 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 α_{1A} channel function.

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