Identification of Three Subunits of the High Affinity \(\omega\)-Conotoxin MVIIIC-sensitive \(\text{Ca}^{2+}\) Channel*

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\(\text{Ca}^{2+}\) channels play a central role in neurotransmitter release and muscle contraction. Several voltage-dependent \(\text{Ca}^{2+}\) channels have been identified which are classified by electrophysiological and pharmacological criteria as T-, L-, N-, P-, and Q-type \(\text{Ca}^{2+}\) channels (1, 2). To date, three different types of \(\text{Ca}^{2+}\) channels (N-, P-, and Q-type) are known to mediate synaptic transmission in the nervous system (3, 4). Biochemical characterization of the brain N-type \(\text{Ca}^{2+}\) channel revealed that it is composed of \(\alpha_{1A}\), \(\alpha_{2\beta}\), and \(\beta\) subunits and a unique 95-kDa protein (5, 6). Among the subunits of brain N-type \(\text{Ca}^{2+}\) channels, \(\alpha_{1A}\) is the pore-forming subunit and the receptor for antagonist drugs (7); \(\beta\) is cytoplasmic and regulates the function of \(\alpha_{1}\) (8–10); \(\alpha_{2\beta}\) is heavily glycosylated and modifies the functional properties of the channel complex (11, 12). Molecular cloning has thus far identified six different \(\alpha_{1}\) genes (S, A, B, C, D, and E), four different \(\beta\) genes (1, 2, 3, and 4), and one \(\alpha_{2\beta}\) gene. Several splice isoforms for \(\alpha_{1}\), \(\beta\), and \(\alpha_{2\beta}\) genes have also been identified (13–18).

Although P- and Q-type \(\text{Ca}^{2+}\) channels seem to play an even greater role than N-type channels in neurotransmission, little is known about the structural aspects of these channels (4, 19).

Voltage-dependent \(\text{Ca}^{2+}\) channels play a central role in neurotransmitter release and muscle contraction. Several voltage-dependent \(\text{Ca}^{2+}\) channels have been identified which are classified by electrophysiological and pharmacological criteria as T-, L-, N-, P-, and Q-type \(\text{Ca}^{2+}\) channels (1, 2). To date, three different types of \(\text{Ca}^{2+}\) channels (N-, P-, and Q-type) are known to mediate synaptic transmission in the nervous system (3, 4). Biochemical characterization of the brain N-type \(\text{Ca}^{2+}\) channel revealed that it is composed of \(\alpha_{1A}\), \(\alpha_{2\beta}\), and \(\beta\) subunits and a unique 95-kDa protein (5, 6). Among the subunits of brain N-type \(\text{Ca}^{2+}\) channels, \(\alpha_{1A}\) is the pore-forming subunit and the receptor for antagonist drugs (7); \(\beta\) is cytoplasmic and regulates the function of \(\alpha_{1}\) (8–10); \(\alpha_{2\beta}\) is heavily glycosylated and modifies the functional properties of the channel complex (11, 12). Molecular cloning has thus far identified six different \(\alpha_{1}\) genes (S, A, B, C, D, and E), four different \(\beta\) genes (1, 2, 3, and 4), and one \(\alpha_{2\beta}\) gene. Several splice isoforms for \(\alpha_{1}\), \(\beta\), and \(\alpha_{2\beta}\) genes have also been identified (13–18).

Although P- and Q-type \(\text{Ca}^{2+}\) channels seem to play an even greater role than N-type channels in neurotransmission, little is known about the structural aspects of these channels (4, 19).

Pharmacologically, both channels can be blocked by \(\omega\)-agatoxin IVA and \(\omega\)-conotoxin (CTX)\(^{3}\) MVIIIC (20–22), although both have different sensitivities to \(\omega\)-agatoxin IVA (22). However, it has not been established with certainty whether the P- and Q-type \(\text{Ca}^{2+}\) channels are encoded by the same \(\alpha_{1}\) subunit gene; or whether they represent different \(\alpha_{1}\) gene products (1, 12, 22, 23). Since the activity of \(\alpha_{1A}\) subunits expressed in Xenopus oocytes can be blocked by both \(\omega\)-agatoxin IVA and \(\omega\)-CTX MVIIIC, it has been proposed that this subunit may be the pore-forming subunit of P- and/or Q-type \(\text{Ca}^{2+}\) channels (1, 23). Lack of specific blockers to distinguish P- and Q-type channels has made it difficult to further characterize these channels by either electrophysiological or biochemical approaches.

The major types of \(\text{Ca}^{2+}\) channels controlling neurotransmission (N-, P-, and Q-type) have been shown to be blocked by \(\omega\)-CTX MVIIIC (24, 25). In this study, we identified a high affinity \(\omega\)-CTX MVIIIC receptor which can be distinguished from the low affinity N-type \(\text{Ca}^{2+}\) channel. Our data demonstrate for the first time that the high affinity \(\omega\)-CTX MVIIIC receptor is composed of at least \(\alpha_{1A}\), \(\alpha_{2\beta}\), and any one of the four different brain \(\beta\) subunits. The association of different \(\beta\) subunits with \(\alpha_{1A}\) and \(\alpha_{2\beta}\) components may produce \(\text{Ca}^{2+}\) channels with distinct functional properties, such as P- and Q-type.

EXPERIMENTAL PROCEDURES

Materials—\(\omega\)-Conotoxin (\(\omega\)-CgTX) GVIA and ECL immunoblotting kit were from Amersham Corp., \(1^{125}\)-\(\omega\)-CTX MVIIIC from DuPont NEN, \(\omega\)-CgTX GVIA from Bachem, protein G-Sepharose from Pharmacia Biotech Inc., TNT coupled reticulocyte lysate system from Promega, Hydrazide Avided from Unisyn Technologies, \(\omega\)-CTX MVIIIC was a kind gift from Neurex Corporation. All chemicals are of reagent grade.

Ligand Binding Analysis of the \(\omega\)-CTX MVIIIC Receptor and the \(\text{Ca}^{2+}\) Channel—Rabbit brain membranes were prepared as detailed elsewhere (26). Protein concentration was determined by the method of Lowry (27). To obtain enough material of \(1^{125}\)-\(\omega\)-CTX MVIIIC, \(1^{125}\)-\(\omega\)-CTX MVIIIC (specific activity, 2200 Ci/mmol) was mixed with unlabelled \(\omega\)-CTX MVIIIC to reach a specific activity of 440 Ci/mmol. Aliquots (50 \(\mu\)g of protein) of membranes were incubated with increasing concentrations (0.05–8 nM) of \(1^{125}\)-\(\omega\)-CTX MVIIIC at 25°C for 1 h in a total volume of 200 \(\mu\)l of buffer A (10 mM HEPES, pH 7.4, 0.2 mM MgCl\(_2\) bovine serum albumin, 100 mM NaCl, 0.75 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride) containing 0.1 mM EDTA, and 0.1 mM EGTA. The receptor-ligand complexes were collected on GF/B filters and washed rapidly with 4 ml of ice-cold buffer A. The counts remaining on the filter represent total binding. Nonspecific binding was determined by the addition of 0.5 \(\mu\)M nonradioactive ligand prior to the addition of radioactive ligand. Specific binding was calculated by sub-

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1 The abbreviations used are: CTX, conotoxin; \(\omega\)-CgTX GVIA, \(\omega\)-conotoxin GVIA; \(\omega\)-CTX MVIIIC, \(\omega\)-conotoxin MVIIIC; PCR, polymerase chain reaction; FP, fusion protein; GST, glutathione S-transferase; mAb, monoclonal antibody.
tracing nonspecific binding from total binding. 125I-ω-CgTX Gvia binding was assayed similarly to 125I-ω-CTX MVIIC but without EGTA and EDTA in buffer A. Binding of 125I-ω-Ctx MVIIC and 125I-ω-CgTX Gvia to the solubilized membrane extracts are the same as described above. All binding data were analyzed with Grafit software (Sigmam) and all results were presented as value ± S.E. Each sample point represents the average of triplicates for all binding and immunoprecipitation experiments in this paper.

Competition of Unlabeled 125I-ω-Ctx MVIIC with 125I-ω-CgTX Gvia Binding—Rabbit brain membranes (4 mg/ml) were solubilized for 1 h on ice in buffer (0.5 mM NaCl, 1% (w/v) digitonin, 10 mM HEPES, pH 7.4) plus protease inhibitors mixture A (0.6 μg/ml pepstatin A, 0.5 μg/ml aprotonin, 0.1 mM phenylmethylsulfonyl fluoride). Bovine serum albumin, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mg/ml leupeptin. Aliquots were mixed with increased concentrations (0.001 μM to 1 μM) of 125I-ω-Ctx MVIIC for 10 min before addition of 125I-ω-CgTX Gvia (42.5 pm). After incubating for 1 h at room temperature, receptor-ligand complexes were collected on GF/B filters and washed as described above. For binding to brain membranes, 50 μg of brain membranes were incubated with increasing concentrations of 125I-ω-Ctx MVIIC for 10 min in 200 μl of buffer (10 mM HEPES, pH 7.4, 62.5 mM NaCl, 0.1% (w/v) bovine serum albumin, 0.1 mg/ml EDTA, 0.1 mg/ml EGTA, 0.5 μg/ml aprotonin, 0.1 μg/ml leupeptin) before addition of 50 μl of beads was washed as described above. The ICP was determined by Graf software using the average of triplicates. Since the concentration of 125I-ω-CgTX Gvia used should not affect the presentation of IC50 in the competition curve, data were normalized by setting 125I-ω-CgTX Gvia binding in the absence of 125I-ω-Ctx MVIIC as 100%.

Primers Used in Polymerase Chain Reaction (PCR) for Production of Fusion Proteins—α1a forward 5′-AGGTTAGCTCGGAGGAAAGCAGAGACAAGCG-3′ (1657–1681 in rat α2a, 1733–1757 in rat α2b, 1759–1783 of GenBank TM no. M67516) and α1c reverse 5′-AGGTTAGCTCGGAGGAAAGCAGAGACAAGCG-3′ (1609–1633 in rat α2a, 1681–1695 in rat α2b, 1705–1729 of GenBank TM no. M85045); β2a reverse 5′-TGACAGTATCCGACAGTGTT-3′ (2053–2077 in rat, 2069–2093 in mouse) for production of L-type Ca2+ channel. The PCR product was subcloned into a pGex 2T vector which had been linearized with NcoI and HindIII and subjected to scintillation counting. The nonspecific binding determined from the control pGex vector was subtracted from the total binding for each antibody to obtain specific binding. Data were presented as values ± S.E.

Immunoprecipitation of Various Types of Ca2+ Channels—Brain membranes (4 mg/ml) were solubilized and diluted as described above. The solubilized protein was labeled with 45 pm 125I-ω-Ctx MVIIC for 1 h at room temperature. The labeled protein was precipitated with 50 μl of antibody-protein G-Sepharose beads. The beads were washed quickly with 1.5 ml of iced-cold buffer A twice, and total binding was quantified by γ counting. Nonspecific binding was determined by washing with 0.5 M NaCl before addition of 125I-ω-Ctx MVIIC prior to the addition of the labeled toxin. Immunoprecipitation of L-type Ca2+ channel was as described elsewhere (26). Saturating immunoprecipitation was achieved with 50 μl of antibody/tube as compared with that of 100 and 150 μl of antibody.

Enrichment of the High Affinity ω-Ctx MVIIC Receptor—Affinity-purified sheep 37 (α1a) antibody was coupled to Hydrazide Avidex AX according to the manufacturer’s instructions. Protein A-conjugated mixtures were present in all buffers during the purification. Rabbit brain membranes (1 g) were solubilized in buffer (1% digitonin, 1 mM NaCl, 10 mM HEPES, pH 7.4) for 1 h on ice, followed by centrifugation at 140,000 × g for 37 min in a Beckman 45 Ti rotor. The solubilized extracts were divided 3-fold with iced-cold distilled and deionized water and subjected to the heparin column extensively with protein A-Sepharose coupled to protein G-Sepharose beads. The heparin column was extensively washed with 100 mM of each protein/g lane were resolved on 3–12% gradient SDS-PAGE gels and transferred to nitrocellulose. Horseradish peroxidase colorimetric detection was performed as described previously (6). An ECL detection system (Amersham Corp.) was used according to the manufacturer’s instructions. The β2 subunit was detected with an ECL immunoblotting technique. All others used horseradish peroxidase colorimetric detection. Since their affinities for different epitopes, immunoblot staining does not necessarily reflect the level of different proteins in a quantitative way.

Immunoprecipitation of 35S-Labeled Full-length β Subunits—The full-length β2a, β2b, β2c and β2 subunits were in vitro translated using a coupled transcription and translation system (Promega) as detailed previously (16). The expression conditions were identical to the immunoprecipitation conditions used for native Ca2+ channels as described in the following section. Brain membranes (4 mg/ml) were solubilized and diluted as indicated under “Immunoprecipitation of Unlabeled ω-Ctx MVIIC with 125I-ω-CgTX Gvia Binding.” Triplicates of 25 μl of each β subunit antibody and control α1c antibody were coupled to protein G-Sepharose beads by overnight incubation in phosphate-buffered saline. Subsequently, the antibodies were washed with phosphate-buffered saline extensively and incubated with aliquots of solubilized brain extract (1 ml) in buffer (0.1% digitonin, 10 mM HEPES, pH 7.4, 100 mM NaCl, 0.5 μg/ml aprotonin, 0.5 μg/ml leupeptin) for 15 min before the addition of 200,000 cpm of 35S-labeled full-length β subunit per tube for overnight incubation at 4°C. The antibody-protein complexes were extensively washed with phosphate-buffered saline and subjected to scintillation counting. The nonspecific binding determined from the control α1c antibody was subtracted from the total binding for each antibody to obtain specific binding. Data were presented as values ± S.E.

Immunoprecipitation of Various Types of Ca2+ Channels—Brain membranes (4 mg/ml) were solubilized and diluted as described above. The solubilized protein was labeled with 45 pm 125I-ω-Ctx MVIIC for 1 h at room temperature. Then aliquots of the labeled extract (0.5–1 ml) were incubated for 3 h with 50 μl of antibody-protein G-Sepharose beads. The beads were washed quickly with 1.5 ml of iced-cold buffer A twice, and total binding was quantified by γ counting. Nonspecific binding was determined by washing with 0.5 M of each protein/g lane and subjected to SDS-polyacrylamide gel electrophoresis analysis. The protein concentration was determined by the method of Bradford (1998) and subjected to 35S-polyacrylamide gel electrophoresis analysis after transcribed RNA mixtures were injected per oocyte (0.5 μg/ml α1a, 100 μg/ml α1c, 1 g/l of each protein/lane) were resolved on 3–12% gradient SDS-PAGE gels and transferred to nitrocellulose. Horseradish peroxidase colorimetric detection was performed as described previously (6). An ECL detection system (Amersham Corp.) was used according to the manufacturer’s instructions. The β2 subunit was detected with an ECL immunoblotting technique. All others used horseradish peroxidase colorimetric detection. Since their affinities for different epitopes, immunoblot staining does not necessarily reflect the level of different proteins in a quantitative way.

Preparation of Xenopus Oocytes and Injection— Mature Xenopus laevis female frogs were purchased from NASCO (Fort Atkinson, WI). Preparation of Xenopus oocytes, dRNA synthesis, and dRNA injection procedures were as described previously (10). Full-length 125I-ω-Ctx MVIIC or transfected RNA mixtures were injected into Xenopus oocytes using a micromanipulator. The oocytes were then incubated in 4°C HEPES (pH 7.4) for 1 h. After washing, the oocytes were solubilized in buffer (1% digitonin, 10 mM HEPES, pH 7.4, 100 mM NaCl) and subjected to SDS-PAGE. The protein concentration was determined by the method of Lowry (27) or a silver binding method by Krystal (31) for measuring nanogram amounts of proteins.

Preparation of Xenopus Oocytes and Injection— Mature Xenopus laevis female frogs were purchased from NASCO (Fort Atkinson, WI). Preparation of Xenopus oocytes, dRNA synthesis, and dRNA injection procedures were as described previously (10). Fluorescent in vitro transfected RNA mixtures were injected per oocyte (0.5 μg/ml α1a and 0.5 μg/ml α1c together with either 0.1 μg/ml rabbit β2a (16) and 0.1 μg/ml β2c in the case of a β2a, β2c ratio of 1:1, or 0.1 μg/ml β2a and 1 μg/ml β2c in the case of a β2a, β2c ratio of 1:10). Electrophysiological recording and data analysis were as described previously (10). Briefly, Ba2+ currents were recorded by a standard two-microelectrode voltage-clamp technique using a Dagan amplifier (TEV-200). Voltage and current electrodes (0.5–2 meqoms) were filled with 3 mM KCl. The bath solution was (in mM): 40 Ba(OH)2, 50 NaOH, 2 KCl, 1 niflumic acid, 0.1
Rabbit Brain Expresses a High Affinity \(\omega\)-CTX MVIIC-sensitive \(\text{Ca}^{2+}\) Channel—Both a high affinity and a low affinity binding site for \(\omega\)-CTX MVIIC were detected in rabbit brain (Fig. 1A). Curve fitting of the direct binding curve and Scatchard analysis (32) reveal a high affinity binding site with a \(K_d\) of 0.37 ± 0.09 nM and a \(B_{\text{max}}\) of 870 ± 92 fmoi/mg; and a low affinity binding site with a \(K_d\) of 4 ± 2 nM and a \(B_{\text{max}}\) of 290 ± 110 fmoi/mg. It is technically difficult to analyze a low affinity site (\(K_d > K_{\text{d1}}\)) with a smaller binding capacity because the high affinity site always forms more ligand-bound receptor than the low affinity site (32). The characteristics of the high affinity \(\omega\)-CTX MVIIC binding site are in approximate agreement with previous results reported for rat and human brain membranes (24, 33, 34). In comparison, \(^{125}\text{I}-\omega\)-conotoxin GVIA (\(\omega\)-CgTX GVIA), a specific blocker for N-type \(\text{Ca}^{2+}\) channel, binds to the same rabbit brain membrane with a \(K_d\) of 45 ± 6 pM and a \(B_{\text{max}}\) of 207 ± 14 fmoi/mg (Fig. 1B). These results demonstrate that the expression level of the high affinity \(\omega\)-CTX MVIIC receptor is higher than that of N-type \(\text{Ca}^{2+}\) channel in rabbit brain; while the expression of the low affinity receptor is similar to that of N-type \(\text{Ca}^{2+}\) channel. In order to further analyze the \(\omega\)-CTX MVIIC binding receptor, unlabeled \(\omega\)-CTX MVIIC was used to compete with \(^{125}\text{I}\) \(\omega\)-CgTX GVIA for binding to rabbit brain membrane and solubilized brain extracts (Fig. 1C). The results reveal an \(IC_{50}\) of 7.7 ± 0.3 (brain membranes) and 7.9 ± 0.3 nM (solubilized brain extracts) for \(\omega\)-CTX MVIIC and a \(K_i\) of 4.4 nM. All of these data suggest that \(\omega\)-CTX MVIIC binds with low affinity (about 4 nM) to the N-type \(\text{Ca}^{2+}\) channel. Notably, \(\omega\)-CTX MVIIC up to 1 nM did not significantly inhibit \(^{125}\text{I}\) \(\omega\)-CgTX GVIA binding to the N-type \(\text{Ca}^{2+}\) channel, while it starts to bind to the N-type \(\text{Ca}^{2+}\) channel at higher than 1 nM concentration (Fig. 1C). This important property of \(\omega\)-CTX MVIIC enabled us to distinguish these two binding sites by specifically labeling only the high affinity receptor with these low toxin concentrations (<1 nM).

\(\alpha_{1A}\) and Any One of the Four \(\beta\) Subunits Are Components of the High Affinity \(\omega\)-CTX MVIIC Receptor—A low concentration of \(^{125}\text{I}\) \(\omega\)-CTX MVIIC (45 pM) was used to specifically label the high affinity \(\omega\)-CTX MVIIC receptor. Polyclonal \(\alpha_{1S}\) subunit antibodies immunoprecipitated 21 fmoi of labeled high affinity \(\omega\)-CTX MVIIC receptor (84% of total labeled receptors) (Fig. 2A). In contrast, monoclonal antibodies raised against the \(\alpha_{1S}\) subunit (IIIC12) and the \(\alpha_{1B}\) subunit (CC18) together with a polyclonal antibody against the \(\alpha_{1C}\) subunit (sheep 41) did not sediment significant amounts of labeled high affinity \(\omega\)-CTX MVIIC receptor (less than 5% of total labeled receptors). In control experiments, however, the mAb against \(\alpha_{1S}\) (IIIC12) immunoprecipitated more than 80% of \(^3\text{H}\)-PN200–110 binding to the skeletal muscle L-type \(\text{Ca}^{2+}\) channel (26); the mAb against \(\alpha_{1B}\) (CC18) immunoprecipitated more than 80% of \(^3\text{H}\)-PN200–110 binding to the brain \(\text{Ca}^{2+}\) channel; and the polyclonal antibody against \(\alpha_{1C}\) (sheep 41) immunoprecipitated 80% of cardiac L-type \(\text{Ca}^{2+}\) channel (data not shown). These results demonstrate that the \(\alpha_{1A}\) subunit is a component of the brain high affinity \(\omega\)-CTX MVIIC receptor, which is in agreement with the results from rat brain (35). Furthermore, the data confirm that low concentrations of \(\omega\)-CTX MVIIC specifically label the brain high affinity \(\omega\)-CTX MVIIC receptor but not the N-type \(\text{Ca}^{2+}\) channel.

To establish which \(\beta\) subunit is associated with the \(\alpha_{1A}\) pore-forming component of \(\text{Ca}^{2+}\) channel, we have produced \(\beta\)
subunit subtype specific antibodies. β subunit genes have two highly homologous central domains, but unique N and C termini (Fig. 3A). Although domains I and II of each β subunit share 65 and 78% amino acid identity, respectively, the C termini of all four brain β subunits share only 3% amino acid identity. The C-terminal regions of four β subunits were thus expressed as GST fusion proteins. Excluding the common GST portion of these fusion proteins, the overall amino acid identity of all four fusion proteins is 1.2%. Antibodies were therefore raised against these fusion proteins in order to produce specific antibodies for each β subunit. As shown in Fig. 3B, the purified β1b, β2, β3, and β4 GST fusion proteins are of molecular mass of 52, 42, 39, and 34 kDa, respectively. The purified fusion proteins were used to generate polyclonal antibodies in rabbits (β1b, β2, and β3) or sheep (β4).

The specificity of each of the four FP antibodies was analyzed by immunoblotting. Crude bacterial lysates expressing the four fusion proteins were loaded on SDS-polyacrylamide gel electrophoresis. Approximately equal amounts of FPβ1b, FPβ2, FPβ3, and FPβ4 were present on the blots as determined by densitometry (corresponding Coomassie Blue-stained gel not shown). Since all four fusion protein antibodies should also contain antibodies against GST, they were removed by preincubation with bacterial GST lysates. Since an excess of fusion proteins were present in the blots, this assay should be very sensitive to detect any cross reactivity of the antibodies. As shown in Fig. 3C, antibodies to FPβ1b, FPβ2, FPβ3, and FPβ4 specifically recognized unique antigens and did not cross react with any of the other three fusion proteins in immunoblots. The antibodies to FPβ1b, FPβ3, and FPβ4 also detected proteolyzed forms of the fusion proteins which were present in low amounts during the production of the fusion proteins.

In addition, the specificity of these antibodies for immunoprecipitation were tested using full-length in vitro translated β1b, β2, β3, and β4 subunits. 35S-Labeled β1b, β2, β3, and β4 subunits were previously shown to be the only radiolabeled proteins present in the translation products (30). The results show that each antibody was able to immunoprecipitate their respective labeled β subunit but did not cross react significantly with the other three in vitro translated β subunits (Fig. 3D).

Immunoprecipitation of 125I-ω-CTX MVIIIC labeled high affinity receptors with the four β subunit specific antibodies was performed (Fig. 2B). β4 immunoprecipitated the maximum amount (48%) of bound receptor followed by β3 36%, β1b 8.4%, and β2 7.2%. The results demonstrate that four different β subunits are present in high affinity ω-CTX MVIIIC receptors.

In contrast, none of the α1A or four brain β subunit antibodies were capable of immunoprecipitating the skeletal muscle L-type Ca2⁺ channel (Fig. 2C). The C-terminal regions of brain β1b, β2, β3, and β4 subunits share little homology with the β1a subunit of skeletal muscle L-type Ca2⁺ channels. The results suggest that none of these antibodies (β1b, β2, β3, and β4) reacted with the β1a subunit of skeletal muscle L-type Ca2⁺ channel. The results also confirm that the nonspecific immunoprecipitation by each of these antibodies is negligible.

Copurification of α1A, α2δ, and Any One of the β1b, β2, β3, and β4 Subunits—The α1A subunit antibody was raised against a 200-amino acid region, which is located on the cytoplasmic loop between domains II and III of the rabbit α1A subunit. This region of α1A shares a 37-amino acid sequence homology with the α1B subunit, but shares little sequence homology with either α1S, α1C, α1D, or α1E. After enrichment of the Ca2⁺ channels on a heparin agarose column (26, 28), we utilized the mAb CC18 which was raised against the II-III loop of the α1B subunit as an immunoaffinity ligand to specifically remove all the N-type Ca2⁺ channel (6), and then subsequently used an α1A antibody affinity column to enrich the high affinity ω-CTX MVIIIC receptor. The void from the mAb α1B column was devoid of 125I-ω-CgTX G VI A binding, showing that over 95% of the N-type Ca2⁺ channel was removed by mAb α1B column (Fig. 4A). Second incubation with mAb α3γ column did not remove any additional ω-CgTX G VI A binding. Notably, the mAb α3γ column did not remove any ω-CTX MVIIIC binding when brain receptors were labeled with 0.1 nM (data not shown) and 0.5 nM ω-CTX MVIIIC, which only labeled the high affinity receptor as previously shown in Fig. 1C. In contrast, α1A antibody affinity column bound greater than 95% of the labeled ω-CTX MVIIIC receptor from the α1B column void. The results demonstrate that the α1A subunit is a major, if not the only, α1 present in the high affinity ω-CTX MVIIIC receptor. Since the eluted receptors were inactive in ω-CTX MVIIIC binding, the enrichment of receptor could not be estimated. The recovery of protein from the α1A column was about 0.3% compared to the total amount.
of protein loaded to the column (the α1B column void) and about 0.02% compared to the quantity of starting brain membranes.

Further analysis of heparin column eluate, α1B column void, and α1A column void by immunoblotting showed that α1A, α2δ, β1β, β2β, and β4 subunits copurified on the α1A immunoaffinity column. As shown in Fig. 4B, a fraction of the α2δ subunit was removed by the α1B column since this subunit is also present in the N-type Ca2+ channel. Importantly, the α1A column further removed another portion of the α2δ subunit and thus this subunit was enriched in the α1A eluate. These results demonstrate that the α2δ subunit is also a component of the high affinity ω-CTX MVIIC receptor. In agreement with this result, wheat germ agglutinin-Sepharose sedimented a significant amount of high affinity 125I-ω-CTX MVIIC receptors probably through its interaction with the heavily glycosylated α2δ subunit (data not shown). Similar to α2δ subunit, β1b, β2, β3, and β4 subunits were also enriched in the eluate of α1A column (Fig. 4C) while they were barely detectable in 100 μg of α1B column void (data not shown). The β1b subunit has a molecular mass of 76 kDa on immunoblot. There is also a higher band running at 80 kDa which may be a modified form or a β1 isofrom. The β2 subunit has a molecular mass...
mass of 84 kDa. The \( \beta_3 \) subunit has a molecular mass of 58 kDa. The \( \beta_4 \) subunits appear to run at 59 and 55 kDa possibly representing differential phosphorylation states or isoforms. These results confirm the immunoprecipitation data with the \( \beta_2 \) subunit antibodies. Furthermore, they demonstrate the specificity of \( \beta \) subunit antibodies on the immunoblot and the copurification of the \( \alpha_2\delta \) and four \( \beta \) subunits during enrichment of high affinity \( \omega \)-CTX MVIIIC receptors.

Association of Various \( \beta \) Subunits with the \( \alpha_{1A} \) Channel Contributes to Its Functional Diversity—Previously all four \( \beta \) subunits have been coexpressed separately with the \( \alpha_{1A} \) and \( \alpha_{2\delta} \) subunit in Xenopus oocytes. This results in several different modifications of the biophysical properties of the \( \alpha_{1A} \) channel (10, 23). Previous results from our laboratory indicate that the current decay of the \( \alpha_{1A}\alpha_{2\delta} \) channel coexpressed with the \( \beta_3 \) subunit is monophasic and fast (10), which resembles the \( \beta \)-type current found in cerebellar granule cells (22). The current inactivation is voltage-dependent but reaches its fastest decay at +10 mV with a time constant of ~120 ms (10). When this channel is coexpressed with \( \beta_2 \) subunit, the current is slowly inactivating with a time constant of ~500 ms (10), which resembles the \( \beta \)-type current detected in cerebellar Purkinje cells (20).

Since an 18-amino acid domain in \( \alpha_3 \) subunit has been identified to be responsible for \( \beta \) subunit association (36), the affinity of various \( \beta \) subunits binding to \( \alpha_{1A} \) interaction domain has been determined (30). Compared to \( \beta_2 \), \( \beta_3 \) and \( \beta_4 \) subunits, \( \beta_3 \) has a 10-fold lower affinity for \( \alpha_{1A} \alpha_{2\delta} \) interaction domain based on in vitro binding assay. To test if \( \beta_3 \) subunit is able to form Q-like channels in the presence of other \( \beta \) subunits in one cell, we coexpressed \( \beta_3 \) subunit with the \( \beta_2 \) subunit. The formation of \( \alpha_{1A}\alpha_{2\delta}\beta_3 \) or \( \alpha_{1A}\alpha_{2\delta}\beta_2 \) channel can be easily distinguished because these channels exhibit distinct inactivation kinetics. When \( \beta_1 \) and \( \beta_2 \) subunits were coexpressed in Xenopus oocytes with cRNA ratio of 1:1, the kinetics of the \( \alpha_{1A} \) channel current showed a biexponential decay (Fig 5A). The biphasic decay can be separated into two components with distinct inactivation kinetics (\( t_1 = 69 \pm 12 \) ms, \( t_2 = 471 \pm 32 \) ms, \( n = 5 \)) which represents the currents carried by \( \alpha_{1A}\alpha_{2\delta}\beta_3 \) or \( \alpha_{1A}\alpha_{2\delta}\beta_2 \) channel. The results suggest the formation of two populations of functionally distinct channels. When \( \beta_3 \) and \( \beta_2 \) subunits were coexpressed in Xenopus oocytes with cRNA ratio of 10:1, the inactivation of the \( \alpha_{1A} \) channel is also biphasic with a \( t_1 \) of 59 \( \pm \) 7 ms and a \( t_2 \) of 338 \( \pm \) 25 ms (\( n = 4 \)). As expected for the formation of two populations of \( \beta \)-type channel complexes, the time constants of the biphasic decay for both cRNA ratios were comparable to that of \( \beta_3 \) and \( \beta_2 \) expressed separately. While the total biexponential current is the sum of the two monoexponential components, the fast component (\( \beta_3 \)) increased from 52 to 77% of the total current when cRNA ratio of \( \beta_2/\beta_3 \) changed from 1:1 to 10:1 (Fig 5B). Although protein levels of \( \beta_2 \) and \( \beta_3 \) subunits could not be determined due to extremely low amounts of expression, these results show that both \( \beta_3 \) and \( \beta_2 \) subunits are able to associate with \( \alpha_{1A}\alpha_{2\delta} \) to form functionally distinct channels for both \( \beta_3/\beta_2 \) cRNA ratios. Furthermore, these results suggest that the expression level of a \( \beta \) subunit may be one factor involved in \( \alpha_{1A}\beta \) complex formation.

**DISCUSSION**

There has been some controversy concerning the specificity of \( \omega \)-CTX MVIIIC in the literature. While some researchers found that this toxin blocks N-, P-, and Q-type \( Ca^{2+} \) channels (24, 25), others obtained specific blockage of P- and Q-type \( Ca^{2+} \) channels but not the N-type channel (19, 37). In this study, we detected both a high affinity and a low affinity binding site for this toxin in rabbit brain. Importantly, there is a critical concentration of \( \omega \)-CTX MVIIIC, below which the toxin specifically binds the high affinity receptor and above which the toxin starts to bind the N-type \( Ca^{2+} \) channel. Kristipati et al. (34) has reported that \( \omega \)-CTX MVIIIC binding is sensitive to concentration changes of monovalent (sodium, potassium) and divalent ions (calcium, barium) commonly used in biochemical and electrophysiological studies. It is conceivable that this critical concentration varies depending on the experimental conditions and different investigators were using variable ranges of the \( \omega \)-CTX MVIIIC concentration, which may explain some of the reported inconsistencies.

Although N-, P-, and Q-type \( Ca^{2+} \) channels are all involved in neurotransmission, P- and Q-type channels seem to be of greater importance than N-type not only in terms of larger \( Ca^{2+} \) currents but also in the efficacy of these \( Ca^{2+} \) currents coupling to neurotransmitter release (4, 22, 25, 38). The high affinity \( \omega \)-CTX MVIIIC receptor is expressed at a higher level than N-type channel which is in agreement with larger current expression of P- and Q-type channels. \( \omega \)-CTX MVIIIC has been used as a potent blocker for P- and Q-type \( Ca^{2+} \) channels (19, 22). Therefore the high affinity \( \omega \)-CTX MVIIIC receptor we detected in this study probably represents P- and Q-type \( Ca^{2+} \) channels.

Although it has been generally assumed that \( \alpha_1 \), \( \alpha_{2\delta} \), and \( \beta \) subunits may be common subunits for high voltage-activated \( Ca^{2+} \) channels, whether or not this holds true for all high voltage-activated channels has not been tested. Among the voltage-dependent \( Ca^{2+} \) channels, subunit composition of skel-
et al. muscle L-type, cardiac muscle L-type and brain N-type channels have been extensively characterized, while brain L-type (α_{1C}- or α_{1D}-containing), P-type, Q-type, and α_{1D}-containing Ca^{2+} channels are poorly understood. Our data demonstrate for the first time that the high affinity ω-CTX MVIIIC receptor is composed of at least α_{1A}, α_{1B}, and any one of the four β subunits. Furthermore, we demonstrate that different β subunits are associated with the high affinity ω-CTX MVIIIC-sensitive Ca^{2+} channel. This heterogenous association of β subunits results in channels with distinct properties such as inactivation kinetics when studied in Xenopus oocytes. Therefore, the association of various β subunits with the α_{1A} subunit in brain may produce some of the functional heterogeneity expected of Ca^{2+} channels sensitive to ω-CTX MVIIIC.

The distinction between P- and Q-type Ca^{2+} channels is poorly defined. To our knowledge, there is no evidence to suggest that ω-CTX MVIIIC has different affinity for brain P- or Q-type Ca^{2+} channels. The difference between inactivation kinetics when studied in a single cell, and the expression level of a receptor is composed of at least an α_{1A}, α_{1D}, and any one of the four β subunits. Furthermore, we demonstrate that different β subunits are associated with the high affinity ω-CTX MVIIIC-sensitive Ca^{2+} channel.

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