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CHARACTERIZATION OF THE PURIFIED N-TYPE Ca^{2+} CHANNEL AND THE CATION SENSITIVITY OF ω-CONOTOXIN GVIA BINDING

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Summary—A functional N-type Ca^{2+} channel (ω-conotoxin GVIA receptor) has been purified from rabbit brain and shown to be composed of four subunits of molecular weights 230 K (α_{1B}), 160 K ($\alpha_2\delta$), 95 K and 57 K (β_3) [Witcher D. R., De Waard M., Sakamoto J., Franzini-Armstrong C., Pragnell M., Kahl S. D. and Campbell K. D. (1993) *Science* **261:** 486–489]. These four subunits migrate on sucrose density gradients as a single complex and are identified by subunit specific polyclonal antibodies. Polyclonal antibodies against the purified receptor complex immunoprecipitate greater than 90% of the [^{125}I]ω-conotoxin GVIA (ω-CgTx) binding sites in solubilized crude rabbit brain membranes. Furthermore, polyclonal antibodies affinity-purified against unique GST fusion proteins from two of the cloned subunits in the complex (α_{1B} and β_3) specifically immunoprecipitated [^{125}I]ω-CgTx binding sites and not [^{3}H]PN200-110 binding sites. Analysis of [^{125}I]ω-CgTx binding to the purified N-type Ca^{2+} channel demonstrated that the equilibrium binding was sensitive to increasing cation concentrations. The IC_{50} for calcium and barium was 2.5 and 5 mM, respectively. [^{125}I]ω-CgTx binding was not significantly reduced within 15 min after the addition of 50 mM barium. However, single channel analysis of the purified N-type Ca^{2+} channel preincubated with 10 μM ω-CgTx demonstrated that in the presence of 50 mM barium and 0.5 μM ω-CgTx, channel activity was detected but at a low open state probability ($P < 0.10$). These data suggest that the Ca^{2+} binding site(s) allosterically regulates the ω-CgTx binding site. Since the channel gating persisted in the presence of ω-CgTx, the ω-CgTx binding site may not be located within the pore of the channel and may be different from intra-pore Ca^{2+} binding sites.

Key words—neuronal Ca^{2+} channels, ω-conotoxin receptor, single channel analysis.

Voltage-sensitive Ca^{2+} channels play a vital role in the regulation of many neuronal processes. On the basis of electrophysiological and pharmacological characteristics, at least four subtypes of voltage-sensitive Ca^{2+} channels (classified as T-, L-, P- and N-type) have been identified (Nowycky, Fox and Tsien, 1985; Fox, Nowycky and Tsien, 1987a, b; Bean, 1989; Llinas, Sugimori, Lin and Cherksey, 1989). However, multiple genes encoding differing α_1 and β subunits have been identified recently (Snutch, Leonard, Gilbert and Davidson, 1990; Hulin, Singer-Lahat, Freichel, Biel, Dascal, Hofmann and Flockerzi, 1992). The N-type Ca^{2+} channels, localized mainly in neurons, are specifically and irreversibly inhibited by the peptide, ω-conotoxin GVIA (Fox *et al.*, 1987a, b; Sher and Clementi, 1991; Tsien, Ellinor and Horne, 1991). ω-CgTx has also been shown to inhibit transmitter release from mammalian neurons (Horne and Kemp, 1991; Hong, Tsuji and Chang, 1992). This supports the hypothesis that the N-type Ca^{2+} channel plays an essential role in controlling neurotransmitter release from different regions of the central nervous system (Smith and Augustine, 1988). Besides the regulation of transmitter release, the N-type Ca^{2+} channel is also thought to direct the migration of immature neurons (Komuro and Rakic, 1992).

The only voltage-sensitive Ca^{2+} channel which has been extensively characterized is the skeletal muscle L-type Ca^{2+} channel or dihydropyridine receptor (Campbell, Leung and Sharp, 1988; Catterall, 1988). This Ca^{2+} channel has been purified and identified as a multisubunit complex composed of four tightly-associated subunits, α_1, $\alpha_2\delta$, β and γ. Recently, we have reported the purification of the N-type Ca^{2+} channel complex (ω-CgTx receptor) from rabbit brain (Witcher *et al.*, 1993). The complex is composed of an α_{1B} subunit of molecular masses 230 kDa (class B as defined by Snutch), an $\alpha_2\delta$ subunit of 160 kDa (nonreduced, which is highly homologous to the skeletal $\alpha_2\delta$ subunit), a unique 95 kDa subunit, and a β_3 subunit of 57 kDa. When the purified receptor is reconstituted in lipid bilayers, it forms a functional Ca^{2+} channel with the same pharmacological properties as those of the native N-type Ca^{2+} channel in neurons (Witcher *et al.*, 1993). In the present work, we have prepared specific polyclonal antibodies

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against the α_{1B}, α_2, 95 K and β_3 subunits of the Ca^{2+} channel complex. The ability of these antibodies to immunoprecipitate [^{125}I]ω-CgTx and [^{3}H]PN200-100 binding sites in solubilized brain membranes was tested. We have also characterized the ω-CgTx binding site on the purified N-type Ca^{2+} channel complex using binding assays and single channel experiments. Our results suggest that the Ca^{2+} binding site(s) allosterically regulates the ω-CgTx binding site. Furthermore in the presence of high divalent cation concentrations, block of single channel activity of the purified complex is incomplete suggesting that the ω-CgTx binding site is distinct from the pore of the Ca^{2+} channel.

METHODS

Purification of the N-type Ca^{2+} channel complex

The ω-conotoxin GVIA receptor was purified from whole rabbit brain as previously described (Witcher *et al.*, 1993). Briefly, digitonin-solubilized rabbit brain membranes were applied to a heparin-agarose column (50 ml). After extensive washing, the heparin–agarose column was eluted with buffer A (10 mM Hepes, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride and 0.75 mM benzamidine) containing 0.7 M NaCl and 0.1% digitonin (w/v). The fractions containing the eluted receptor were incubated overnight with 9 ml of VD2$_1$ (monoclonal antibody against the β subunit) agarose resin, washed extensively, eluted with 50 mM CAPS (pH 10) containing 0.6 M NaCl and 0.1% digitonin, and neutralized immediately. The VD2$_1$ eluted receptor was then preabsorbed with an immunoaffinity resin prepared with polyclonal antibodies against a 46 kDa contaminating protein. Polyclonal antibodies against this contaminating protein did not immunoprecipitate the [^{125}I]ω-CgTx receptor. The ω-CgTx receptor was then placed on a 5–30% linear sucrose density gradient and centrifuged at 4°C in a Beckman VTi65.1 rotor for 100 min at 215,000 *g*. The purified receptor was located in fractions 8 through 12.

ω-Conotoxin GVIA binding assays

The purified ω-conotoxin GVIA receptor ($5 \times 10^{-2}\ \mu g$) was incubated in buffer B (10 mM HEPES, pH 7.4, containing 0.25 nM [^{125}I]labeled ω-conotoxin GVIA, 10 mM NaCl, 0.5% digitonin, 0.75 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride and 0.2 mg/ml bovine serum albumin), with or without 0.25 μM ω-conotoxin GVIA for 45 min at 25°C. The amount of [^{125}I]ω-conotoxin bound was determined by filtration on Whatman GF/B filters (presoaked in 0.5% polyethylenimine) using a buffer containing 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 0.2 mg/ml bovine serum albumin. Specific binding was calculated by subtracting nonspecific binding determined in the presence of 0.25 μM unlabeled ω-conotoxin from the total binding. For individual experiments, the concentration of calcium and barium were varied while keeping all other conditions constant.

Production of antibodies against the N-type Ca^{2+} channel complex

Specific polyclonal antibodies against the different components of the receptor complex were produced in sheep using the purified ω-conotoxin receptor. Approximately 0.1 mg of pure receptor emulsified with Freund's complete adjuvant was administered subcutaneously into a sheep at numerous sites. Four weeks after the initial injection the animal was boosted with 0.1 mg of pure receptor intravenously. Seven days later a blood sample was taken and tested for the presence of antibodies against specific subunits of the receptor. Specific antibodies against α_{1B}, α_2, 95 K and β_3 were affinity purified from Immobilon-P transfer strips of individual components of the receptor complex as previously described (Witcher *et al.*, 1993).

SDS–PAGE and immunoblot analysis

Samples from each purification step and purified receptor were analyzed by SDS–PAGE (3–12% gradient gels) using the buffer system of Laemmli (1970). Gels were stained with Coomassie blue or transferred to nitrocellulose for immunoblot analysis as previously described (Witcher *et al.*, 1993).

Sucrose density gradients

Purified receptor was layered onto a linear 5–30% sucrose density gradient (12.5 ml). Gradients were centrifuged at 4°C in a Beckman VTi65.1 vertical rotor for 100 minutes at 215,000 *g*. Fractions (0.6 ml) were collected from the top of the gradients using an ISCO Model 640 density gradient fractionator. Gradient fractions (100 μl) were separated by SDS–PAGE (3–12% gradient gels) and transferred to nitrocellulose. Nitrocellulose blots were stained with affinity-purified antibodies against the subunits of the complex.

Glutathione-S-transferase (GST) fusion proteins

pGEX vectors (Smith and Johnson, 1988) were used to produce the α_{1B} subunit GST fusion protein (α_{1B}) and two β_3 subunit GST fusion proteins (β_{3C} and β_{3N}). The α_{1B} GST fusion protein consisted of amino acid residues 720 to 1139 of the unique intracellular loop between the second and third transmembrane domain of the clone α_1 class B subunit (Dubel, Starr, Hell, Ahlijanian, Enyeart, Catterall and Snutch, 1992). The carboxyl-terminal β_3 subunit fusion protein (β_{3C}) consisted of amino acid residues 369 to 484 which are unique to the β_3 subunit. The amino-terminal β_3 subunit GST fusion protein (β_{3N}) consisted of amino acid residues 9 to 296 which contained regions highly homologous to all of the cloned β subunits. The amino acid sequence for these GST fusion proteins was based on the

cloned rabbit β_3 subunit (Witcher *et al.*, 1993) which is highly homologous to the β_3 subunit cloned and sequenced by Hullin *et al.* (1992). Each fusion protein construct was transformed into *E. coli* DH5$_\alpha$ cells. Overnight cultures were grown at 37°C. These cultures were diluted 1 to 10, incubated for 1 hr and induced for 2.5 hr with 1 mM IPTG. The cells were resuspended in PBS and sonicated for 30 sec. The supernatant was then placed on glutathione-Sepharose. The column was extensively washed with PBS and the fusion proteins eluted with 5 mM glutathione in 50 mM Tris, pH 8.0.

Immunoprecipitation analysis

Crude rabbit brain membranes (2 mg/ml) were labeled with 0.5 nM [^{125}I]ω-CgTx or 10 nM [^{3}H]PN200-110 as previously described (Sakamoto and Campbell, 1991). Labeled membranes were pelleted at 100,000 *g* for 15 min, resuspended in buffer A containing 1 M NaCl and 1% digitonin, and mixed for 1 hr at 4°C. The insoluble material was removed by centrifugation at 100,000 *g* for 15 min at 4°C. The supernatant was diluted 10-fold and the specific binding of each compound was determined as described above. Aliquots of the diluted material were added to increasing amounts of ω-CgTx receptor polyclonal antibodies conjugated to protein G-Sepharose. Polyclonal antibodies were conjugated to protein G-Sepharose beads by incubation with the sheep serum overnight at 4°C. The beads were then washed 3 times in buffer A containing 0.1 M NaCl and 0.1% digitonin. The labeled aliquots were incubated with the polyclonal antibody beads for 3 hr at 4°C. After incubation, the beads were washed three times in buffer A containing 0.1 M NaCl and 0.1% digitonin. The percent of [^{125}I]ω-CgTx or [^{3}H]PN200-110 binding sites immunoprecipitated was determined by either γ or scintillation counting. Nonspecific binding of [^{3}H]PN200-110 was measured in the presence of 10 μM nitrendipine.

Single channel analysis

One μl of purified receptor was diluted into 10 mM HEPES, pH 7.4, 10 mM NaCl, 0.75 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride and incubated for at least 20 min with or without 10 μM ω-CgTx. The receptor was then diluted to a final concentration of 1 pM into a 2 ml extracellular bath containing 50 mM $Ba(OH)_2$, 5 mM Hepes, pH 7.5. A mixture of phosphatidylserine and phosphatidylethanolamine (30 mg/ml, Avanti Polar Lipids, 1:1 ratio) was spread at the surface of the bath. Electrode resistance was between 2 and 10 MΩ, and only seal resistances between 10 and 80 GΩ were considered. A magnetic stirrer was used in the bilayer to assist channel incorporation into the lipid monolayer. Lipid bilayers were formed at the tip of heat-polished and sylgard-coated patch electrodes by dipping the tip of the electrode twice through the monolayer. If channel activity was not obtained within 10 min of the receptor dilution into the barium solution used for recordings, the procedure was repeated to avoid prolonged exposure of the channel to barium. Single channel data were digitally stored on tape (DTR 1202, Biologic), then post filtered at 2–3 kHz before computer acquisition at 5–10 kHz. Channel data were analyzed using PClamp software. Amplitude histograms were made after separation of open- and closed-states of the channels using the threshold set at 0.5 of the predominant open channel amplitude. A non-linear Levenberg–Marquardt least squares curve fitting procedure was used for fitting Gaussian curves. First order least square fits to the data were used for conductance measurements.

Materials

Horseradish peroxidase-conjugated secondary antibodies were from Boehringer Mannheim, U.S.A. [^{125}I]ω-conotoxin GVIA was from Amersham, U.S.A. and ω-conotoxin GVIA was from Bachem California, U.S.A. Molecular weight standards and heparin–agarose were from Sigma. Protein G-Sepharose was obtained from Pharmacia, U.S.A. All other chemicals were of reagent grade.

RESULTS

Subunit composition of the purified N-type Ca^{2+} channel complex

We have recently purified the ω-conotoxin GVIA receptor (N-type Ca^{2+} channel) from solubilized rabbit brain membranes by a combination of heparin chromatography, immunoaffinity chromatography, and sucrose density gradient centrifugation. We also have demonstrated that the reconstituted receptor complex forms a functional calcium channel with the same pharmacological properties and conductance as those of the native N-type Ca^{2+} channel in neurons (Witcher *et al.*, 1993). The purified N-type Ca^{2+} channel complex consists of several subunits. Samples from each of the purification steps were separated on a 3–12% linear gradient SDS–polyacrylamide gel and stained with Coomassie blue (Fig. 1). The N-type Ca^{2+} channel is composed of a 230 kDa subunit, which was identified with specific polyclonal antibodies to the cloned α_{1B} subunit, a 140 kDa subunit, highly homologous to the skeletal muscle DHP receptor α_2 subunit, a 95 kDa subunit and a 57 kDa subunit, which was identified with specific polyclonal antibodies to the cloned β_3 subunit (Witcher *et al.*, 1993). The position of these subunits are indicated by the arrows in Fig. 1, lane 6 (α_{1B}, α_2, 95 K and β_3). A few minor proteolytic fragments were detected on SDS–PAGE which varied in amount and were identified with affinity-purified antibodies against the individual subunits. The purified N-type Ca^{2+} channel co-migrated on sucrose density gradients with the peak of [^{125}I]ω-conotoxin binding activity (Witcher *et al.*, 1993). Western blot analysis of the sucrose density gradient fractions with affinity-purified sheep

polyclonal antibodies to each subunit demonstrated that all four subunits of the receptor complex co-migrate on the sucrose density gradients and are immunologically distinct (Fig. 2). Each subunit of the N-type Ca^{2+} channel also co-immunoprecipitated with the receptor complex using purified antibodies against individual subunits (not shown).

Characterization of polyclonal antibodies specific for the N-type Ca^{2+} channel complex

To determine the specificity of the polyclonal antibodies against the purified N-type Ca^{2+} channel complex, rabbit brain membranes were labeled with either [^{3}H]PN200-110 or [^{125}I]ω-CgTx, solubilized and immunoprecipitated with whole sheep serum (which contained antibodies against the N-type Ca^{2+} channel complex; α_1, α_2, 95 K and β subunits) or affinity-purified antibodies to various GST fusion proteins of the cloned α_1 class B subunit and the β_3 subunit (α_{1B}, β_{3C} and β_{3N}). A dose-dependent immunoprecipitation of brain [^{125}I]ω-CgTx binding sites shows that more than 90% of the solubilized [^{125}I]ω-CgTx receptor was immunoprecipitated by 10 μl of sheep serum [Fig. 3(A), solid line]. Preimmune serum did not immunoprecipitate [^{125}I]ω-CgTx binding sites (not shown).The amount of [^{125}I]ω-CgTx binding sites immunoprecipitated was not significantly reduced by preincubation of the polyclonal antibody-protein G beads with 5 μg of a full length brain β_1 subunit GST fusion protein (Pragnell, Sakamoto, Jay and Campbell, 1991) [Fig. 3(B), solid line]. In comparison, the same maximum volume of sheep serum only immunoprecipitated 34% of the specific brain [^{3}H]PN200-110 binding sites [Fig. 3(A), dashed line]. This immunoprecipitation of brain [^{3}H]PN200-110 binding sites by the serum could be significantly reduced by preincubation of the polyclonal antibody-protein G beads with 5 μg of the brain β_{1b} subunit fusion protein before immunoprecipitation [Fig. 3(B), dashed line]. These results suggest that common epitopes identified by the polyclonal antibodies on the brain dihydropyridine (DHP) receptor and the ω-CgTx receptor are mainly located on the β subunit.

Sheep affinity-purified polyclonal antibodies to specific GST fusion proteins from different regions of the β_3 subunit (β_3 COOH-terminal, β_{3C} and β_3 NH_3-terminal, β_{3N}) and the intracellular loop between the second and third transmembrane domains of the α_{1B} subunit of the N-type Ca^{2+} channel complex were also used to immunoprecipitate [^{125}I]ω-CgTx and [^{3}H]PN200-110 binding sites. Affinity-purified polyclonal antibodies to the α_{1B} fusion protein immunoprecipitated 83% of the [^{125}I]ω-CgTx binding sites while these same antibodies did not precipitate any significant amount of the brain DHP receptor [Fig. 3(C)]. Similarly, affinity-purified polyclonal antibodies to the β_{3C} fusion protein, which is specific for the β_3 subunit of the N-type Ca^{2+} channel complex, immunoprecipitated 85% of the [^{125}I]ω-CgTx binding sites and no significant amount of brain [^{3}H]PN200-110 binding sites. Affinity-purified polyclonal antibodies to the β_{3N} fusion protein, which contains regions homologous to all β subunits, immunoprecipitated 85 and 63% of the [^{125}I]ω-CgTx and [^{3}H]PN200-110 binding sites respectively.

Characterization of [^{125}I]ω-CgTx binding to the purified N-type Ca^{2+} channel

We have previously demonstrated that the purified N-type Ca^{2+} channel binds [^{125}I]ω-CgTx with a K_d of 0.06 nM which is in agreement with the reported affinity for this peptide to the membrane bound receptor (Cruz and Olivera, 1986; Wagner, Snowman, Biswas, Olivera and Snyder, 1988; Abe, Koyano, Saisu, Nishiuchi and Sakakibara, 1986; Sakamoto and Campbell, 1991). To further characterize the ω-CgTx binding site on the N-type Ca^{2+} channel complex, binding and electrophysiological experiments were performed on the purified complex. It has previously been shown that cations affect the binding of ω-CgTx to its membrane-bound receptor. Physiological concentrations of calcium were also shown to inhibit the peptide-receptor binding noncompetitively with an IC_{50} of 0.2–4 mM (Barhanin, Schmid and Lazdunski, 1988; Wagner et al., 1988; Sakamoto and Campbell, 1991). Cations were tested for their effects on ω-CgTx binding to the purified N-type Ca^{2+} channel complex. The effects of calcium and barium were assessed by measuring the specific [^{125}I]ω-CgTx binding in the presence of increasing cation concentrations. Both calcium and barium have a potent inhibitory effect on the binding of ω-CgTx to the purified N-type Ca^{2+} channel [Fig. 4(A)]. The half-maximal displacement of [^{125}I]ω-CgTx binding occurs at 2.5 mM for calcium and 5 mM for barium. These values are very similar to what has been demonstrated for the membrane bound receptor.

The dissociation of [^{125}I]ω-CgTx in the presence of divalent cations was examined by incubating the peptide with the purified N-type Ca^{2+} channel to maximum equilibrium binding for 1 hr at room temperature. Dissociation was initiated by the addition of barium chloride to the binding reaction such that the final concentration of barium was 50 mM. The amount of remaining [^{125}I]ω-CgTx binding was determined at 0.5, 1 and 1.5 hr after the addition of barium. Only approximately 5% of [^{125}I]ω-CgTx was dissociated from the purified receptor in the first 15 min after the addition of 50 mM barium. At 0.5 hr, the percent of binding was 89% of the maximum level [Fig. 4(B)]. A small dissociation of ω-CgTx from its membrane bound receptor has also been demonstrated in the presence of 2 mM $CaCl_2$ and 140 mM NaCl (Barhanin, Schmid and Lazdunski, 1988). The dissociation in the presence of barium appears to be slightly greater than in the absence of barium (not shown).

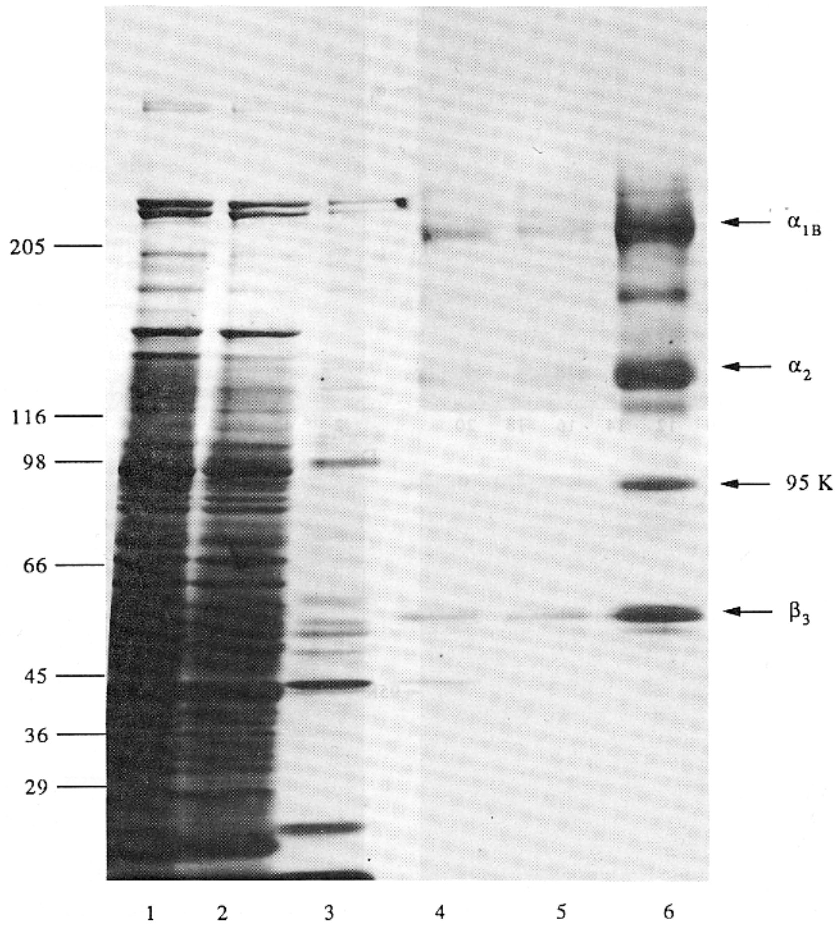

Fig. 1. Summary of the N-type Ca^{2+} channel purification. Samples from each purification step were analyzed on Coomassie blue-stained 3–12% SDS–polyacrylamide gels. The samples are described as follows; 100 μg of rabbit brain membranes (1), 100 μg of digitonin-solubilized brain membranes (2), 50 μg of heparin–agarose pooled fractions (3), 8 μg of $VD2_1$ elution (4), 8 μg void from anti-46 kDa polyclonal column (5), 30 μg of sucrose density gradient fractions 10 and 11 (6). The positions of the subunits of the N-type Ca^{2+} channel on the SDS-polyacrylamide gel are indicated by the arrows on the right (α_{1B}, α_2, 95 K and β_3). Molecular weight standards ($\times 10^{-3}$) are indicated on the left.

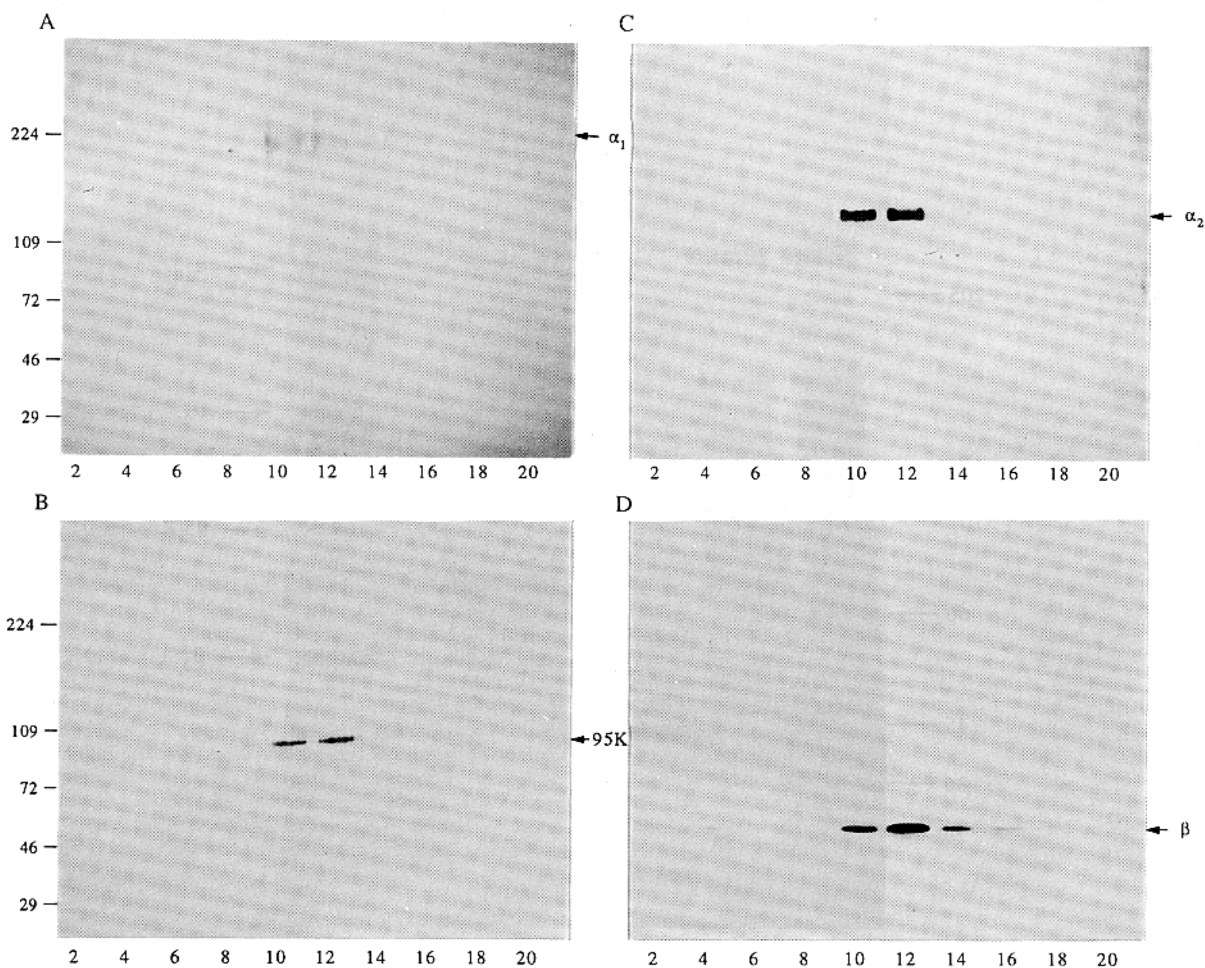

Fig. 2. Immunoblot analysis of the N-type Ca^{2+} channel. (A–D) Sucrose density gradient fraction 2–20 (even numbered fractions) were separated on 3–12% polyacrylamide gels and transferred to nitrocellulose. Nitrocellulose transfers were stained with: (A) affinity-purified sheep polyclonal antibodies against the α_1 subunit; (B) affinity-purified sheep polyclonal antibodies against the 95 K subunit; (C) affinity-purified sheep polyclonal antibodies against the α_2 subunit; or (D) affinity-purified sheep polyclonal antibodies against the β subunit. The molecular weight standards ($\times 10^{-3}$) are indicated on the left and the subunit components of the N-type Ca^{2+} channel complex are indicated on the right (α_1, α_2, 95 K, β).

Mechanism of ω-CgTx block of the purified N-type Ca^{2+} channel

Reconstitution of the purified ω-CgTx receptor into bilayers shows that the receptor is a functional channel (Figs 5 and 6). The channel is active over a wide range of potentials and does not show any signs of inactivation (Witcher *et al.*, 1993). In 67% of control recordings, channel activity was characterized by an extremely high open state probability. Figure 5(A) shows a typical example of such a recording at a holding potential of +50 mV in symmetrical 100 mM Ba^{2+}. The mean channel amplitude was 1.25 ± 0.22 pA, yielding an estimated conductance of 25 pS (not shown). The opening probability of the channel was 83% as estimated over 17 sec of recording. The high incidence of channels with high open state probability allowed us to test for the mechanism of ω-CgTx block at the single channel level. Previously we have shown that ω-CgTx binding is inhibited by high divalent cation concentrations, therefore precluding a direct comparison of the properties of single channel activity in the bound and unbound states in high divalent cation concen-

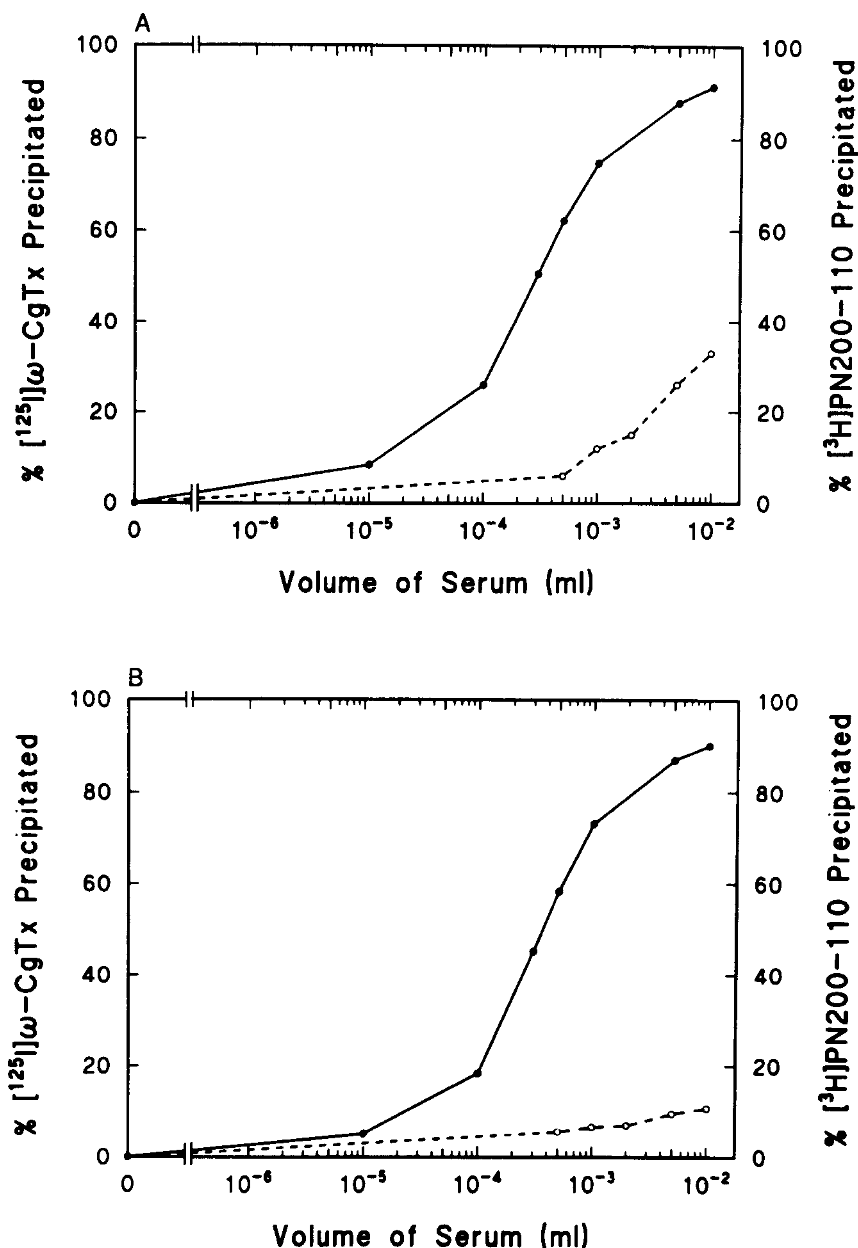

Fig. 3—*continued overleaf.*

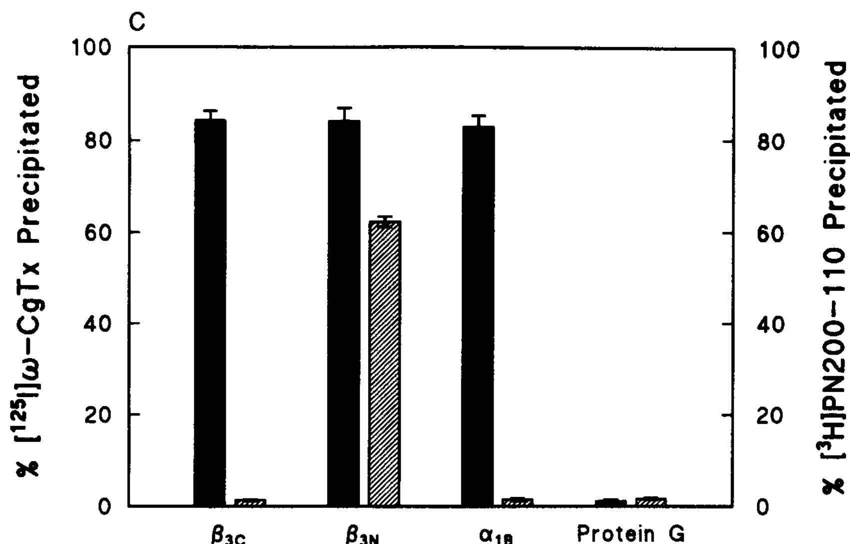

Fig. 3. Immunoprecipitation of [^{125}I]ω-CgTx and [^{3}H]PN200-110 binding sites from solubilized brain membranes. (A) Shown is the immunoprecipitation of [^{125}I]ω-CgTx binding sites with increasing volumes (0.001–10 μl) of sheep polyclonal antibodies against the purified N-type Ca^{2+} channel complex coupled to protein-G beads. The dashed line represents the amount of [^{3}H]PN200-110 binding sites immunoprecipitated by the polyclonal antibody-protein G beads. (B) Immunoprecipitation of [^{125}I]ω-CgTx binding sites with increasing volumes of sheep polyclonal antibody-protein G beads preincubated with 5 μg of purified bacterially expressed GST fusion protein containing a full length neuronal β_{1b} subunit. The dashed line represents the amount of [^{3}H]PN200-110 binding sites immunoprecipitated with polyclonal antibody-protein G beads preincubated with the same β_{1b} GST fusion protein. (C) Immunoprecipitation of both [^{125}I]ω-CgTx (solid bars) and [^{3}H]PN200-110 (hatch bars) binding sites by affinity-purified polyclonal antibodies to a β_3 COOH-terminal GST fusion protein (β_{3C}), a β_3 NH_3-terminal GST fusion protein (β_{3N}), an α_{1B} GST fusion protein (α_{1B}) and protein G beads alone (Protein G). Data are presented as the mean of three experiments, and the error bars represent the standard deviation.

trations. However, we now have taken advantage of the fact that once ω-CgTx was bound to its receptor, the association is not quickly reversed by the subsequent addition of 50 mM barium. To minimize partial dissociation of the toxin, single channel activity was assessed in the presence of symmetrical 0.5 μM ω-CgTx within 10 min of the addition of barium. Fifty millimolar barium was used since it was the lowest concentration achievable without significantly affecting the channel conductance (mean conductance in 100 mM barium is 18.0 ± 5.3 pS, $n = 26$ and in 50 mM is 15.2 ± 2.4 pS, $n = 6$). Figure 5(B) demonstrates that under these conditions, channel activity was not completely abolished ($n = 5$). Interestingly, channel activity in the presence of ω-CgTx and barium was characterized by the switching between two gating modes, a high open-state probability mode ($P_0 > 0.9$) to a low open-state probability mode ($P_0 < 0.2$) [Fig. 5(B), left panel]. This is different from control recordings where no switching between open-state probability modes could be detected ($n = 26$). The systematic observation of channels switching to a low open-state probability in the presence of the toxin suggests that the binding of ω-CgTx to its site favors the induction of a lower open-state probability. A plot of the open-state probabilities of the channel over 5 sec episodes demonstrates the switching of P_0 modes over a 17 min period at −60 mV [Fig. 5(C)]. An amplitude histogram of high and low P_0 events revealed a single Gaussian distribution with a mean channel amplitude of −0.97 pA [Fig. 5(C), left panel]. A plot of channel amplitude versus open time distribution for each event revealed that long open-state events (found more commonly in the high P_0 mode) had the same amplitude as short open-state events (mostly found in the low P_0 mode). These data suggest that ω-CgTx affected the open-state probability of the channel without altering single channel amplitude. Because channel activity was seen in the presence of bound ω-CgTx, the toxin does not appear to act as a pore blocker.

The dependence of ω-CgTx block to voltage and direction of barium flux relative to channel orientation was tested. We analyzed the amplitude and conductance of the channel over a wide range of potentials and in conditions that favored barium flux from both sides of the channels. Figure 6(A) shows representative traces at various holding potentials (pipette) of the channel in the low P_0 mode. Amplitude measurements at various holding potentials yielded a single channel conductance of 16.7 pS [Fig. 6(B)]. The absence of any sign of rectification suggests that the effects of ω-CgTx

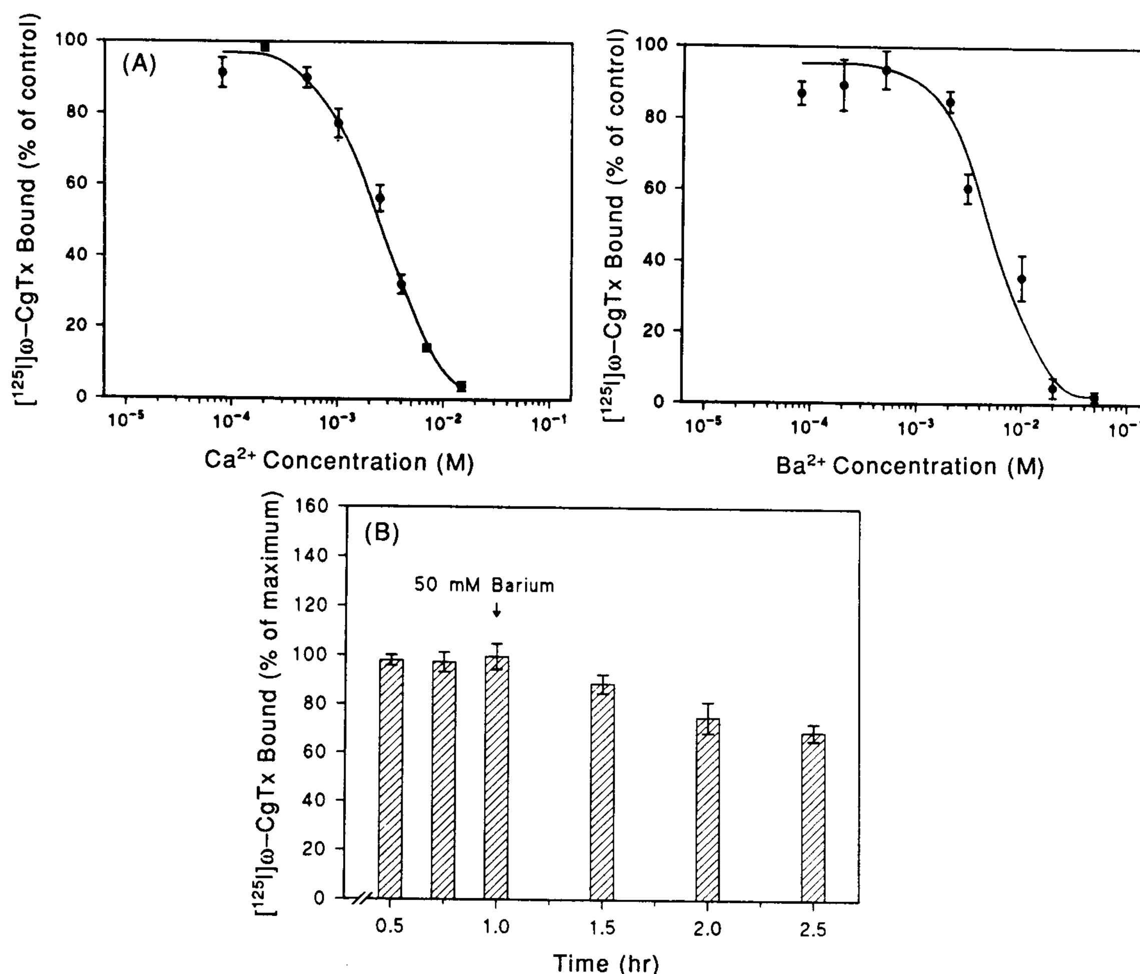

Fig. 4. Characterization of $[^{125}I]\omega$-CgTx binding to the N-type Ca^{2+} channel complex. (A) Effects of calcium and barium ions on $[^{125}I]\omega$-CgTx binding. Purified N-type Ca^{2+} channel complex was incubated with either 0.08–15 mM $CaCl_2$ (left) or 0.08–50 mM $BaCl_2$ (right). Specific binding of 0.25 nM $[^{125}I]\omega$-CgTx was determined at each cation concentration and the results are expressed as the percentage of binding in the absence of cations (control). Data are presented as the mean of three experiments, and the error bars represent the standard deviation. (B) Dissociation time-course of $[^{125}I]\omega$-CgTx from its receptor after the addition of 50 mM $BaCl_2$. Specific binding of 0.25 nM $[^{125}I]\omega$-CgTx was determined at each time after the addition of 50 mM $BaCl_2$. The results are expressed as the percentage of maximum binding at 1 hr in the absence of barium.

were solely on the open-state probability of the channel and that these effects lacked voltage-dependency.

DISCUSSION

The N-type Ca^{2+} channel has been purified from rabbit brain membranes (Witcher *et al.*, 1993). The channel is composed of an α_{1B} subunit, a $\alpha_2\delta$ subunit similar to that of the skeletal muscle DHP-receptor $\alpha_2\delta$ subunit, a novel 95 K subunit, and a β_3 subunit. In the present work, we have prepared polyclonal antibodies specific to each of the subunits of the receptor complex and have used these antibodies to biochemically characterize the N-type Ca^{2+} channel complex. Our results demonstrated that the subunits of the N-type Ca^{2+} channel migrate on sucrose density gradients as a single complex. Polyclonal antibodies against the whole complex immunoprecipitate solubilized $[^{125}I]\omega$-CgTx binding sites from brain membranes. Furthermore, affinity-purified polyclonal antibodies to unique GST fusion proteins from both α_{1B} and β_3 subunits immunoprecipitate only solubilized $[^{125}I]\omega$CgTx binding sites and not $[^3H]$PN200-110 binding sites. These data demonstrate that the purified N-type Ca^{2+} channel is a multisubunit complex. It has been previously shown that a neuronal L-type Ca^{2+} channel also exists as a multisubunit complex (Takahashi and Catterall, 1987; Yoshida, Takahashi, Fujimoto, Takisawa and Nakamura, 1990; Ahlijanian, Westenbroek and Catterall, 1990). Since the preincubation of the sheep anti-N-type Ca^{2+} channel polyclonal antibody beads with β_{1b} GST fusion protein significantly reduced the small amount of $[^3H]$PN200-110 binding sites immunopre-

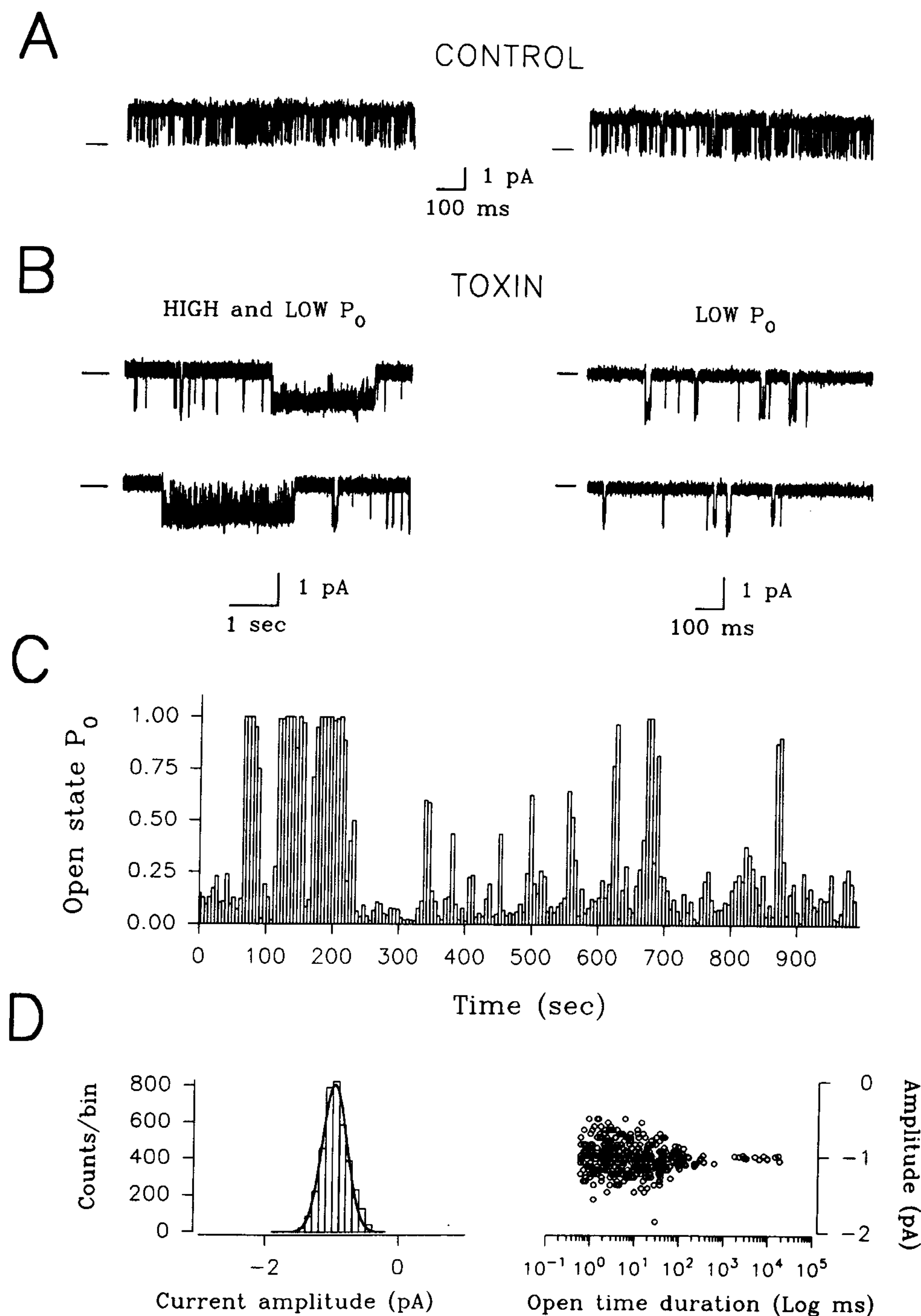

Fig. 5. Effect of high divalent cation concentration of the block of barium permeability by ω-CgTx. (A) Control traces of the purified reconstituted N-type Ca^{2+} channel in a high P_0 state. Holding potential is +50 mV and filter was set at 3 kHz. (B) Traces of reconstituted receptor preincubated 30 min with 10 μM ω-CgTx, then reconstituted in the presence of symmetrical 50 mM Ba^{2+} and 0.5 μM ω-CgTx. Left panel: channel activity from two different gating modes (high and low-opening probability of the channel). Right panel: channel activity in low P_0 mode. Note the difference in the time scale for both panels. The holding potential for this recording is −60 mV and traces are filtered at 2 kHz. (C) Fluctuations of open-state probability versus time. The open-state probability of the channel illustrate in (B) was sampled every 5 sec for a period exceeding 16 min. The plot illustrates the oscillatory activity of the channel. (D) Amplitude histogram and plot of the open-time duration versus amplitude for each event. The mean current amplitude is $\mu = -0.967 \pm \tau = 0.184$ ($n = 3775$). Amplitude histogram is fitted by $N = (W)\exp[-(A - \mu)^2/2 \times \tau^2]/(2\pi)^{0.5} \times \mu$ with W constant equal to 1951, and N the counts at each amplitude A. Bin width is 0.1 pA/division.

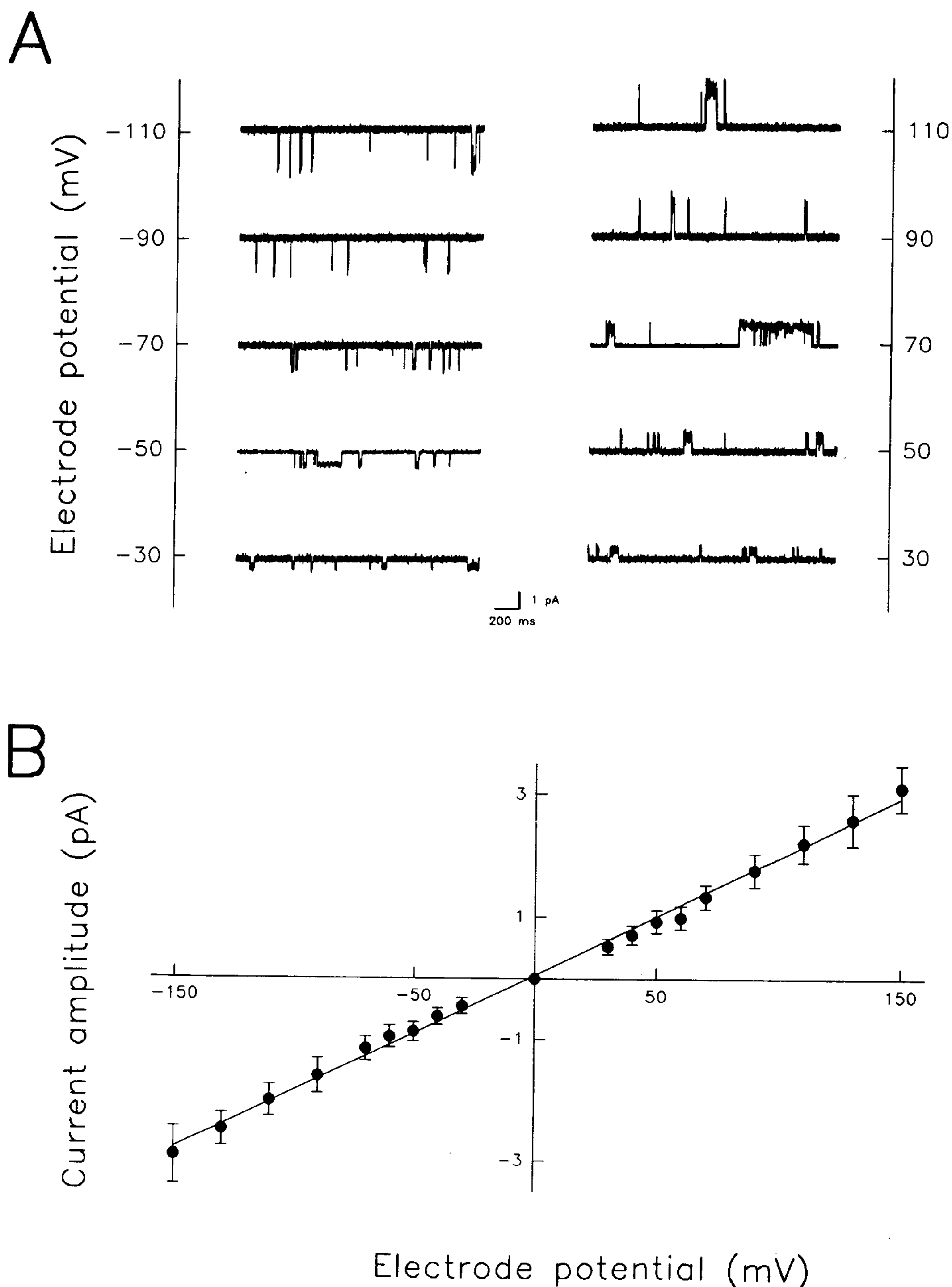

Fig. 6. Conductance and voltage-independence of N-type channel activity in the presence of ω-CgTx. (A) Representative channel activity at various holding potentials. Scales on left and right side show the potential at which the channel was held. Negative pipette potentials (left panel) result in negative Ba flow with openings downward and positive potentials (right panel) result in openings with upward deflections. In symmetrical ionic conditions, increases in driving forces (positive or negative) result in increases in single channel amplitudes. All traces were filtered at 2 kHz except at −50 mV (1 kHz) and +70 mV (1.5 kHz). (B) Single channel conductance of the channel. Each single point is the mean from amplitude histograms at each holding potential. Amplitude histograms contained between 62 and 3775 events. Conductance of the channel is 16.7 pS as estimated from a first order regression fit to the data.

cipitated, the neuronal DHP-receptor may contain a β subunit with similar epitopes as the β_{1b} subunit.

Previously, we have demonstrated that the purified N-type Ca^{2+} channel contains a very high-affinity binding site for $[^{125}I]$-ω-CgTx (Witcher *et al.*, 1993). Our recent results demonstrate that the ω-CgTx binding site of the purified receptor has properties that are very similar to that of the membrane-bound receptor. The purified N-type Ca^{2+} channel bound $[^{125}I]\omega$-CgTx with a K_d of 60 pM. Other investigators have described a similar high-affinity binding site in brain membranes (Cruz and Olivera, 1986; Abe *et al.*, 1986; Feigenbaum, Garcia and Kaczorowski, 1988; Wagner *et al.*, 1988). Our results demonstrate that cations have a potent inhibitory effect on the binding of $[^{125}I]\omega$-CgTx on the purified N-type Ca^{2+} channel complex. Since it is known that the potency of cation block of ω-CgTx binding follows the rank order: $Na^+ < Ba^{2+} < Ca^{2+} < Cd^{2+}$ (Abe *et al.*, 1986) and the opposite order exists for permeability, it is possible that the Ca^{2+} binding sites(s) which regulates the ω-CgTx binding is located in the pore of the channel.

Single channel analysis of the purified N-type Ca^{2+} channel in the presence of 1 μM ω-CgTx and 10 mM barium demonstrated that the toxin was able to block the barium current (Witcher *et al.*, 1993). Our recent results show that in the presence of high divalent cation concentrations (50 mM barium) ω-CgTx does not completely block the single channel activity of the reconstituted channel. Our data show that ω-CgTx did not affect the conductance of the channel and therefore the toxin can not be considered as a partial pore blocker. Instead, ω-CgTx had a significant effect on the open-state probability of the channel. These observations are consistent with the data of Carbone and Lux (1988) who showed that Ca^{2+} flux in chick sensory neurons could be blocked by ω-CgTx but not Na^+ flux in the absence of Ca^{2+}. It has previously been shown that calcium is a noncompetitive inhibitor of $[^{125}I]\omega$-CgTx binding to the membrane-bound receptor (Wagner *et al.*, 1988). Since 50 mM barium does not substantially reduce the amount of $[^{125}I]\omega$-CgTx bound to the purified receptor in 30 min, barium may be allosterically regulating the extent of ω-CgTx block of single channel activity. With these results, we conclude that ω-CgTx can not be considered as a complete inhibitor of the purified N-type Ca^{2+} channel at high divalent cation concentrations. Single channel analysis of the receptor in high barium revealed that a likely mechanism of action of ω-CgTx was to dramatically decrease the open probability of the channel and not to block the pore.

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