Subunit Identification and Reconstitution of the N-Type Ca^{2+} Channel Complex Purified from Brain

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Calcium channels play an important role in regulating various neuronal processes, including synaptic transmission and cellular plasticity. The N-type calcium channels, which are sensitive to ω-conotoxin, are involved in the control of transmitter release from neurons. A functional N-type calcium channel complex was purified from rabbit brain. The channel consists of a 230-kilodalton subunit (α_{1B}) that is tightly associated with a 160-kilodalton subunit ($\alpha_2\delta$), a 57-kilodalton subunit (β_3), and a 95-kilodalton glycoprotein subunit. The complex formed a functional calcium channel with the same pharmacological properties and conductance as those of the native ω-conotoxin–sensitive calcium channel in neurons.

In neurons, the calcium influx that triggers vesicle fusion to the presynaptic membrane and subsequent neurotransmitter release is the result of the activation of voltage-sensitive Ca^{2+} channels in the plasma membrane (*1*). Using freeze-fracture electron microscopy, investigators have identified active zone particles in the presynaptic membrane that have been proposed to be voltage-sensitive Ca^{2+} channels (*2*). These Ca^{2+} channels may be the antigen or may be associated with the antigen recognized by pathogenic autoantibodies in small cell lung carcinoma involved in Lambert-Eaton myasthenic syndrome (*3*). The N-type Ca^{2+} channels are distinguished from L-, T-, and P-type voltage-dependent Ca^{2+} channels by electrophysiological and pharmacological properties (*4*). The peptide ω-conotoxin GVIA, isolated from the snail *Conus geographus*, selectively blocks N-type Ca^{2+} channels, whereas the L-type Ca^{2+} channels are inhibited by dihydropyridines (DHPs) (*5*). The DHP-sensitive Ca^{2+} channel from skeletal muscle has been purified and is composed of four subunits: α_1 (molecular weight 175 kD), $\alpha_2\delta$ (160 kD), β (52 kD), and γ (32 kD) (*6*). At least four genes encoding Ca^{2+} channel α_1 subunits from the brain share homology with the α_1 subunit of the skeletal muscle DHP receptor (*7, 8*). Recently, the complementary DNA (cDNA) encoding the human neuronal class B α_1 subunit (*7*) has been transiently expressed to produce ω-conotoxin–sensitive currents (*9*). Although there has been recent progress in molecular biological studies of brain Ca^{2+} channel subunits (*7, 8*), little is known about the native structure and function of neuronal Ca^{2+} channels.

We have purified the ω-conotoxin receptor (N-type Ca^{2+} channel) from digitonin-solubilized rabbit brain membranes by heparin chromatography, immunoaffinity chromatography, and sucrose density gradient centrifugation (*10*). The receptor complex migrated as a single peak on the sucrose density gradient (Fig. 1A) and contained four subunits of molecular weight 230 kD, 140 kD (reduced), 95 kD, and 57 kD, all of which comigrated with the peak of binding to ^{125}I-labeled ω-conotoxin (Fig. 1B) and were in a stoichiometric ratio of 1:1.0:0.9:1.3. In more than 70 purifications, these four subunits were consistently observed.

The isolated ω-conotoxin receptors appeared as globular complexes (Fig. 1C). Most of the complexes were within a narrow size range, indicating the purity of the preparation. The approximate diameter was 16 nm, which is similar to the size of active zone particles in the presynaptic membranes, as visualized by freeze-fracture electron microscopy (*2*). The few larger complexes in each image were possibly aggregates of receptors.

Rabbit brain membranes bound ^{125}I-labeled ω-conotoxin with a dissociation constant (K_d) of 0.08 nM, in agreement with other reports (*11, 12*), and a maximum binding capacity (B_{max}) of 305 fmol per milligram of protein. A 2400-fold purification of the receptor was achieved, yielding 60 μg of purified N-type Ca^{2+} channel. The purified receptor bound ^{125}I-labeled ω-conotoxin with a K_d of 0.06 nM and a B_{max} of 423 pmol/mg (Fig. 1D); however, it did not bind [^{3}H]PN200-110, a specific blocker of L-type Ca^{2+} channels. Only a single binding site was observed for ω-conotoxin both in brain membranes and for the purified receptor.

To confirm the subunit composition of the receptor, we generated sheep antibodies

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against the purified receptor complex. Antibodies were produced against each subunit of the purified receptor. Immunoblot analysis of the fractions obtained from the purification of the ω-conotoxin receptor demonstrated that all four subunits copurified with ^{125}I-labeled ω-conotoxin binding activity. Analysis of the sucrose density gradient fractions (*13*) showed that all four subunits comigrated on the sucrose gradient and were immunologically distinct.

To compare the subunit composition of the ω-conotoxin receptor channel to that of the neuronal DHP receptor, we labeled rabbit brain membranes with either [^{3}H]PN200-110 or ^{125}I-labeled ω-conotoxin and solubilized and immunoprecipitated them with affinity-purified antibodies to the various subunits of the ω-conotoxin receptor (*14*). Affinity-purified antibodies to the ω-conotoxin receptor α_1 and 95K subunits immunoprecipitated 79 and 74% of the ^{125}I-labeled ω-conotoxin binding sites, respectively, and did not precipitate any significant amount of the brain DHP receptor (Table 1). Polyclonal antibodies against the β subunit and monoclonal antibody VD2$_1$ each immunoprecipitated 84% of the ^{125}I-labeled ω-conotoxin binding sites. They also immunoprecipitated 19 and 68% of the [^{3}H]PN200-110 binding sites, respectively (Table 1). This suggests that the β subunit of the two brain receptors share similar epitopes, whereas the α_1 and 95K subunits of the ω-conotoxin receptor appear to be unique.

Immunoblot analysis showed that affinity-purified polyclonal antibodies to the α_1 and 95K subunits recognized only the brain ω-conotoxin receptor and not the skeletal muscle DHP receptor (*15*) (Fig. 2). Also, polyclonal antibodies to the β subunit of the ω-conotoxin receptor weakly identified the β_1 subunit of the skeletal muscle DHP receptor. These results, along with the immunoprecipitation data, suggest that the β subunit of the ω-conotoxin receptor is different from the β subunit of the brain and skeletal muscle DHP receptor. Affinity-purified polyclonal antibodies to the ω-conotoxin receptor α_2 subunit identified the α_2 subunit of both the ω-conotoxin receptor and skeletal muscle DHP receptor (*16*) (Fig. 2). The α_2 subunits of both receptors have been identified as glycoproteins that bind wheat germ agglutinin (WGA) (Fig. 2). By treating the ω-conotoxin receptor complex with *N*-glycosidase F (*16*), we demonstrated that the α_2 subunit as well as the 95K subunit contained N-linked sugars. Thus, the purified ω-conotoxin receptor consists of an α_1, $\alpha_2\delta$, β, and also a 95K subunit.

Because genes encoding different α_1 and β subunits exist in neuronal tissue, it was important to further identify the subtype of α_1 and β subunits in the receptor complex. Polyclonal antibodies affinity-purified against a fusion protein made from the unique intracellular loop between the second and third transmembrane domains of the cloned class B α_1 subunit (*16*) recognized only the α_1 subunit of the ω-conotoxin receptor (Fig. 2). Affinity-purified sheep polyclonal antibodies against the β_3-specific COOH-terminal fusion protein identified only the ω-conotoxin receptor β subunit (*16*). However, affinity-purified antibodies against the NH_2-terminal β_3 fusion protein, containing regions homologous to all β subunits, identified the β subunits of both the ω-conotoxin receptor and the DHP receptor (Fig. 2).

The ω-conotoxin receptor was reconstituted in phospholipid bilayers (*17*) made from the apposition of monolayers at the tips of patch pipettes (*18*) (Fig. 3). Channel activity showed little voltage dependency under these experimental conditions (Fig. 3A), a result

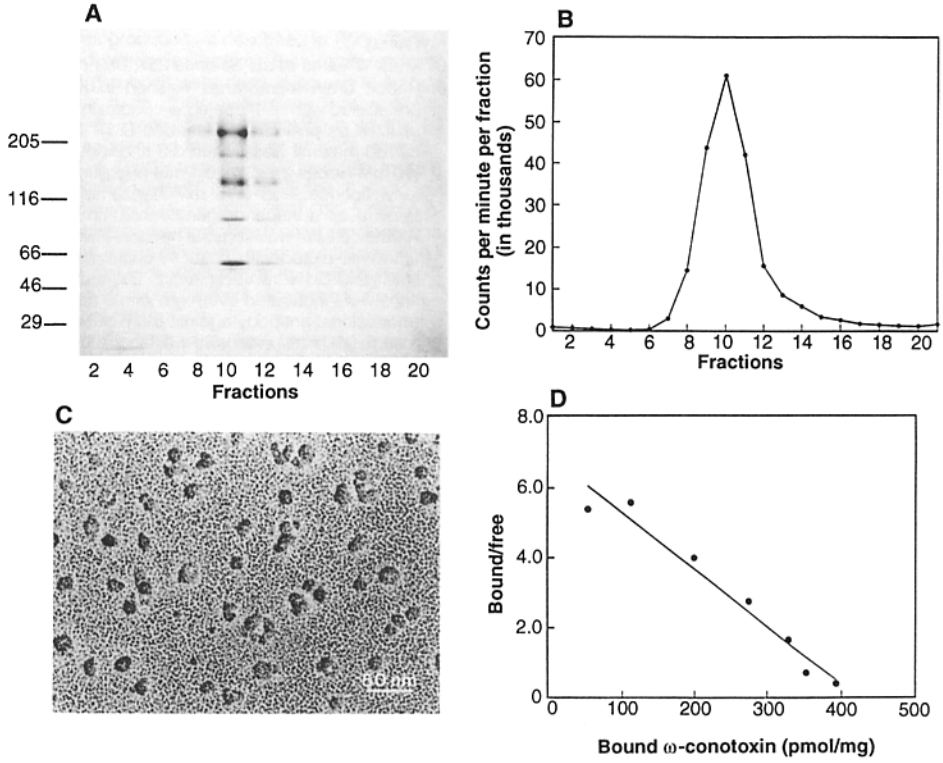

Fig. 1. Structural characterization of the purified ω-conotoxin receptor. (**A**) Sucrose density gradient fractions of the purified ω-conotoxin receptor stained with Coomassie blue. Molecular weight markers (in kilodaltons) on left. (**B**) Distribution of ^{125}I-labeled ω-conotoxin bound to the purified receptor. (**C**) Rotary-shadowing electron microscopy of the purified ω-conotoxin receptor (*25*). (**D**) Scatchard analysis of ^{125}I-labeled ω-conotoxin binding to the purified receptor (in picomoles per milligram of protein).

Table 1. Immunoprecipitation of ω-conotoxin and neuronal DHP receptor. The percentage of ^{125}I-labeled ω-conotoxin and [^{3}H]PN200-110 immunoprecipitated by various antibodies as described (*14*). Values are mean ± SD from n = 3 experiments, except for those marked with asterisk.

Antibody	ω-Conotoxin (%)	[^{3}H]PN200-110 (%)
Anti-α_1 (*13*)	79.1 ± 0.6*	1.7 ± 0.7*
Anti-95kD (*13*)	73.7 ± 2.2	2.5 ± 1.4
Anti-β (*13*)	83.5 ± 1.1*	19.0 ± 0.8
VD2$_1$ (*10*)	83.7 ± 1.3	68.2 ± 1.3
No antibody	1.1 ± 0.1	1.1 ± 0.1

*From n = 4 experiments.

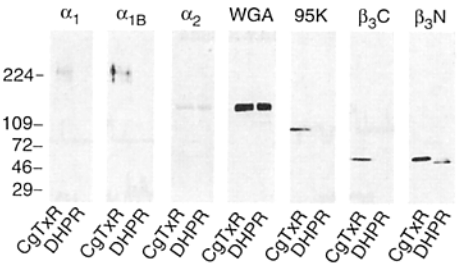

Fig. 2. Identification of the ω-conotoxin receptor subunits by immunoblot analysis. Nitrocellulose transfers of purified ω-conotoxin receptor (CgTxR) and skeletal muscle DHP receptor (DHPR) were stained with affinity-purified sheep polyclonal antibodies to the α_1 subunit (α_1) of the ω-conotoxin receptor, affinity-purified polyclonal antibodies to an α_1 class B fusion protein (α_{1B}), affinity-purified antibodies to the α_2 subunit (α_2) of the ω-conotoxin receptor, 1 μg of peroxidase-conjugated WGA per milliliter (WGA), affinity-purified antibodies to the 95-kD subunit (95K), and affinity-purified antibodies to the COOH- (β_3C) and NH_2- (β_3N) terminal fusion proteins derived from a β_3 clone. Molecular weight markers (in kilodaltons) on left.

similar to that described from the reconstitution of the skeletal muscle DHP receptor (*19*). No channel activity was recorded with no channels ($n = 7$), addition of 0.1% digitonin ($n = 4$), heat-inactivated channels ($n = 5$), or trypsin-digested channels ($n = 11$). Thus, channel activity was a result of the incorporation of receptors into the bilayer. Moreover, no difference in channel activity could be detected whether the channels were purified with digitonin ($n = 43$) or CHAPS ($n = 10$). The unphosphorylated purified ω-conotoxin receptor complex exhibited a conductance between 14 and 25 pS in symmetrical 100 mM barium ($n = 6$), similar to that of the native N-type Ca^{2+} channel (*20*), whereas the phosphorylated receptor displayed more complex properties. The channel did not conduct Na^+, and Ba conductance was three times higher than Ca^{2+} conductance of the channel. When recording in symmetrical 10 mM Ba, 1 μM ω-conotoxin was required to inhibit the average unitary current by 100% ($n = 3$) (Fig. 3B). Furthermore, no channel activity was detected when the receptor was preincubated with 5 μM ω-conotoxin ($n = 10$, asymmetrical ionic conditions). However, channel activity was seen in 9 of 10 experiments in similar ionic conditions but in the absence of the toxin. Finally, the unitary currents were insensitive to DHP agonist and antagonist (Fig. 3C). The addition of 1 μM BAY K 8644 or 1 μM nitrendipine did not significantly affect the single-channel activity and the average unitary current. These results, along with the biochemical data, indicate the absence of DHP-sensitive channels in the purified receptor preparation and further confirm the lack of sensitivity of the ω-conotoxin receptor to DHPs.

Our results demonstrate that the ω-conotoxin receptor complex is the N-type Ca^{2+} channel and is composed of four immunologically distinct subunits. Comigration of the receptor subunits on sucrose gradients and immunoprecipitation experiments with subunit-specific polyclonal antibodies demonstrate that the complex is tightly associated. Recent biochemical studies have suggested that a few of the proteins that compose the ω-conotoxin receptor may have molecular weights similar to those of subunits of the DHP receptor (*21*). Our data suggest that the subunit composition of the ω-conotoxin receptor is similar but distinct from the DHP receptor. Our results illustrate that the reconstituted ω-conotoxin receptor forms a Ca^{2+} channel with pharmacological properties similar to that of the native N-type Ca^{2+} channel. Finally, it is known that different β subunits (β_1, β_2, and β_3) modulate the same α_1 subunit with different efficiencies in expression studies (*22*). We have identified the β_3 subunit as a component of the N-type Ca^{2+} channel complex.

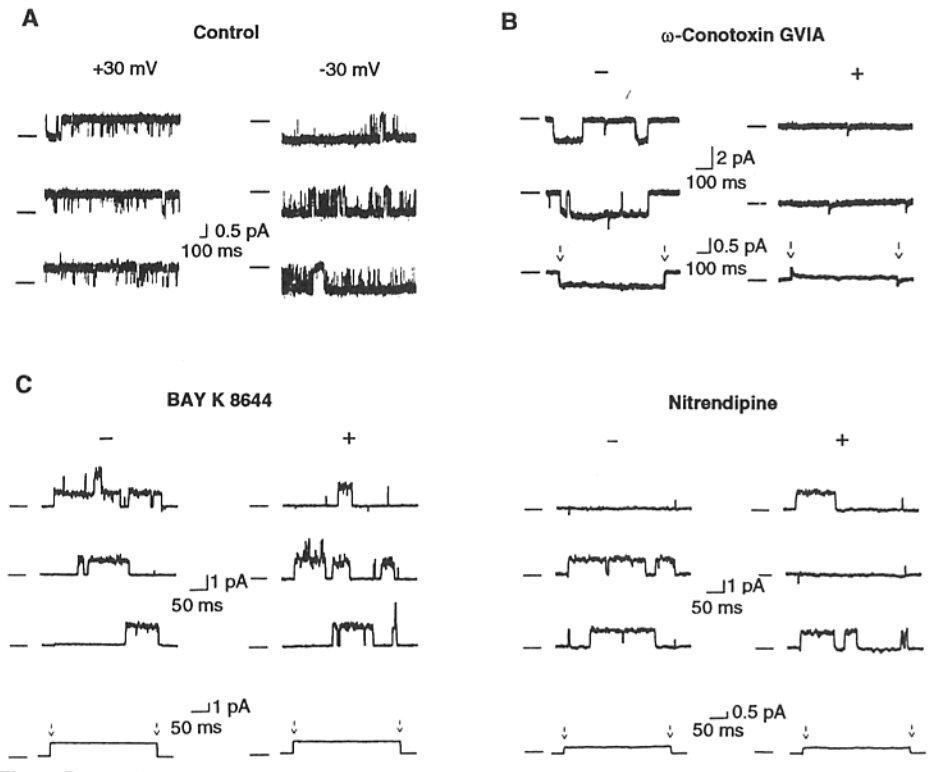

Fig. 3. Reconstitution of the ω-conotoxin receptor into phospholipid bilayers. (**A**) Control recordings of channel activity in symmetrical barium condition. Solid line represents the closed state of the channel. (**B**) Presence of ω-conotoxin inhibits Ba unitary currents. Currents without (−) and 4 min after addition of (+) 1 μM ω-conotoxin into the bath. Top two rows: control recordings at a pipette voltage of −100 mV; bottom row: leak-subtracted average current (between arrows) of 100 episodes after stepping from 0 to −100 mV. Transients under the arrows are capacitance artifacts. Other transients between arrows are capacitor feedback resets from the amplifier. (**C**) Absence of the effects of DHPs BAY K 8644 (1 μM) and nitrendipine (1 μM) on barium unitary currents. Top three rows: individual control recordings without (−) or 1 min after the addition of (+) DHP into the bath after pulsing the pipette voltage from 0 to 100 mV. Bottom row: leak-subtracted average current of 400 and 100 episodes for BAY K 8644 and nitrendipine, respectively.

REFERENCES AND NOTES

1. S. J. Smith and G. J. Augustine, *Trends Neurosci.* **11**, 458 (1988); R. Robitaille, E. M. Adler, M. P. Charlton, *Neuron* **5**, 773 (1990); M. W. Cohen, O. T. Jones, K. J. Angelides, *J. Neurosci.* **11**, 1032 (1991).
2. H. Fukunaga, A. G. Engel, M. Osame, E. M. Lambert, *Muscle Nerve* **5**, 687 (1982).
3. E. Sher *et al.*, *Lancet* **335**, 640 (1989); C. Leveque *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3625 (1992).
4. R. W. Tsien *et al.*, *Trends Neurosci.* **11**, 431 (1988); R. W. Tsien, P. T. Ellinor, W. A. Horne, *Trends Pharmacol. Sci.* **12**, 349 (1991); B. P. Bean, *Annu. Rev. Physiol.* **51**, 367 (1989); M. C. Nowycky, A. P. Fox, R. W. Tsien, *Nature* **316**, 440 (1985).
5. D. Swandulla, E. Carbone, H. D. Lux, *Trends Neurosci.* **14**, 46 (1991); E. Sher and F. Clementi, *Neuroscience* **42**, 301 (1991).
6. K. P. Campbell, A. T. Leung, A. H. Sharp, *Trends Neurosci.* **11**, 425 (1988).
7. T. P. Snutch *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3391 (1990).
8. T. P. Snutch, J. Tomlinson, J. P. Leonard, M. Gilbert, *Neuron* **7**, 45 (1991); Y. Mori *et al.*, *Nature* **350**, 398 (1991); M. E. Williams *et al.*, *Neuron* **8**, 71 (1992).
9. M. E. Williams *et al.*, *Science* **257**, 389 (1992).
10. Rabbit brain membranes washed in KCl were prelabeled with ^{125}I-labeled ω-conotoxin and solubilized as previously described (*23*). The solubilized material was diluted 3.3-fold with buffer A [10 mM Hepes (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, and 0.75 mM benzamidine] and applied to a heparin-agarose column (50 ml). After extensive washing, the heparin-agarose column was eluted with buffer A containing 0.7 M NaCl and 0.1% (w/v) digitonin. The eluted fractions were incubated overnight with 9 ml of $VD2_1$ (monoclonal antibody against the β subunit agarose resin). After extensive washing, the ω-conotoxin receptor was eluted with 50 mM CAPS (pH 10) containing 0.6 M NaCl and 0.1% digitonin and was neutralized immediately. The $VD2_1$-agarose purified receptor contained a contaminating protein (46 kD), which was removed by first adsorbing the $VD2_1$-agarose eluted receptor with immunoaffinity resin prepared with polyclonal antibodies raised against the 46-kD protein. Anti–46-kD polyclonal antibodies did not immunoprecipitate ^{125}I-labeled ω-conotoxin receptor. The preadsorbed receptor was concentrated to 0.6 ml and layered onto a linear 5 to 30% sucrose density gradient (12.5 ml). Gradients were centrifuged at 4°C in a Beckman VTi 65.1 rotor for 100 min at 215,000*g*. Fractions (0.6 ml) were collected using an Isco Model 640 density-gradient fractionator and counted in a gamma counter. Gradient fractions were concentrated and separated by 3 to 12% (reducing) SDS–polyacrylamide gel electrophoresis (SDS-PAGE). [U. K. Laemmli, *Nature* **227**, 680 (1970)] and stained with Coomassie blue. A few minor proteolytic fragments of the subunits that were detected on SDS-PAGE varied in amount and were identified with polyclonal antibodies affinity-purified against the four subunits. Gel lanes were scanned with a Molecular Dynamics 300S scanning densitometer.

11. T. Abe, K. Koyano, H. Saisu, Y. Nishiuchi, S. Sakakibara, *Neurosci. Lett.* **71**, 203 (1991); L. J. Cruz and B. M. Oliveria, *J. Biol. Chem.* **261**, 6230 (1986).
12. Scatchard binding analysis was performed on the purified receptor as described (*23*) with use of polyethylenimine-presoaked Whatman GFB filters. Nonspecific binding (less than 10%) was determined in the presence of 5 μM unlabeled ω-conotoxin.
13. Sucrose gradient fractions were separated by SDS-PAGE on 3 to 12% linear gradient gels and transferred to nitrocellulose (150 μl per lane) [K. P. Campbell and S. D. Kahl, *Nature* **338**, 259 (1989)]. Specific polyclonal antibodies against the α_1, α_2, 95K, and β subunits were affinity-purified as described by J. M. Ervasti and K. P. Campbell [*Cell* **66**, 1121 (1991)].
14. Monoclonal and affinity-purified sheep polyclonal antibodies were coupled to protein G–agarose. Rabbit brain membranes (2 mg per milliliter of solution) labeled with 0.5 nM ^{125}I-labeled ω-conotoxin or 10 nM [^{3}H]PN200-110 were solubilized as previously described (*23*). The percent of ^{125}I-labeled ω-conotoxin or [^{3}H]PN200-110 binding sites immunoprecipitated was determined by either gamma or scintillation counting. Nonspecific binding of [^{3}H]PN200-110 was measured in the presence of 10 μM nitrendipine.
15. The SDS-PAGE was carried out on 3 to 12% gradient gels in the presence of 1% 2-mercaptoethanol. Skeletal muscle DHP receptor was purified as described by S. D. Jay *et al.* [*J. Biol. Chem.* **266**, 3287 (1991)].
16. Four peptide sequences from the 140-kD subunit (reduced form of the 160-kD subunit) of the ω-conotoxin receptor clearly identified it as a neuronal α_2 subunit. Deglycosylation of the ω-conotoxin receptor complex was performed as previously described [J. B. Parys *et al.*, *J. Biol. Chem.* **267**, 18776 (1992)]. The α_{1B} glutathione-S-transferase (GST) fusion protein consisted of amino acid residues 720 to 1139 [S. J. Dubel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5058 (1992)] and was produced as described in (*24*). Affinity-purified antibodies against the ω-conotoxin receptor β subunit were used to screen a lambda gt11 rat brain cDNA expression library. A full-length β_3 clone, highly homologous to the β_3 clone sequenced by Hullin *et al.*, (*22*), was obtained. A COOH-terminal (residues 369 to 484) GST fusion protein unique to the β_3 and an NH_2-terminal (residues 9 to 296) GST fusion protein, which contains regions highly homologous to all of the β subunits, were produced as described in (*24*). Polyclonal antibodies to a peptide specific to the β_1 subunit did not recognize the ω-conotoxin receptor.
17. The tip-dip technique was preferred over the standard technique of painted lipid bilayers because it has a smaller membrane capacitance and an improved signal-to-noise ratio, which makes the tip-dip method better suited for resolving fast gating and low-amplitude channels. A bovine brain phospholipid mixture (10 μl) (phosphatidylethanolamine:phosphatidylserine with a weight ratio of 1:1; Avanti Polar Lipids) at 30 mg per milliliter of decane was spread on the surface of a 2-ml bath. Bilayer membranes were formed by the apposition of two monolayers at the tip of fire-polished and Sylgard-coated patch electrodes of mean resistance 7 megohms (n = 27). Bilayers with seal resistances below 10 gigohms were discarded to avoid possible artifacts. The solution on both sides of the bilayer (symmetrical ionic conditions) contained (Fig. 3, A and C) 100 mM $BaCl_2$ and 10 mM Hepes-tris (pH 7.4) or (Fig. 3B) 10 mM $BaCl_2$ and 1 mM Hepes-tris (pH 7.4). Before reconstitution, the purified receptor was phosphorylated by calcium-calmodulin dependent protein kinase II (CaM kinase II) to increase channel activity. The reaction medium contained 10 mM Hepes (pH 7.4), 0.3 M NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA, 1 mM adenosine triphosphate, 7% sucrose, 0.05% digitonin, and 10 μg of calmodulin, 0.5 μg of CaM kinase II, and 12.5 nmol of receptor per milliliter of solution. The potential across the bilayer was controlled by clamping the electrode potential with respect to the bath. By convention, as provided by the Axopatch 200A (Axon Instruments), a negative current is defined by the net cation flow from the bath to the pipette. Chloride current flow in the opposite direction was excluded by current-reversal potential measurements after a decrease in $BaCl_2$ concentration in the bath. Average unitary currents were constructed before and after drug application with the following protocol applied every three seconds: the steady-state voltage [(B) −100 mV, (C) +100 mV] was transiently stepped to 0 mV (reversal potential) for 50 ms and stepped back to the same potential for 300 ms [between arrows, last traces of (B) and (C)]. Recordings were low-pass–filtered at a corner frequency of 1 kHz with an 8-pole Bessel filter and were computer-digitized at 5 kHz. Traces were leak subtracted, and electrode capacitance was compensated.
18. R. Coronado and R. Latorre, *Biophys. J.* **43**, 231 (1983).
19. V. Flockerzi *et al.*, *Nature* **323**, 66 (1986).
20. A. P. Fox, M. C. Nowycky, R. W. Tsien, *J. Physiol.* (*London*) **394**, 173 (1987); M. R. Plummer, D. E. Logothetis, P. Hess, *Neuron* **2**, 453 (1989).
21. A. J. Wagner, A. M. Snowman, A. Biswas, B. M. Olivera, S. H. Snyder, *J. Neurosci.* **8**, 3354 (1988); M. K. Ahlijanian, J. Striessnig, W. A. Catterall, *J. Biol. Chem.* **266**, 20192 (1991); M. W. McEnery, A. M. Snowman, A. H. Sharp, M. E. Adams, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11095 (1991).
22. R. Hullin *et al.*, *EMBO J.* **11**, 885 (1992).
23. J. Sakamoto and K. P. Campbell, *J. Biol. Chem.* **266**, 18914 (1991).
24. O. Ibraghimov-Beskrovnaya *et al.*, *Nature* **355**, 696 (1992).
25. P. S. McPherson *et al.*, *Neuron* **7**, 17 (1991).
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