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EVIDENCE FOR DISTINCT SITES COUPLED TO HIGH AFFINITY ω-CONOTOXIN RECEPTORS IN RAT BRAIN SYNAPTIC PLASMA MEMBRANE VESICLES

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SUMMARY. The neuronal Ca^{2+} channel blocker ω-conotoxin (GVIA) binds with very high affinity (K_d of 0.8 pM) to a single class of receptors in purified rat brain synaptic plasma membrane vesicles. Three types of agents have been found to modulate toxin binding. The affinity of ω-conotoxin is decreased by metal ions or organic cations which interact at the pore of voltage-dependent Ca^{2+} channels. Dynorphin A [1-13] and related peptides stimulate ω-conotoxin binding by increasing toxin affinity through a non-opiate allosteric mechanism. Venom of the spider Plectreurys tristes inhibits ω-conotoxin binding (IC_{50} of 30 ng protein/ml) by a noncompetitive allosteric mechanism. These results suggest that ω-conotoxin binding sites exist in a complex with distinct receptors for other agents, all of which may be functionally associated with neuronal Ca^{2+} channels.

Introduction. Voltage-dependent Ca^{2+} channels can be categorized into three groups: L-, T-, and N-types depending on physical and pharmacological properties (reviewed in 1). N-type channels have only been identified in neuronal cells (2). These channels are not sensitive to organic L-type channel modulators, but are blocked by a family of peptide toxins, termed ω-conotoxins, isolated from venom of fish hunting cone snails (3). One of these toxins derived from Conus geographus, ω-conotoxin GVIA (ω-CgTX), has been shown to inhibit irreversibly N-type channels by a direct mechanism (4). ω-CgTX also suppresses neurotransmitter release from nerve terminals (5-7), implying that toxin-sensitive channels are involved in neurotransmission (1). Since ω-CgTX blockable N-type channels can be expressed in Xenopus oocytes (8), there appears to be close coupling between the toxin receptor and the channel pore structure. However, ω-CgTX will also inhibit neuronal L-type channels (4,9).

To define better the properties of the ω-CgTX receptor and demonstrate functional association with a specific type of neuronal Ca^{2+}

Abbreviations used: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; Tris, Tris(hydroxymethyl)-aminomethane; ω-CgTX, ω-conotoxin (GVIA).

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channel, compounds were sought that might modulate toxin binding to synaptic membranes. Three types of agents were identified: metal ions and organic cations which interact at the pore of voltage-dependent Ca^{2+} channels (10,11); dynorphin A and related peptides which block neuronal Ca^{2+} channel function (12-14); and crude venom of the spider Plectreurys tristes which interferes with transmitter release by inhibiting presynaptic Ca^{2+} entry (15). These data indicate that the ω-CgTX receptor is allosterically coupled to binding sites for other agents, all of which exist in a complex that may be part of the N-type Ca^{2+} channel.

EXPERIMENTAL PROCEDURES

Materials - ω-[^{125}I]CgTX GVIA (2200 Ci/mmol), [^{3}H]PN 200-110 (80 Ci/mmol), [^{3}H]D-600 (87 Ci/mmol), and d-cis-[^{3}H]diltiazem (61 Ci/mmol) were purchased from New England Nuclear, while ω-CgTX GVIA, dynorphin A [1-13] and related peptides were bought from Peninsula Laboratories. Crude venom of the spider Plectreurys tristes was purchased from Spider Pharm. (Black Canyon City, Arizona). Other drugs were obtained from the Merck sample collection.

Preparation of Brain Synaptic Plasma Membrane Vesicles - Rats (Wistar, 150-250 g) were sacrificed by guillotine and their brains quickly removed. A crude synaptosomal membrane fraction was prepared essentially as described (16). Synaptosomes in homogenization medium were collected by centrifugution (18,500g, 10 min) and suspended in 5 mM Tris-HCl, pH 7.4. This suspension was stirred for 45 min (4°C), then diluted 1:1 with 2 M sucrose, layered within a discontinuous gradient consisting of 1.2 M, 1 M (synaptosomal fraction), and 0.6 M sucrose, and finally subjected to centrifugation (96,000g, 90 min). The membrane fraction at the 0.6/1 M interface was collected, washed with 50 mM Tris-HCl, pH 7.4, and stored at -70°C in this buffer at 4 mg protein/ ml after rapid freezing in liquid nitrogen. Binding activities were stable for at least 2 months.

Ligand Binding Assays - To assess ω-[^{125}I]CgTX binding, purified synaptic vesicles (1 µg) were incubated with ligand at 25°C in 50 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin (2 ml volume). At appropriate times, samples were diluted with 4 ml of ice-cold 10 mM Tris-Hepes, pH 7.4, 0.1% bovine serum albumin, and membranes were collected under reduced pressure on GF/C glass fiber filters (presoaked in 0.3% polyethylenimine) and washed twice. Nonspecific binding was determined with 10 nM ω-CgTX present. Triplicate assays were routinely performed and the data were averaged (standard error of mean $\leq$3%). Stock solutions of peptides were prepared in 50 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin. Binding of organic Ca^{2+} entry blockers was monitored as previously described (17). Protein was determined according to Lowry et al (18) using bovine serum albumin as a standard.

Analysis of Data - Data from saturation experiments were subjected to Scatchard analysis, and linear regression was performed to yield the equilibrium dissociation constant (K_d) and maximum receptor density (B_{max}) (correlation coefficients typically $\geq$ 0.95). K_i values were determined from competition experiments as described (19). The kinetics of ligand binding were determined from a linear plot of k_{obs} vs toxin concentration, where k_{obs} is the slope of the pseudo-first order plot $\ln [[LR]_e/([LR]_e-[LR]_t)]$ vs time, and $[LR]_e$ and $[LR]_t$ are concentrations of the toxin-receptor complex at equilibrium and time t, respectively.

RESULTS AND DISCUSSION. Since low (20-23), intermediate (22,24), and high affinity (25,26) ω-CgTX receptors have been reported to exist in brain membrane preparations, we re-examined the properties of ω-CgTX binding using purified rat brain synaptic plasma membrane vesicles. Incubation of

vesicles with ω-[^{125}I]CgTX until equilibrium is established results in concentration-dependent association of toxin which is markedly suppressed by 10 nM ω-CgTX (Fig. 1A). Scatchard analysis indicates the presence of a single class of very high affinity ω-CgTX receptors with a K_d of 0.74 pM and B_{max} of 1 pmol/mg protein (Fig. 1A-inset). Average K_d and B_{max} values from four different preparations are 0.78±0.08 pM and 0.995±0.334 pmol/mg protein, respectively. No indication of a lower affinity site is evident. For comparison, the site density of L-type channel receptors, based on PN 200-110, D-600 and diltiazem binding, is 0.1 pmol/mg protein: a 10-fold lower density. Competition binding experiments confirm that ω-[^{125}I]CgTX and non-labeled peptide have identical binding activities. These K_d and B_{max} values are not affected by temperature (4-37°C). However, increasing ionic strength of the medium lowers the affinity of ω-CgTX (e.g., in 145 mM NaCl, 5 mM KCl, 1.4 mM $MgCl_2$, 100 µM $CaCl_2$, 20 mM MOPS Tris, pH 7.4, K_d is increased 400-fold), which may explain why biological effects of ω-CgTX in physiological salines require nM concentrations (5).

Kinetics of toxin binding have been determined by monitoring ligand association at different concentrations of ω-[^{125}I]CgTX (Fig. 1B). Logarithmic transformations of these data are linear with slopes representing k_{obs} values. A plot of these rate constants vs ω-CgTX concentration gives a straight line whose slope is k_1 and y-intercept is k_{-1} (Fig.

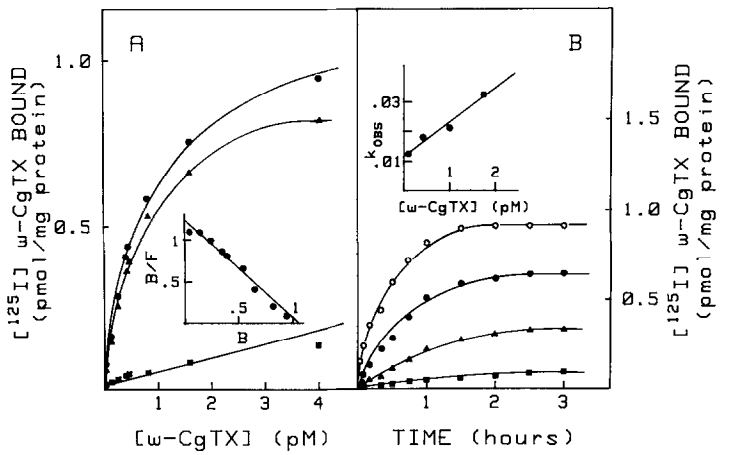

Fig. 1. **Binding of ω-[^{125}I] CgTX to purified rat brain synaptic plasma membrane vesicles** (A). Vesicles were incubated in the presence of increasing concentrations of ω-[^{125}I]CgTX until equilibrium was achieved. Total binding (●), nonspecific binding determined with 10 nM ω-CgTX (■), and specific binding (▲) are illustrated. Insert: Specific binding data are presented in the form of a Scatchard representation. Units of the y- and x-axes are pmol/mg protein/pM and pmol/mg protein, respectively. (B). Membrane vesicles were incubated with the indicated concentrations of ω-[^{125}I]CgTX for different periods of time. Insert: The slope of the semilogarithmic representation of each pseudo-first order association reaction (k_{obs}) is plotted vs toxin concentration. Units of the y-axis are min^{-1}.

1B-inset). Corresponding kinetic constants are: k_1 = 1.3 X 10^{10} $min^{-1}M^{-1}$ and k_{-1} =0.011 min^{-1}. These yield a K_d of 0.86 pM; a value identical to that determined in equilibrium measurements. The fast on-rate of toxin may reflect an electrostatic interaction since ω-CgTX is highly positively charged. Although k_{-1} can be calculated in this way, all attempts to determine k_{-1} experimentally by direct toxin dissociation measurements give much lower values (i.e., a $t_{1/2}$ of 18 hr; k_{-1} of 0.0006 min^{-1}: cf. Fig. 3B). This finding contrasts with other reports (26), and suggests that multiple steps are involved in the binding reaction.

Distribution of ω-CgTX receptor sites was determined by measuring toxin binding to plasma membranes derived from different excitable cells including porcine left ventricle, bovine aortic smooth muscle and GH_3 rat anterior pituitary cells. All these preparations contain high levels of binding sites for L-type channel modulators, but ω-[^{125}I]CgTX binding was not observed at low concentrations of ligand. Low affinity binding can be detected in GH_3 membranes when ligand concentration is increased to 1 nM, perhaps correlating with the observation that ω-CgTX will block L-type channels in this cell line (27). Therefore, high affinity ω-CgTX binding sites are present only in neural tissue. Given that N-type channels are found exclusively in neuronal cells, and that the site density of ω-CgTX receptors is 10-fold greater than that of L-type channels in brain, high affinity ω-CgTX sites may be associated with N-type channels.

To test this prediction, various agents which affect Ca^{2+} channel activity were investigated for their ability to modulate ω-CgTX binding. High affinity toxin binding is inhibited by mono-, di- and trivalent metal ions which are either channel substrates or blockers. K_i values (mM) for various ions are: Ca^{2+}, 0.45; Ba^{2+}, Sr^{2+}, 1.4; Ni^{2+}, 0.33; Cd^{2+}, 0.11; Co^{2+}, 0.28; La^{3+}, 0.02; Mg^{2+}, 1.9; Na^+, K^+, 50. Saturation experiments indicate that Ca^{2+} inhibits binding by decreasing affinity of peptide for its receptor (Fig. 2A). These results suggest that agents which interact at the pore of Ca^{2+} channels modulate ω-CgTX binding directly. Consistent with this, several structurally different organic cations which are also Ca^{2+} channel pore blockers, including amiloride analogs (11) and aminoglycosides (10), inhibit ω-CgTX binding. K_i values (μM) for agents representing these structural classes are: 3,4-dichlorobenzamil, 10.2; N^5-(propyl, butyl)-amiloride, 9.2; neomycin, 1.8. Scatchard analyses indicate that 3,4-dichlorobenzamil and neomycin (Fig. 2A) decrease toxin affinity. These data imply that ω-CgTX interacts directly with Ca^{2+} channels in brain membranes. However, the agents described above affect all types of Ca^{2+} channels. Nonetheless, L-type channel modulators (i.e., PN 200-110, D-600, diltiazem, fluspirilene; 10 μM) have no effect on ω-CgTX binding and ω-CgTX (100 nM) has no effect on PN 200-110, D-600 or diltiazem binding in brain. There-

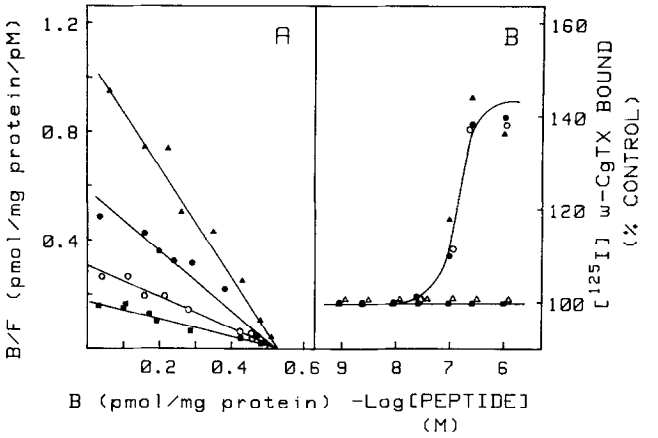

Fig. 2. **Effect of Ca^{2+} channel modulators on ω-CgTX binding** (A). Brain synaptic membrane vesicles were incubated with increasing concentrations of ω-[^{125}I]CgTX in the absence (●) or presence of either 1 mM $CaCl_2$ (■), 2 μM neomycin (○), or 250 nM dynorphin A [1-13] (▲) until equilibrium was achieved. Specific binding data in each case are presented in the form of a Scatchard analysis. (B). Membrane vesicles were incubated with 0.4 pM ω-[^{125}I]CgTX in the absence or presence of increasing concentrations of either dynorphin A [1-13] (●), dynorphin A [3-13] (▲), dynorphin A [1-5] (■), morphine (△), or dynorphin A (1-13) with 1 μM naloxone (○), until equilibrium was achieved. Specific binding data are presented relative to an untreated control.

fore, there is little evidence that high affinity ω-CgTX receptors are functionally associated with L-type channels.

The neuropeptide dynorphin A affects neuronal Ca^{2+} channel activity (e.g., it inhibits N-type channels; 12) and blocks neurotransmission (13,14). Both opiate and non-opiate related mechanisms have been implicated. To determine if a relationship exists between these findings and ω-CgTX binding in brain, binding was monitored with dynorphin A present. Dynorphin A [1-13] causes concentration-dependent stimulation of ω-[^{125}I]CgTX binding with a K_a of 140 nM (Fig. 2B). Higher concentrations of peptide (10 μM) reverse stimulation of ω-CgTX binding. Identical results are obtained with dynorphin A [1-17], as well as with Dynorphin A [3-13], which does not bind to κ opiate receptors (Fig. 2B). Neither the κ opiate agonists dynorphin A [1-5], dynorphin A [1-8], morphine or cyclazocine, nor the antagonist naloxone have any effect (1 μM) on ω-CgTX binding and naloxone (1 μM) does not prevent stimulation of toxin binding by dynorphin A [1-13] (Fig. 2B). These findings rule out involvement of an opiate-related mechanism. Dynorphin A peptides increase receptor affinity as illustrated in the Scatchard analysis of Fig. 2A; 250 nM dynorphin A [1-13] decreases K_d of ω-[^{125}I]CgTX from 0.8 to 0.4 pM. Kinetic analyses indicate that dynorphin A [1-13] increases k_1 of the binding reaction. Since exogenous cofactors are absent, the present findings suggest direct allosteric coupling between the ω-CgTX receptor and a non-κ related receptor

which recognizes the carboxyl-terminal region of dynorphin A. Synthetic κ opiate agonists affect dihydropyridine binding to L-type channels in rat brain (28), and dynorphin A [1-13] inhibits PN 200-110 binding in brain synaptic vesicles (K_i = 10 μM). However, dynorphin A [3-13] is without effect (50 μM). Therefore, dynorphin A-like peptides have multiple effects on neuronal Ca^{2+} channels, but interaction between dynorphin A and ω-CgTX receptors most likely does not involve L-type channels.

Venom of the spider Plectreurys tristes contains peptides which interfere with synaptic transmission in drosophila by apparently blocking presynaptic Ca^{2+} channels (15). This venom caused potent concentration-dependent inhibition of ω-CgTX binding with an IC_{50} of 30 ng venom protein/ml (Fig. 3A). Saturation experiments indicate that inhibition is noncompetitive (Fig. 3A-inset). In separate experiments, venom was found to affect kinetics of ω-CgTX binding: k_{-1} is increased (Fig. 3B), as is k_1. Thus, it is probable that a peptidyl-venom component binds to a site which is allosterically coupled to the ω-CgTX receptor. Importantly, this venom (5 μg protein/ml) has no effect on PN 200-110, D-600 or diltiazem binding in brain, indicating that interaction between venom and L-type channels is unlikely. Moreover, crude venom (50 μg protein/ml) has no effect on L- or T-type channel activity in GH_3 cells as determined by voltage clamp protocols (G. Suarez-Kurtz, and G. Kaczorowski, unpublished). These data

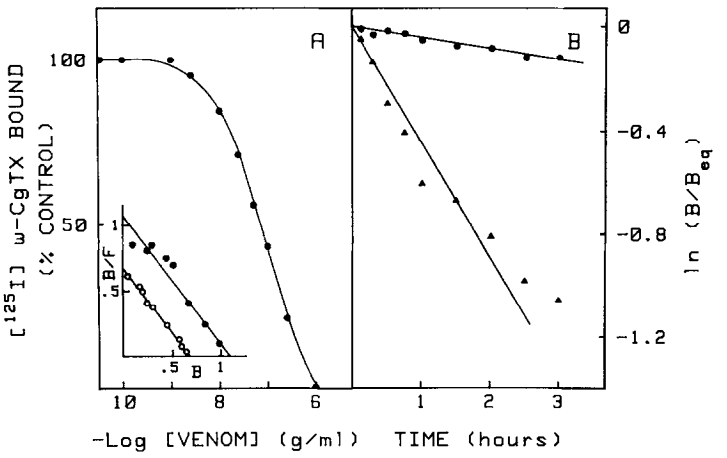

Fig. 3. **Effect of venom of Plectreurys tristes on ω-CgTX binding** (A). Membrane vesicles were incubated with 1 pM ω-[^{125}I]CgTX in the absence or presence of increasing concentrations of venom of Plectreurys tristes. Specific binding data is presented relative to an untreated control. Insert: Scatchard representation of ω-[^{125}I]CgTX binding in the absence (●) or presence (○) of 25 ng/ml venom of Plectreurys tristes. Units of the y- and x-axes are pmol/mg protein/pM and pmol/mg protein, respectively. (B). Dissociation kinetics of ω-[^{125}I]CgTX from its receptor were measured by addition of 10 nM ω-CgTX alone (●) or with 0.5 μg/ml of venom of Plectreurys tristes present (▲) and incubating at 25°C for different periods of time. A semilogarithmic plot of the first-order dissociation reaction is presented.

suggest that the complex containing ω-CgTX and spider-neurotoxin receptors is not associated with L-type channels.

In summary, this study demonstrates that ω-CgTX binding in rat brain is affected by three different classes of agents: metal ions and hydrophobic cations which interact at the pore of voltage-dependent Ca^{2+} channels; dynorphin A and related peptides which block neuronal Ca^{2+} channels; and neurotoxins of a spider which are potent presynaptic blockers. The latter two groups of agents interact at distinct sites which are allosterically linked to the ω-CgTX receptor. The receptor itself could be localized at or near the pore of a Ca^{2+} channel, given the effects of cations on toxin binding. Several lines of evidence suggest that these receptors are functionally associated with N- rather than L-type Ca^{2+} channels. Density and tissue distribution studies reveal no correlation between ω-CgTX sites and markers of L-type channels. If low affinity ω-CgTX binding (GH_3) is to the L-type channel, then the low density of these channels in our brain preparation may preclude any direct demonstration of such binding activity in synaptic vesicles. L-type channel modulators do not affect ω-CgTX binding, nor do they block ω-CgTX sensitive transmitter release that is mediated by N-type channel action (1). Dynorphin A-like peptides affect neuronal Ca^{2+} channels through both opiate and non-opiate related mechanisms: the opiate-related mechanism appears coupled to L-type channels while the interaction with ω-CgTX receptors is not. Finally, Plectreurys tristes neurotoxins have no effect on L-type channels, yet they are potent inhibitors of ω-CgTX binding. Taken together, these data are consistent with the idea that high affinity ω-CgTX receptors are associated with N-type Ca^{2+} channels in neuronal tissue. Nevertheless, direct demonstration of this link will require purification of the channel protein.

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REFERENCES

1. Miller, R.J. (1987) Science 235, 46-52.
2. Nowycky, M.C., Fox, A.P., and Tsien, R.W. (1985) Nature 316, 440-443.
3. Olivera, B.M., Cruz, L.J., de Santos, V., Le Cheminant, G.W., Griffin, D., Zeikus, R., McIntosh, J.M., Galyean, R., Varga, J., Gray, W.R., and Rivier, J. (1987) Biochemistry 26, 2086-2090.
4. McCleskey, E.W., Fox, A.P., Feldman, D.H., Cruz, L.J., Olivera, B.M., Tsien, R.W., and Yoshikami, A. (1987) Proc. Natl. Acad. Sci. USA 84, 4327-4331.
5. Reynolds, I.J., Wagner, J.A., Snyder, S.H., Thyer, S.A.,Olivera, B.M., and Miller, R.J. (1986) Proc. Natl. Acad. Sci. USA 83, 8804-8807.
6. Koyana, K., Abe, T., Nishiuchi, Y., and Sakakibara, S. (1987) Eur. J. Pharmacol. 135, 337-343.
7. Sano, K., Enomoto, K.I., and Maeno, T. (1987) Eur. J. Pharmacol. 141, 235-241.

8. Umbach, J.A., and Gunderson, C.B. (1987) Proc. Natl. Acad. Sci. USA 84, 5464-5468.
9. Feldman, D.H., Olivera, B.M., and Yoshikami, D. (1987) FEBS Lett. 214, 295-300.
10. Suarez-Kurtz, G., and Reuben, J.P. (1987) Pfluger Arch. 410, 517-523.
11. Suarez-Kurtz, G., and Kaczorowski, G.J. (1988) J. Pharmacol. Exp. Therap. submitted.
12. Gross, R.A., and MacDonald, R.L. (1987) Proc. Natl. Acad. Sci. USA 84, 5469-5473.
13. Walker, J.M., Moises, H.C., Coy, D.H., Baldrighi, G., and Akil, H. (1982) Science 218, 1136-1138.
14. Stevens, C.W., Weigner, M.B., and Yaksh, T.L. (1987) Eur. J. Pharmacol. 138, 299-302.
15. Branton, W.D., Kolton, L., Jan, Y.N., and Jan, L.Y. (1987) J. Neurosci. 7, 4195-4200.
16. Lai, J.C.K., Walsh, J.M., Dennis, S.C., and Clark, J.B. (1977) J. Neurochem. 28, 625-631.
17. Garcia, M.L., King, V.F., Siegl, P.K.S., Reuben, J.P., and Kaczorowski, G.J. (1986) J. Biol. Chem. 261, 8146-8157.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Cheng, Y.C., and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099-3108.
20. Cruz, L.J., and Olivera, B.M. (1986) J. Biol. Chem. 261, 6230-6233.
21. Cruz, L.J., Johnson, D.S., and Oliveria, B.M. (1987) Biochemistry 26, 820-8234.
22. Abe, T., Koyano, K., Saisu, H., Nichiuchi, Y., and Sakakibara, S. (1986) Neuroscience Lett. 71, 203-208.
23. Abe, T., and Saisu, H. (1987) J. Biol. Chem. 262, 9877-9882.
24. Wagner, J.A., Snowman, A.M., Biswas, A., Olivera, B.M., and Snyder, S.H. (1988) J. Neurosci. in press.
25. Knaus, H.G., Striessnig, J., Koza, A., and Glossmann, H. (1987) Naunyn-Schmiedbergs' Arch. Pharmacol. 336, 583-586.
26. Barhanin, J., Schmid, A., and Lazdunski, M. (1988) Biochem. Biophys. Res. Commun. 150, 1051-1062.
27. Suzuki, N., and Yoshioka, T. (1987) Neuroscience Lett. 75, 235-239.
28. Gandhi, V.C., and Ross, D.H. (1987) Biochem. Biophys. Res. Commun. 149, 1042-1048.