Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11095–11099, December 1991
Neurobiology

Purified ω-conotoxin GVIA receptor of rat brain resembles a dihydropyridine-sensitive L-type calcium channel

(voltage-dependent calcium channels/ω-agatoxin IIIA/photoaffinity labeling)

MAUREEN W. MCENERY*, ADELE M. SNOWMAN*, ALAN H. SHARP*, MICHAEL E. ADAMS†, AND SOLOMON H. SNYDER*‡

*Departments of Neuroscience, Pharmacology and Molecular Sciences, Psychiatry and Behavioral Sciences, Johns Hopkins Medical Institutions, 725 North Wolfe Street, Baltimore, MD 21205; and †Department of Entomology, University of California, Riverside, Riverside, CA 92521

Contributed by Solomon H. Snyder, September 13, 1991

ABSTRACT **The ω-conotoxin GVIA (CTX) receptor has been purified 1900-fold to apparent homogeneity by monitoring both reversible binding of ^{125}I-labeled CTX (^{125}I-CTX) and photoincorporation of *N*-hydroxysuccinimidyl-4-azidobenzoate-^{125}I-CTX (HSA-^{125}I-CTX). Photoincorporation of HSA-^{125}I-CTX into a 230-kDa protein exhibits a pharmacologic and chromatographic profile indicating that the 230-kDa protein is the CTX-binding subunit of the receptor. The pharmacologic specificity of ^{125}I-CTX binding to the purified CTX receptor closely resembles that of the native membrane-bound form with respect to sensitivity towards CTX (K_d = 32 pM) and other peptide toxin antagonists. The purified CTX receptor comprises the 230-kDa protein (α_1) and four additional proteins with apparent molecular masses of 140 (α_2), 110, 70 (β_2), and 60 (β_1) kDa. This subunit structure closely resembles that of the 1,4-dihydropyridine-sensitive L-type calcium channel.**

Biophysical studies have discriminated several types of voltage-gated calcium channels (designated L-, T-, P-, and N-type) in neurons (1–3) and other excitable tissues (4, 5). The L-type calcium channel is labeled by 1,4-dihydropyridine (DHP) and phenylalkylamine drugs (6–8), permitting its purification to homogeneity from skeletal muscle (9–12) and heart (13, 14). The purified receptor comprises four distinct subunits (α_1, α_2-δ, β, and γ). Molecular cloning of the DHP-binding α_1 subunit from several tissues (15–17) indicates a common protein structure. In the brain, molecular cloning reveals a heterologous family of calcium channels with homology to the α_1 subunit of the skeletal muscle DHP receptor (18–22).

The N-type calcium channel, which regulates neurotransmitter release from synaptic endings, can be distinguished from the neuronal L-type calcium channel (23, 24). The N-type channel is selectively inhibited by ω-conotoxin GVIA (CTX) isolated from the venom of the snail *Conus geographus* (25, 26) and can be labeled with ^{125}I-labeled CTX (^{125}I-CTX) (27). CTX receptors are more abundant than neuronal L-type channels (27, 28), but their instability has precluded purification. A photoaffinity derivative of CTX, *N*-hydroxysuccinimidyl-4-azidobenzoate-^{125}I-CTX (HSA-^{125}I-CTX) labels a discrete 230-kDa band in brain membranes (29, 30). As the DHP-binding protein represents only one of several L-type channel subunits, so the band labeled by HSA-^{125}I-CTX may be only a portion of the CTX receptor.

We now report purification to homogeneity of the CTX receptor with the isolated receptor protein retaining reversible ligand binding. The purified rat brain CTX receptor comprises a 230-kDa protein that is the CTX recognition site and four additional subunits. The subunit compositions of the CTX receptor and the L-type calcium channel are closely similar.

MATERIALS AND METHODS

Materials. Calpain inhibitors I and II and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) used for CTX receptor solubilization were obtained from Calbiochem; CHAPS used in column buffers, heparin-Sepharose, arginine-Sepharose (5–10 μmol/ml), metal-chelate-Sepharose (imidodiacetic acid-Sepharose 6B fast flow), and 22-kDa polylysine were from Sigma; ^{125}I-CTX [labeled on Tyr-22, specific activity 2200 Ci (81.4 TBq)/mmol] was from NEN/DuPont; Hepes buffer was from Research Organics; asolectin (soybean lecithin) was from Associated Concentrates, Woodside, NY; succinylated wheat germ agglutinin (sWGA) was from EY Laboratories; HSA was from Pierce; unlabeled CTX was from Peninsula Laboratories; and the UV lamp was from Ultraviolet Products, San Gabriel, CA. All other materials were obtained from Sigma. Adult male Sprague–Dawley rats (135–175 g) were obtained from Harlan–Sprague–Dawley. ω-Conotoxin MVIIA (CTX-MVIIA) was a generous gift of B. M. Olivera (University of Utah).

Solubilization and Purification of CTX Receptor. Adult male rats (8–10 animals) were killed by cervical dislocation, and the forebrains were removed, dissected, and placed immediately upon ice. One brain (1.2–1.5 g) was homogenized in 25 ml of 50 mM Hepes, pH 7.4/1 mM dithiothreitol (DTT)/1 mM EGTA for 5 sec. The brain homogenate was centrifuged at 4°C for 10 min at 48,000 × *g*. To each membrane pellet, 5 ml of 3 M urea in 50 mM Hepes, pH 7.4/1 mM DTT/1 mM EGTA was added. The samples were mixed in a Vortex mixer and incubated on ice for 15 min. Samples were diluted with 30 ml of 50 mM Hepes, pH 7.4/200 mM potassium phosphate, pH 6.5/1 mM DTT/1 mM EGTA and incubated on ice for 10 min. The urea/salt-washed membranes were centrifuged as before, resuspended in 50 mM Hepes, and washed again. The final membrane pellets were resuspended in 10 vol/g original tissue weight in 50 mM Hepes, pH 7.4. Resuspended urea-washed membranes were mixed with equal volumes of 2× CTX buffer [50 mM Hepes, pH 7.4/1.5% CHAPS/2 mM EGTA, 10% (vol/vol) glycerol/2 mM DTT containing benzamidine at 360 μg/ml, leupeptin at 4 μg/ml, aprotinin at 4 μg/ml, pepstatin at 4 μg/ml, 0.4 mM phenylmethanesulfonyl fluoride, and calpain inhibitor I and II at 2 μg/ml] and

Abbreviations: CTX, ω-conotoxin GVIA; CTX-MVIIA, ω-conotoxin MVIIA; AgaIIIA, ω-agatoxin IIIA; DHP, 1,4-dihydropyridine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PEG, polyethylene glycol; HA, hydroxylapatite; HSA, *N*-hydroxysuccinimidyl-4-azidobenzoate; sWGA, succinylated wheat germ agglutinin.

‡To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

incubated on ice for 15 min. The material was centrifuged for 1 hr at 230,000 × g. The soluble CHAPS extract was removed and used immediately.

CHAPS extract was poured sequentially over preadsorbed heparin-Sepharose, arginine-Sepharose, and metal-chelate (Zn)-Sepharose columns and the flow-through was collected. In each case a ratio of 100 ml of extract per 15 ml of packed gel was not exceeded. The metal chelate column was prepared immediately prior to use by rinsing it in 1× CTX buffer plus 0.4% $ZnCl_2$ followed by washing with 1× CTX buffer. Dry hydroxylapatite (HA) (4 g/200 ml) was added to the Zn flow-through fraction and the sample was batch incubated with constant mixing for 1 hr at 4°C. The flow-through of the HA column was collected. The HA column was washed with 1× CTX buffer. The HA column was eluted sequentially with 1.5 packed column volumes of 1× CTX buffer + potassium phosphate (0–400 mM potassium phosphate). The peak of ^{125}I-CTX binding was eluted with 1× CTX buffer plus 200–400 mM potassium phosphate, pH 9.5. The pooled fraction from the HA column was incubated 4 hr at 4°C with 5 ml of sWGA-Sepharose previously equilibrated with 20 ml of 1× CTX buffer plus 200 mM potassium phosphate, pH 6.5. The flow-through from the sWGA-Sepharose column was collected and the column was washed with 250 ml of 1× CTX buffer plus 200 mM sodium chloride. ^{125}I-CTX binding activity was eluted with 3.0 column volumes of 0.5 M *N*-acetylglucosamine in 1× CTX buffer plus 200 mM sodium chloride and phospholipids prepared as described (31) at 0.3 mg/ml. Dry polyethylene glycol (PEG) (M_r 8000) was added (30%, wt/vol) to pooled sWGA eluate and incubated 2 hr with constant mixing. The sample was centrifuged at 4°C for 30 min at 200,000 × g. The CTX receptor pellet was resuspended in 1× CTX buffer plus 200 mM sodium chloride.

Derivatization of ^{125}I-CTX with HSA and Photoaffinity Labeling of Rat CTX Receptor. HSA was prepared as a 10 mM stock solution in dimethyl sulfoxide and stored at −40°C in the dark. ^{125}I-CTX (10–20 μl; total cpm 475,000) was added to 0.2 mM boric acid, pH 9.5, in a final volume of 75 μl in the dark. HSA (25 μl) was added to the ^{125}I-CTX solution and incubated for 60 min at room temperature, whereupon 470 μl of Tris·HCl, pH 7.7, was added. The photoaffinity-derivatized ^{125}I-CTX was designated HSA-^{125}I-CTX. Rat forebrain membranes, CHAPS extract, and metal chelate flow-through were diluted to 80,000 cpm of ^{125}I-CTX specifically bound per 200-μl sample with 50 mM Hepes, pH 7.4, to standardize ^{125}I-CTX binding sites. HSA-^{125}I-CTX (25 μl; total cpm 20,000) was added and incubated for 45 min in the dark. The samples were then exposed to UV light (254 nm) at a distance of 1 cm from the light source for 30 min on ice. Labeling was quenched with 50 μl of 5× Laemmli sample buffer [15% SDS/0.825 M sucrose/0.325 M Tris·HCl, pH 6.8/5% (vol/vol) 2-mercaptoethanol/0.002% bromophenol blue], and samples were resolved by SDS/PAGE on 7–20% gradient gels (32). ω-Agatoxin IIIA (AgaIIIA) was purified as described (33). Protein assays (34, 35) and silver staining (36) were according to standard procedures.

RESULTS AND DISCUSSION

Workers who previously attempted to covalently label the CTX receptor in membranes reported divergent molecular weights (37–39). HSA-^{125}I-CTX appears superior to other cross-linkers, as photoactivation occurs after high-affinity binding to the receptor (29, 30). To maximize selectivity of the photoaffinity labeling, we used subsaturating concentrations of HSA-^{125}I-CTX. A discrete 230-kDa band is labeled, and the labeling is displaced by 500 pM but not 50 pM unlabeled CTX (Fig. 1*A*). We further evaluated the specificity of photolabeling with HSA-^{125}I-CTX by using previously identified potent inhibitors of reversible ^{125}I-CTX binding

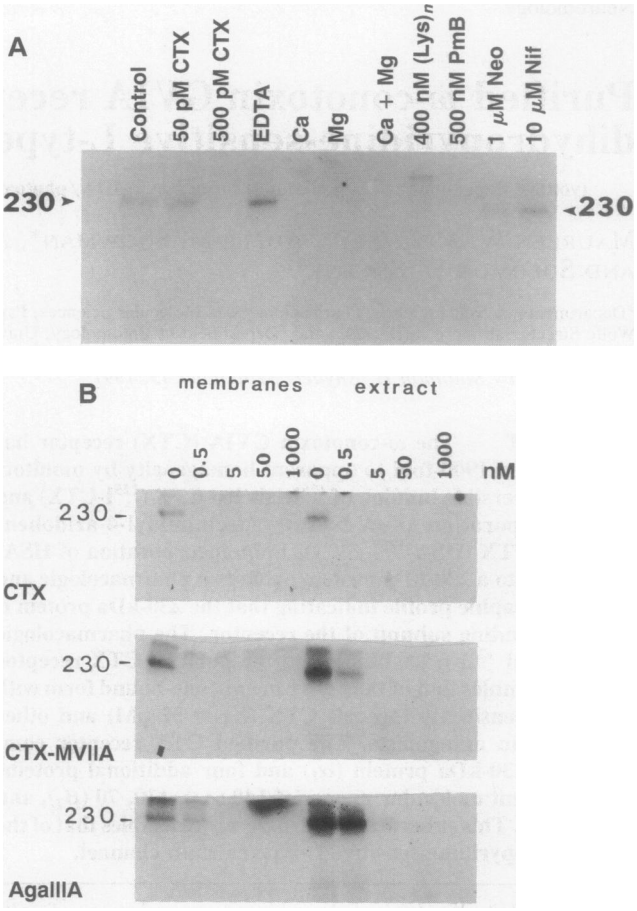

FIG. 1. Photoaffinity labeling of rat forebrain membranes with HSA-^{125}I-CTX identifies a single 230-kDa protein. (*A*) Effect of divalent metals, polylysine, and aminoglycosides on the photoincorporation of HSA-^{125}I-CTX. Rat forebrain membranes were incubated with the stated concentration of reagent and HSA-^{125}I-CTX. Lane 1, control (no additions); lane 2, 50 pM unlabeled CTX; lane 3, 500 pM unlabeled CTX; lane 4, 2 mM EDTA; lane 5, 2 mM $CaCl_2$; lane 6, 2 mM $MgCl_2$; lane 7, 2 mM $CaCl_2$ + 2 mM $MgCl_2$; lane 8, 400 nM polylysine (22 kDa); lane 9, 500 nM polymixin B; lane 10, 1 μM neomycin; lane 11, 10 μM nifedipine. (*B*) Unlabeled CTX and the peptide toxins CTX-MVIIA and AgaIIIA inhibit the photoincorporation of HSA-^{125}I-CTX in crude membranes and CHAPS extract. Lanes 1–5, rat forebrain membranes; lanes 6–10, CHAPS extract; lanes 1 and 6, control (no additions). Toxin concentrations (nM) are given above the lanes. Aggregated receptor is seen at top of resolving gel.

(27, 40, 41). EDTA enhances labeling intensity, presumably by removing divalent metals, while calcium and magnesium

Table 1. Purification of the CTX receptor from rat brain

Fraction	% activity	Protein, mg	^{125}I-CTX bound, cpm × 10^{-2}/μg	Purification, -fold
Extract	100	339	3.2	—
Heparin	93	141	7.3	2.2
Arginine	59	86	7.6	2.3
Metal chelate	64	70	10.1	3.1
HA pool	29	4.5	71.0	22
sWGA eluate pool	10	0.05	2210	680 1226*
PEG precipitate	10	0.035	3150	972 1940*

The purification, according to *Methods*, was replicated more than 10 times with less than 10% variation.
*Based on recovery of HSA-^{125}I-CTX covalently modified CTX receptor.

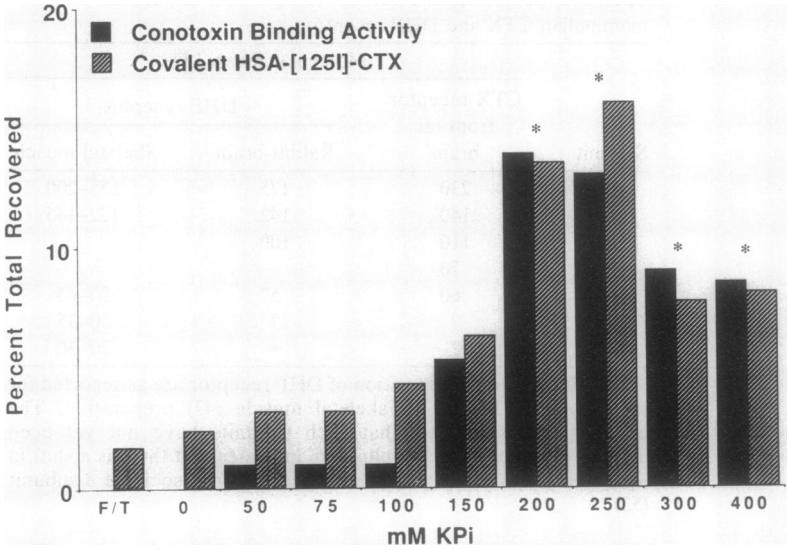

FIG. 2. Copurification of active CTX receptor with covalently labeled CTX-binding protein by HA chromatography. Solid bars show purification of the ^{125}I-CTX receptor by HA chromatography. Metal-chelate (Zn) flow-through (approximately 200 ml) was passed over 4 g of dry HA. The column was eluted with increasing concentrations of potassium phosphate (20 ml per step). Fraction F/T indicates flow-through of HA column; the subsequent fractions are as indicated. Each fraction was assayed for reversible ^{125}I-CTX binding by the standard method. Fractions marked with asterisks were pooled for subsequent chromatography on sWGA. Hatched bars show covalent modification of Zn flow-through by HSA-^{125}I-CTX. Zn flow-through was incubated in 20 1-ml aliquots with HSA-^{125}I-CTX. The pooled samples were passed over 0.4 g of dry HA and chromatographed as described above with each column elution comprising 2 ml. Each fraction was assayed for irreversibly bound HSA-^{125}I-CTX by diluting 50-to 100-μl aliquots to a final volume of 1 ml with 50 mM Hepes, pH 7.4, and removing unbound HSA-^{125}I-CTX by filtration over glass fiber filters (as described in *Methods* for ^{125}I-CTX filter binding assay).

(2 mM) inhibit photolabeling of the 230-kDa protein. Polylysine, polymixin B, and neomycin also inhibit incorporation of HSA-^{125}I-CTX, while the DHP nifedipine is inactive.

HSA-^{125}I-CTX labels the receptor in solubilized as well as intact membrane preparations (Fig. 1*B*). The soluble CTX receptor retains its high affinity for CTX. The solubilized receptor can be distinguished further by the potency of two CTX antagonists. The peptide toxins CTX-MVIIA, from the venom of *Conus magus* (42, 43), and AgaIIIA, from the funnel-web spider *Agelenopsis aperta* (33), are potent competitive inhibitors of ^{125}I-CTX binding to rat forebrain membranes (33, 44). CTX-MVIIA inhibits photolabeling of the native receptor, but it appears to be somewhat weaker in soluble preparations (Fig. 1*B*). AgaIIIA (0.5 nM) reduces HSA-^{125}I-CTX binding in intact membranes and is inactive in detergent-solubilized preparations, suggesting that solubilization of the receptor diminishes its ability to respond to AgaIIIA. AgaIIIA inhibits both neuronal N-type and cardiac L-type calcium channel activity (45), suggesting similarities of DHP and CTX receptors. Despite the inhibitory effect of AgaIIIA on L-type channel activity, high concentrations (1 nM) of AgaIIIA do not inhibit the binding of either DHP ((+)-[^{3}H]PN20010) or phenylalkylamines ([^{3}H]desmethoxyverapamil) to the neuronal L-type channel (M.W.M., A.M.S., A.H.S., and S.H.S., unpublished observation).

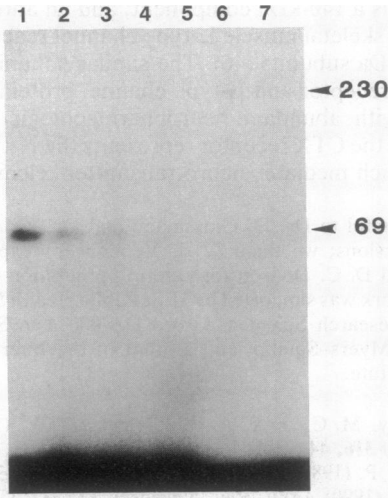

FIG. 3. HSA-^{125}I-CTX-labeled 230-kDa protein copurifies with ^{125}I-CTX receptor: SDS/PAGE analysis of purified fractions. Lane 1, HSA-^{125}I-CTX-labeled Zn flow-through; lane 2, 50 mM + 75 mM potassium phosphate pooled fraction from HA column; lane 3, 100 mM + 150 mM + 200 mM potassium phosphate (pH 6.5) pooled fractions from HA column; lane 4, 200 mM + 250 mM + 300 mM potassium phosphate (pH 9.5) pooled fractions from HA column; lane 5, flow-through from sWGA column; lane 6, *N*-acetylglucosamine (0.5 M) eluate from sWGA column; lane 7, second *N*-acetylglucosamine (0.5 M) eluate from sWGA column. The HSA-^{125}I-CTX-labeled protein was visualized by autoradiography on Kodak X-Omat 5 film for 2 weeks at −70°C.

In initial attempts to purify active CTX receptor, the receptor aggregated and precipitated when applied to various columns. Aggregation is time dependent (<2 hr) and enhanced by all salts examined (less than 20 mM). ^{125}I-CTX binding in soluble preparations is inhibited by Tris buffers and most salt buffers with an IC_{50} of about 20 mM. To obviate these problems, we have utilized Hepes buffers containing glycerol and dithiothreitol and negative chromatography on heparin-Sepharose, arginine-Sepharose, and metal-chelate-Sepharose, which proceeds rapidly and removes contaminants sufficiently to greatly diminish protein aggregation (Table 1).

HA provides a 7- to 8-fold enhancement of reversible CTX binding, with the peak of reversible binding migrating identically to the peak of HSA-^{125}I-CTX photolabel (Fig. 2). The pattern of photolabeling of the Zn flow-through fraction indicates a very minor contribution of the 230-kDa protein at this stage (Fig. 3, lane 1). Fractionation on HA clearly resolves the 69-kDa contaminant from the 230-kDa CTX receptor, which is eluted at 200–300 mM potassium phosphate (Fig. 3, lane 4). Following the HA step, sWGA provides a further substantial increase in specific activity (Table 2). Approximately half of reversible CTX binding is lost in the

Table 2. Comparison of CTX receptor recovery for reversibly bound and photoaffinity ligands

Fraction	^{125}I-CTX binding, % recovery	HSA-^{125}I-CTX binding, % recovery
sWGA chromatography		
HA fraction	100	100
sWGA flow-through	20	20
sWGA eluate	35	80
PEG precipitation		
sWGA eluate	100	100
PEG supernatant	5	5
PEG pellet	85	95

The experiment was replicated three times with less than 5% variation.

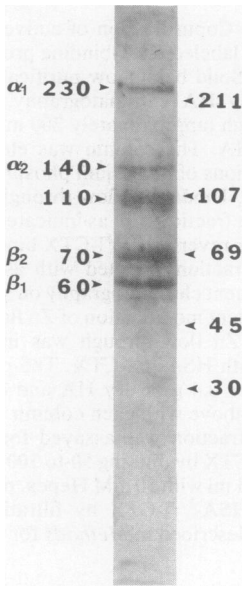

FIG. 4. Subunit composition of the rat forebrain CTX receptor. The CTX receptor eluted from the sWGA column (200 μl) was prepared in 5× Laemmli sample buffer and resolved on a 7–20% polyacrylamide gel, which was silver stained. The apparent molecular masses of the CTX receptor subunits are α_1, 230; α_2, 140; β_1, 60; and β_2, 70 kDa; and there is a 110-kDa doublet. For additional explanation of nomenclature see *Results and Discussion*. The apparent molecular masses (kDa) of the prestained standards are myosin, 211; phosphorylase b, 107; bovine serum albumin, 69; ovalbumin, 45; and carbonic anhydrase, 30.

sWGA purification step. The majority of this loss appears related to inactivation of the binding activity of the protein, as recovery of the photolabeled CTX receptor is substantially greater (Fig. 3, lanes 6 and 7; Table 2). The 230-kDa photolabeled band copurifies with reversible ligand binding in all stages of the purification process. The sWGA eluate can be concentrated and further purified by precipitation with PEG (Table 2), the final preparation being purified almost 2000-fold with a recovery of 10% of the original binding activity, approaching theoretical purity for the CTX receptor.

Our structural evidence indicates a close similarity of the purified CTX receptor and the DHP binding L-type channel. The silver-stained SDS/PAGE profile comprises a 230-kDa CTX-binding protein and four additional bands: 140, 110, 70, and 60 kDa; thus, the nomenclature adopted in Fig 4. Several lines of evidence indicate that all five bands are discrete subunits of the CTX receptor and not proteolytic fragments. First, all purification steps employ a substantial number of protease inhibitors. Second, in sucrose gradient fractionation, reversible CTX binding emerges as a single peak that includes all five bands present in that fraction (data not shown). Further evidence that all five subunits make up a CTX receptor comes from experiments of Sakamoto and Campbell (46) in which an antibody to a β subunit of the DHP receptor immunoprecipitated a ^{125}I-CTX binding protein, and the immunoprecipitate contained five protein bands identical in size to those resulting from our procedure.

The 230-kDa component of the CTX receptor, containing the recognition site for HSA-^{125}I-CTX, resembles in size the unprocessed 212-kDa α_1 subunit of the DHP receptor (15). Drug recognition sites for all three classes of L-type channel antagonists reside on the α_1 subunit (47). α_1 subunits of the channel with apparent molecular masses greater than 200 kDa are found in brain (48) and skeletal muscle (49, 50). The α_2 subunit of the L-type channel is quite similar in apparent mobility to the 140-kDa component of the CTX receptor. The CTX receptor contains a 110-kDa subunit present in L-type channels from brain (50) but not in skeletal and cardiac muscle. The CTX receptor contains 60- and 70-kDa subunits. In brain and skeletal and cardiac muscle the DHP receptor contains a single β subunit of about 60 kDa (47). We do not detect any major subunits of the CTX receptor smaller than 60 kDa. The L-type channel of skeletal and cardiac muscle possesses γ and δ subunits of 30 and 20 kDa, respectively. Interestingly, the brain L-type channel may also lack a γ subunit (51). The δ subunit, which is not visible in brain CTX or DHP receptor preparations, is inferred to be present by the shift in α_2 mobility in SDS/PAGE upon reduction (51).

Table 3. Comparison of subunit composition of purified mammalian CTX and DHP receptors

Subunit	Molecular mass, kDa: CTX receptor from rat brain	DHP receptor: Rabbit brain	DHP receptor: Skeletal muscle
α_1	230	175	155–200
α_2	140	142	122–145
	110	100	
β_2	70		
β_1	60	57	50–65
γ	?	?	30–35
δ	?	+	20–30

Data for subunit composition of DHP receptor are as reported for rabbit brain (51) and rat skeletal muscle (47) preparation. The question marks indicate that such subunits have not yet been definitively identified. The plus sign indicates that there is a shift in α_2 mobility upon reduction that implicates an associated δ subunit (52).

The CTX receptor and L-type channels also may share immunologic epitopes. Antibodies to the DHP receptor immunoprecipitate ^{125}I-CTX binding along with five proteins (46) of the same apparent molecular masses as the subunits of the CTX receptor we have isolated. An antibody to the cardiac L-type channel α_1 subunit identifies a 230-kDa subunit, an antibody to the skeletal muscle L-type channel α_2 subunit labels a 140-kDa component, and an antibody to the β subunit of skeletal muscle L-type channel reacts with both 60- and 70-kDa subunits (46). The similar subunit structures of the CTX receptor and L-type channel proteins (Table 3), combined with abundant neuropharmacologic data (1–3), indicate that the CTX receptor represents the N-type calcium channel, which mediates neurotransmitter release.

We are grateful to Dr. H. Glossmann and Dr. K. Angelides for helpful discussions; we thank V. J. Venema for expert technical assistance and D. C. Dodson for careful preparation of the manuscript. This work was supported by U.S. Public Health Service Grant MH-18501, Research Scientist Award DA-00074 to S.H.S., a gift from Bristol-Myers-Squibb, and a grant of the International Life Sciences Institute.

1. Nowycky, M. C., Fox, A. P. & Tsien, R. W. (1985) *Nature (London)* **316**, 443–446.
2. Bean, B. P. (1989) *Annu. Rev. Physiol.* **51**, 367–384.
3. Hess, P. (1990) *Annu. Rev. Neurosci.* **13**, 337–356.
4. Reuter, H. (1983) *Nature (London)* **301**, 969–974.
5. Tsien, R. W., Bean, B. P., Hess, P., Lansman, J. B., Nilius, B. & Nowycky, M. C. (1986) *J. Mol. Cell Cardiol.* **18**, 691–710.
6. Reynolds, I. J. & Snyder, S. H. (1988) in *Ion Channels*, ed. Narahashi, T. (Plenum, New York), Vol. 1, pp. 213–249.
7. Fossett, M., Jaimovich, E., Delpoint, E. & Lazdunski, M. (1983) *J. Biol. Chem.* **258**, 6086–6092.
8. Glossmann, H., Ferry, D. R. & Boschek, C. B. (1983) *Naunyn-Schmiedebergs Arch. Pharmakol.* **323**, 1–11.
9. Takahashi, M., Seagar, M. J., Jones, J. F., Reber, B. F. & Catterall, W. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5478–5482.

10. Sharp, A. H., Imagawa, T., Leung, A. T. & Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 12309–12315.
11. Striessnig, J., Knaus, H.-G., Grabner, M., Moosburger, K., Seitz, W., Lietz, H. & Glossmann, H. (1987) *FEBS Lett.* **212,** 247–253.
12. Sieber, M., Nastainczyk, W., Zubor, V., Wernet, W. & Hofmann, F. (1987) *Eur. J. Biochem.* **167,** 117–122.
13. Schneider, T. & Hofmann, F. (1988) *Eur. J. Biochem.* **174,** 369–380.
14. Hasse, H., Striessnig, J., Holtzhauer, M., Vetter, R. & Glossmann, H. (1991) *Eur. J. Pharmacol.* **207,** 51–59.
15. Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1987) *Nature (London)* **328,** 313–318.
16. Ellis, S. B., Williams, M. E., Ways, N. R., Brenner, R., Sharp, A. H., Leung, A. T., Campbell, K. P., McKenna, E., Koch, W. J., Hui, A., Schwartz, A. & Harpold, M. M. (1988) *Science* **241,** 1661–1664.
17. Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S. & Numa, S. (1989) *Nature (London)* **340,** 230–233.
18. Snutch, T. P., Leonard, J. P., Gilbert, M. M., Lester, H. A. & Davidson, N. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3391–3395.
19. Snutch, T. P., Tomlinson, J., Leonard, J. P. & Gilbert, M. (1991) *Neuron* **7,** 45–57.
20. Hui, A., Ellinor, P. T., Krizanova, O., Wang, J.-J., Diebold, R. J. & Schwartz, A. (1991) *Neuron* **7,** 35–44.
21. Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofmann, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. & Numa, S. (1991) *Nature (London)* **350,** 398–402.
22. Starr, T. V., Prystay, W. & Snutch, T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5621–5625.
23. Plummer, M. R., Logothetis, D. E. & Hess, P. (1989) *Neuron* **2,** 1453–1463.
24. Hirning, L. D., Fox, A. P., McCleskey, E. W., Olivera, B. M., Thayer, S. A., Miller, R. J. & Tsien, R. W. (1988) *Science* **239,** 57–61.
25. Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A. & Gray, W. R. (1984) *Biochemistry* **23,** 3090–3095.
26. McCleskey, E. W., Fox, A. P., Feldman, D. H., Cruz, L. J., Olivera, B. M. & Tsien, R. W. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4327–4331.
27. Wagner, J. A., Snowman, A. M., Biswas, A., Olivera, B. M. & Snyder, S. H. (1988) *J. Neurosci.* **8,** 3354–3359.
28. Takemura, M., Fukui, H. & Wada, H. (1987) *Biochem. Biophys. Res. Commun.* **149,** 982–988.
29. Glossmann, H. & Striessnig, J. (1988) *ISI Atlas Sci. Pharmacol.* **2,** 202–210.
30. McEnery, M. W., Snowman, A. M. & Snyder, S. H. (1991) *Ann. N.Y. Acad. Sci.* **635,** 435–438.
31. McEnery, M. W., Buhle, E. L., Jr., Aebi, U. & Pedersen, P. L. (1984) *J. Biol. Chem.* **259,** 4642–4651.
32. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
33. Venema, V. J., Swiderek, K., Lee, T. D. & Adams, M. E. (1991) *J. Biol. Chem.*, in press.
34. Jacobs, E. E., Jacobs, M., Sanadi, D. R. & Bradley, L. B. (1956) *J. Biol. Chem.* **223,** 147–156.
35. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
36. Wray, W., Boulikast, T., Wray, V. P. & Hancock, R. (1981) *Anal. Biochem.* **118,** 197–203.
37. Marqueze, B., Martin-Moutot, N., Leveque, C. & Courad, F. (1989) *Mol. Pharmacol.* **34,** 87–90.
38. Barhanin, J., Schmid, A. & Lazdunski, M. (1988) *Biochem. Biophys. Res. Commun.* **150,** 1051–1062.
39. Abe, T. & Saisu, H. (1987) *J. Biol. Chem.* **262,** 9877–9882.
40. Knaus, H. G., Striessnig, J., Koza, A. & Glossmann, H. (1987) *Naunyn-Schmiedebergs Arch. Pharmakol.* **336,** 583–586.
41. Stumpo, R. J., Pullan, L. M. & Salama, A. I. (1991) *Eur. J. Pharmacol.* **206,** 155–158.
42. Olivera, B. M., Cruz, L. J., de Santos, V., LeCheminant, G. W., Griffin, D., Zeikus, R., McIntosh, J. M., Galyean, R., Varga, J., Gray, W. R. & Rivier, J. (1987) *Biochemistry* **26,** 2086–2090.
43. Yeagar, R. E., Yoshikami, D., Rivier, J., Cruz, L. J. & Miljanich, G. P. (1987) *J. Neurosci.* **7,** 2390–2396.
44. Venema, V. J. & Adams, M. E. (1990) *Neurosci. Abstr.* **16,** 956.
45. Mintz, I. M., Venema, V. J., Adams, M. E. & Bean, B. P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6628–6631.
46. Sakamoto, J. & Campbell, K. P. (1991) *Physiologist* **34,** 109 (abstr.).
47. Glossmann, H. & Striessnig, J. (1990) *Rev. Physiol. Biochem. Pharmacol.* **114,** 1–105.
48. Striessnig, J., Knaus, H.-G. & Glossmann, H. (1988) *Biochem. J.* **253,** 39–47.
49. De Jongh, K. S., Merrick, D. K. & Catterall, W. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8585–8589.
50. Lai, Y., Seagar, M. J., Takahashi, M. & Catterall, W. A. (1990) *J. Biol. Chem.* **265,** 20839–20848.
51. Ahlijanian, M. K., Westenbroek, R. E. & Catterall, W. A. (1990) *Neuron* **4,** 819–832.
52. Curtis, B. M. & Catterall, W. A. (1984) *Biochemistry* **23,** 2113–2118.