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SEARCH FOR LIGANDS OF NEURONAL α-BUNGAROTOXIN RECEPTORS

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SUMMARY Extracts of calf brain were analyzed for substances capable of blocking the binding of [^{125}I]-α-bungarotoxin to chick brain membrane preparations, and shown to contain blocking activity that was insensitive to heating and trypsin. Fractionation on Sephadex G-25 yielded two components, one representing nonspecific inhibition by inorganic cations, the other identified as choline by co-chromatography experiments and analysis of the purified inhibitor using thin layer chromatography and mass spectrometry. These results support the notion that the α-bungarotoxin binding macromolecule in the central nervous system is an acetylcholine receptor.

INTRODUCTION α-Bungarotoxin (αBuTX), the principal component of the venom of Bungarus multicinctus, blocks acetylcholine receptors in striated muscle (1). Several lines of evidence suggest that it may also bind to nicotinic acetylcholine receptors on nerve cells: (a) in the central as well as autonomic nervous system specific binding sites exist for αBuTX and related peptides; (b) neuronal toxin receptors are enriched in nerve ending fractions and occur in synapses displaying cholinergic morphology; (c) receptors are found wherever nicotinic-cholinergic neurotransmission is presumed to operate, within the whole body as well as within the central nervous system; and (d) receptors preferentially bind nicotinic drugs (2).

However, synaptic transmission across interneuronal junctions in the central and autonomic nervous system of higher vertebrates does not seem to be affected by the presence of α-bungarotoxin (for review see ref. 2). Conceivably the peculiarities of the interactions between neuronal receptor, toxin and agonist may account for the physiological ineffectiveness (3); however the possibility that these receptors do not

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function as acetylcholine receptors in the conventional sense has to be seriously considered. Experiments on a sympathetic nerve cell line led Patrick and Stallcup to suggest that αBuTX binding sites in sympathetic ganglia - and by implication on all nerve cells - are distinct from acetylcholine receptors and may serve as receptors for trophic signals instead (4). The notion of neuronal neurotoxin receptors responding to an endogenous ligand, possibly of peptide nature, is not entirely implausible. Studies on phylogenetic differences among acetylcholine receptors prompted Burden et al. (5) to postulate that the curarimimetic snake toxins may have evolved from nontoxic precursors subserving a trophic function.

Here we report on a search for endogenous compounds capable of binding to neuronal αBuTX receptors.

EXPERIMENTAL [^{14}C]-choline (46 mCi/mmole) and [^{3}H]-acetylcholine (49.5 mCi/mmole, labeled in the acetyl moiety) were purchased from New England Nuclear. [^{125}I]-α-Bungarotoxin ([^{125}I]-αBuTX; approximately 10^{6} Ci/mole, depending on the age of the preparation) was prepared as described previously (6). Fresh calf brains were obtained from a local butcher (kept at 0° to 4° for approximately 12-16 hrs after slaughter). Several experiments were done on rats and chicks whose brains were removed and processed immediately after sacrifice.

Aqueous extracts were obtained by homogenizing fresh tissue in 9 volumes of 10 mM sodium phosphate, 1 mM EDTA, 0.02% sodium azide and 0.4 mM phenylmethylsulfonyl fluoride, pH 7.2 ('extraction buffer'), containing 46 TIU aprotinin and 1 mg pepstatin per liter. The homogenate was centrifuged at 100,000xg for 1 hr, and the supernatant removed and lyophilized.

Acidic acetone extraction was carried out by either of two procedures: A mixture of acetone-1 M HCl (100:3, v/v) was added in a ratio of three parts of acidic acetone to one part sample, either to fresh whole brain or to an aqueous extract thereof (see above). In either case, insoluble material was removed by passing the suspension twice through Whatman #4 paper. The filtrate was then extracted twice with one volume of petroleum ether, and the aqueous phase collected and lyophilized.

The ligand detection assay is based on the assumption that the endogenous ligand of the neuronal αBuTX receptor competitively inhibits binding of the toxin. Brains of newly-hatched chicks were used as receptor source. Membrane-bound receptor was prepared by homogenizing freshly dissected brain stems in 9 volumes of 'extraction buffer' followed by centrifugation at 100,000xg for 1 hr. The sediment was resuspended in 10 volumes (with respect to original tissue) of 'extraction buffer', and radioactive αBuTX was added to yield an assay 'cocktail' containing 4.5×10^{-10} M of toxin receptor and 1.3×10^{-9} M of [^{125}I]-αBuTX. Samples to be tested for ligand (0.1 ml) were added to 0.1 ml of 'cocktail' in 1.5-ml microfuge tubes and incubated for 2-3 hrs. at 37° with shaking. Maximal binding was determined with 'extraction buffer' or the appropriate chromatography buffer replacing

the sample, and nonsepcific background measured by inclusion of 6.3×10^{-6} M α-cobrotoxin (Naja naja siamensis) in the buffer control. After incubation, 1.0 ml of 0.2 M sodium chloride was added, the suspension mixed vigorously and membranes sedimented by centrifugation in an Eppendorf model 3200 microcentrifuge. The supernatant was carefully removed by aspiration and the tip of the tube containing the sediment was cut off and counted in a Beckman model 8000 gamma counter.

Protein was measured by the Lowry method. Conductivity measurements were carried out with an Industrial Instruments Model RC16B2 conductivity meter calibrated with sodium chloride. For thin layer chromatography precoated Silica Gel G (Analtech) and a mixture of acetone: methanol: water: glacial acetic acid (50:40:5:5) were employed; after development the plates were sprayed with Dragendorff's reagent to identify choline and choline derivatives (7). The mass spectrometric analysis of the purified ligand was performed by Dr. Charles Iden, Department of Chemistry, S.U.N.Y. Stony Brook; the material was analyzed at high temperature under high vacuum without chemical pretreatment (8).

RESULTS: Preliminary experiments indicated that rat brain extract contains a substance (or substances) capable of preventing the binding of αBuTX to brain membrane preparations. Similar observations were made with chick and calf brain. The calf brain material was found to be insensitive to heating (95° for 10 min) and to digestion by trypsin or pepsin. For a more detailed analysis, an acidic acetone extract of calf brain was prepared and chromatographed on Sephadex G-25 (Fig. 1). Except for a slight inhibition near the void volume inhibitory activity eluted in a narrow range at about two void volumes (I) and a broad shoulder coincident with the position of compounds of low molecular weight (II).

Material from region I of the acetone extract was subjected to further fractionation on carboxymethyl cellulose (Fig. 2). Inhibitory activity eluted at a position devoid of proteins or peptides, as indicated by absorbance readings or the phenol reaction. This, together with the protease and heat insensitivity of the inhibitory factor, suggested that the endogenous ligand might in fact be acetylcholine. [^{3}H]-acetylcholine was therefore added as a marker to samples prior to chromatography. These co-chromatography experiments revealed that the inhibitor was different from acetylcholine; they also indicated that acetylcholine was unstable under the chromatographic conditions employed. On the other hand, inclusion of choline as a tracer showed

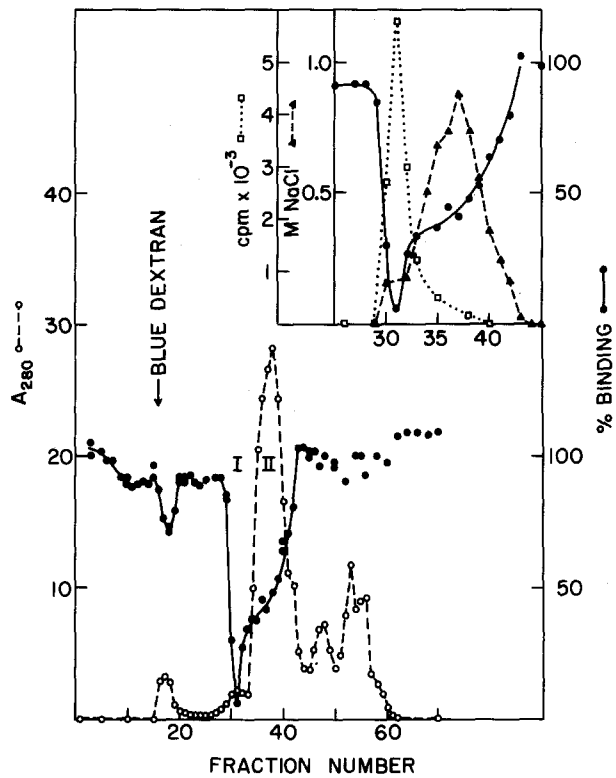

Fig. 1 Fractionation of acetone extract on Sephadex G-25

Two fresh calf brains (733 gm wet weight) were processed as described in the text, and the acetone extract lyophilized. The dried material was redissolved in 20 ml 50 mM sodium phosphate, and the pH of the sample adjusted to 7.2, using 1 M NaOH. After addition of [^{14}C]-choline, the mixture was applied to a Sephadex G-25 "fine" column (2.5x91 cm), equilibrated in 50 mM sodium phosphate pH 7.2. Fractions of 13 ml were collected and assayed for inhibitory activity. Radioactive choline was detected by counting 0.2 ml aliquots in 4 ml Bray's solution. Ionic strength is indicated in arbitrary units, obtained by converting conductivity measurements to sodium chloride equivalents. Data displayed in the insert were obtained in a separate experiment.

that choline might fully account for inhibitory activity in region I of the Sephadex G-25 eluate (Inset in Fig. 1); more convincingly, blocking activity and [^{14}C]-choline co-chromatographed on carboxymethyl cellulose (Fig. 2). After purification by ion exchange chromatography the endogenous ligand was passed through a Sephadex G-10 column equilibrated in 0.1 M acetic acid and lyophilized. A portion of this material was analyzed by thin layer chromatography; it was found to co-migrate with [^{14}C]-choline (R_F=0.24) and upon treatment with the Dragendorff spray gave the purple reaction characteristic of choline. Some of the

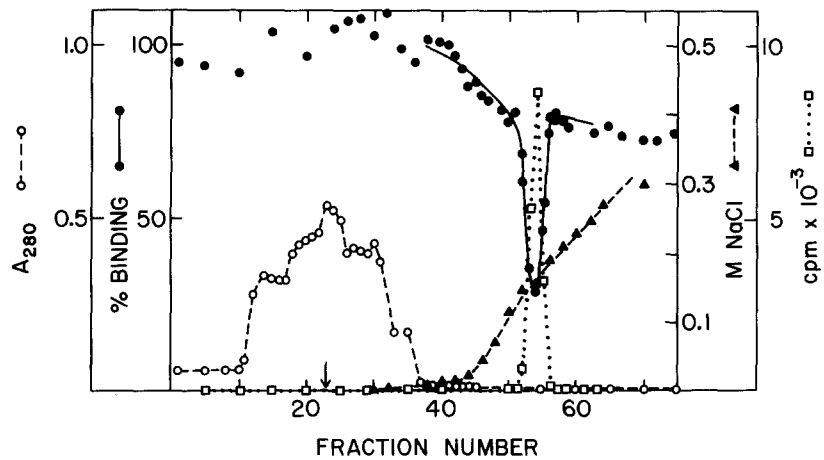

Fig. 2 Carboxymethyl-cellulose chromatography

Fractions 30 to 32 ("region I") of the Sephadex G-25 eluate (Fig. 1) were pooled and diluted five-fold with distilled water. [^{14}C]-choline was added, and the mixture applied to a carboxymethyl cellulose column (2.5x62 cm, CM52 Whatman). Elution was carried out with a linear salt gradient, with 0.5 l 10 mM sodium phasphate pH 7.2 in the mixing vessel and 0.5 l 0.5 M sodium chloride in 10 mM sodium phosphate pH 7.2, in the reservoir. Fractions of 13 ml were collected and assayed as described in the legend to Fig. 1.

lyophilized material was subjected to mass spectrometry and identified as choline acetate.

Inhibitory activity in region II was found to overlap with inorganic salts. It was not investigated further, since the measured conductivity corresponds to a concentration of sodium chloride that is easily sufficient to account for the observed inhibition.

Aqueous calf brain extracts were also analyzed after passage through sephadex G-25. Over 90% of the inhibitory activity was found to co-chromatograph with [^{14}C]-choline.

DISCUSSION: Brains of newly-hatched chicks were selected as a receptor source, and calf brains as a source of endogenous ligand for reasons of practicality. The former contain high levels of αBuTX binding sites (3) while the latter provide the bulk deemed necessary for ligand characterization. Clearly an analysis involving receptor and ligand from the same species would have been preferable. Preliminary experiments showed that extracts of both chick and rat brain contain a

substance that inhibits toxin binding to chick brain receptors and behaves like choline on gel filtration. Furthermore, calf brain contains BuTX binding sites at a concentration of approximately $2x10^{-9}$M, depending on region (unpublished observations); toxin binding sites in bovine brain have also been reported by Mahler and associates (9). The use of a heterologous assay system thus appears justified.

The general conclusion from the findings presented here is that brain-derived BuTX receptors, when incubated with aqueous extracts of whole brain, bind primarily choline; in vivo, they presumably interact with acetylcholine. Several reasons can be given for why choline rather than acethylcholine was identified as the principal endogenous ligand. First, no specific attempt was made to inactivate acetylcholinesterase during ligand extraction. Further, in rat brain choline occurs at concentrations 3 to 10 times higher than acetylcholine, and the same is likely to hold for calf brain (10). Finally, the BuTX receptor from chick brain has a fairly high affinity for choline ($K_D^{apparent}$= $1.9x10^{-4}$ M, ref. 3).

Besides choline, a macromolecular material capable of inhibiting toxin binding may exist. However, it is either not very stable, in spite of the protease inhibitors employed in the extraction, or not very abundant. The peptide region of the Sephadex G-25 column eluate, i.e. material eluting after one complete column volume, and comprising the bulk of the Lowry-positive substances of the acetone extract, is devoid of inhibitory activity. The inhibitory effect of simple inorganic cations has been amply documented with receptor from Torpedo californica electric tissue (11) as well as rat brain (12) and chick brain (3).

Attempts to identify nicotinic acetylcholine receptors in the CNS by means of small ligands date back to 1967, when DeRobertis and his colleagues employed [^{14}C]- d-tubocurarine (1,3). Later Schleifer and Eldefrawi (14) measured binding of [^{3}H]-acetylcholine and [^{3}H]-nicotine to mouse brain preparations. More recently Romano and Goldstein (15)

used [^{3}H]-nicotine, and Schwartz et al. (16) [^{3}H]-acetylcholine, of high specific activity, to characterize high-affinity binding sites with nicotinic-cholinergic properties. The physiological significance of these sites and their relationship to each other and to receptors for curarimimetic toxins is at present unknown.

The results presented here do not establish the neuronal αBuTX receptor as a nicotinic acetylcholine receptor, but they suggest that, in vivo, the toxin binding sites may be binding acetylcholine, either as a neurotransmitter in the conventional sense or as a neurohumor whose precise function remains to be determined.

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