Polypeptide Composition of the α-Latrotoxin Receptor

HIGH AFFINITY BINDING PROTEIN CONSISTS OF A FAMILY OF RELATED HIGH MOLECULAR WEIGHT POLYPEPTIDES COMPLEXED TO A LOW MOLECULAR WEIGHT PROTEIN

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α-Latrotoxin is a vertebrate neurotoxin from black widow spider venom that causes massive neurotransmitter release. In order to gain insight into the mechanism of action of α-latrotoxin, we have studied α-latrotoxin-binding proteins from bovine and rat brain. Proteins purified by affinity chromatography on immobilized α-latrotoxin were investigated. Two sets of proteins were isolated: 1) three polypeptides of Mr 79,000, 65,000, and 43,000 that were eluted from immobilized α-latrotoxin by increasing KCl concentrations in the presence of Ca2+, and 2) a family of related proteins ranging in molecular weight from 160,000 to 220,000 and a low molecular weight component of Mr 29,000 that were eluted from immobilized α-latrotoxin only after removal of Ca2+. Amino acid sequences of these proteins demonstrated that all of these proteins represent novel proteins except for the Mr 65,000 polypeptide, which is identical with synaptotagmin (Petrenko, A. G., Perin, M. S., Davletov, B. A., Ushkaryov, Y. A., Geppert, M., and Sudhof, T. C. (1991) Nature 353, 65–68). Surprisingly, the Mr 79,000 and 43,000 proteins were also found in tissues insensitive to α-latrotoxin action. Since these proteins do not bind 125I-α-latrotoxin with high affinity, their purification probably is not physiologically significant. On the other hand, the fractions containing the Mr 160,000–220,000 and 29,000 polypeptides bound α-latrotoxin with high affinity. Sucrose gradient centrifugations and anion exchange chromatography suggested that most of the Mr 160,000–220,000 proteins were complexed with the Mr 29,000 protein. α-Latrotocin binding correlated with the presence of the Mr 160,000–220,000 proteins and Mr 29,000 polypeptide, and α-latrotoxin formed stable complexes with the Mr 160,000–220,000 proteins. Accordingly, the α-latrotoxin receptor consists of a high molecular weight protein (Mr 160,000–220,000) that is complexed with one or several copies of an Mr 29,000 polypeptide. In addition, the receptor is found in a less tight association with synaptotagmin but not with other polypeptides.

Black widow spider venom contains a complex mixture of neurotoxins that act on synapses (1, 2). One of these toxins, α-latrotoxin, causes extensive neurotransmitter release from the presynaptic nerve terminals of vertebrates (3). α-Latrotoxin is a Mr 116,000 protein that binds to brain membranes with high affinity in a specific and saturable manner (4, 5). Indirect immunofluorescence suggests that α-latrotoxin only binds to the presynaptic plasma membrane (6), but its mechanism of action in causing neurotransmitter release is unknown.

Extensive studies on the neuromuscular junction demonstrated that α-latrotoxin has a specific effect on the nerve terminal (reviewed in Ref. 7). At low doses, the toxin causes extensive continuous neurotransmitter release from vesicular stores but does not interfere with the recycling of synaptic vesicles or result in significant morphological changes in the exposed tissues. The stimulatory effect of the toxin on neurotransmitter release is independent of Ca2+. However, in the absence of Ca2+ the endocytosis of synaptic vesicles is inhibited, suggesting that Ca2+ is necessary for synaptic vesicle endocytosis (8). Additional and slightly different results were obtained with central nervous system synaptosomes. With these, α-latrotoxin used at relatively high concentrations causes nonspecific leakage of cytosolic components from nerve endings (9, 10). It has also been shown that α-latrotoxin induces nonspecific ion channels in black lipid membranes in a comparatively slow process (11).

Based on these results, two alternative hypotheses about the action of α-latrotoxin have been proposed (7, 10–12). According to the first hypothesis, nerve terminals contain specific α-latrotoxin receptors solely as a means to fix the toxin close to the plasma membrane. This would allow the toxin to insert into the nerve terminal plasma membrane and to form nonspecific ion channels. The second alternative hypothesis suggests that binding of α-latrotoxin to its receptor activates the receptor. The receptor would then transmit a signal into the cytosol that causes Ca2+-independent neurotransmitter release.

Neither hypothesis fully explains all available data. The fact that low toxin doses at the neuromuscular junction allow full recycling of synaptic vesicles and do not cause tissue damage in spite of stimulating massive neurotransmitter release argues against the nonspecific channel hypothesis. However, the action of the toxin even at the neuromuscular junction is very slow. It is hard to imagine how the toxin could cause Ca2+-independent neurotransmitter release without some direct action in the cytosol.

One approach to understanding α-latrotoxin action consists in the study of its receptor. The purification of high affinity α-latrotoxin-binding proteins has been reported (13, 14).
One study, a single disulfide-linked protein complex containing M, 66,000 and 54,000 polypeptides was purified. In the second study, α-latrotoxin-binding proteins with major components of M, 200,000 and 160,000 were isolated; in addition, several proteins were observed that appeared to co-purify but eluted at lower ionic strengths from the affinity matrix than the actual receptor. One of these associated polypeptides was shown to be identical with synaptotagmin (15), suggesting that the receptor may have a docking function in the nerve terminal. Although these studies clearly demonstrate the feasibility of purifying high affinity α-latrotoxin receptors from brain, the nature of the different purified polypeptides and their relation to each other as well as the subunit composition of the α-latrotoxin binding receptor are currently unclear. Since these questions are of great importance for any understanding of how α-latrotoxin acts, we have embarked on a study of the α-latrotoxin receptor.

**EXPERIMENTAL PROCEDURES**

α-Latrotoxin Binding Assays—α-Latrotoxin was purified from black widow spider venom glands and iodinated in active form as described (16). 125I-α-Latrotoxin binding to solubilized membrane proteins was measured using a nitrocellulose filter binding assay as reported (17). The difference in binding observed in absence and presence of unlabeled α-latrotoxin as a competitor. Usually 0.5 nM 125I-α-latrotoxin was used for binding and specific sites were competed with 20 nM unlabeled α-latrotoxin. These concentrations were chosen because of their relation to the previously determined Kd of 0.8 × 10^-9 M for the solubilized binding site (14).

α-Latrotoxin Receptor Purification—α-Latrotoxin receptor purification was performed essentially as described (14, 15). Briefly, purified α-latrotoxin was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.). Bovine and rat brain membrane proteins were solubilized in 0.5% (w/v) Triton X-100 (Sigma) or CHAPS3 (Calbiochem) in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride. After extensive washing, proteins bound to the column were first eluted with a 0.13-0.6 M KCl gradient in the presence of 2 mM CaCl2 and 20 mM Tris-HCl, pH 7.4 (first gradient). The remaining bound proteins were then eluted with a step gradient of 1.0 M KCl and 10 mM EDTA (second gradient). All fractions were analyzed for 125I-α-latrotoxin binding as described above. Only the proteins in the peak fractions collected during the second gradient showed significant α-latrotoxin binding activity. These proteins are therefore collectively referred to as α-latrotoxin receptor.

Anion-exchange Chromatography of α-Latrotoxin-binding Proteins—Proteins eluted from immobilized α-latrotoxin by the second gradient were dialyzed against 0.1 M Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% Triton X-100. 0.5 ml (~15 μg of protein) were applied to a Mono Q HR5/5 column (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 7.4, and 0.1% (w/v) Triton X-100 buffer. The column was washed with 4 ml of the equilibration buffer and eluted with 25 ml linear gradient of 0.0-1.0 M NaCl in the same buffer. 0.5-ml fractions were collected and analyzed by binding measurements, SDS-PAGE, and immunoblotting.

Preparative Gel Filtration of α-Latrotoxin-binding Proteins—Purified α-latrotoxin receptor proteins (approximately 0.4 mg of protein) were precipitated by chloroform/methanol extraction. The pellet was washed with acetone and dissolved in 0.3 ml of 3% (w/v) SDS, 50 mM dithiothreitol, and 0.2 M Tris-HCl, pH 8.2. The solution was boiled, centrifuged briefly, and applied to a Superose 12 column (Pharmacia) equilibrated in 0.1% SDS, 2 mM EDTA, and 100 mM Tris-HCl, pH 8.2. The column was eluted with the same buffer at 0.1 ml/min, and 0.4-ml fractions were collected.

Sucrose Density Gradient Centrifugation—Two types of experiments were performed. In the first, the formation of complexes between α-latrotoxin and its putative receptor were analyzed by studying their apparent sizes on sucrose gradients. For this purpose, α-latrotoxin receptor was purified in the presence of CHAPS and further dialyzed against 1% (w/v) CHAPS, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.4. Linear sucrose gradients (6-20% (w/v)) were formed in 1% CHAPS, 2 mM CaCl2, and 75 mM Tris-HCl, pH 7.4. Samples (0.2-0.5 ml, approximately 5-15 μg of protein), consisting either of α-latrotoxin receptor proteins or of 125I-α-latrotoxin or of mixtures of the two in different ratios, were loaded on sucrose gradients and run in a SW 41 rotor (Beckman) at 38,000 rpm for 16 h at 4°C. After centrifugation, 0.54-ml fractions were collected and analyzed by SDS-PAGE followed by autoradiography for 125I-α-latrotoxin or by immunoblotting for the M, 160,000-220,000 proteins. Marker proteins (apparent molecular weights in parentheses) centrifuged in parallel gradients migrated at the following positions: carbonic anhydrase (29,000), fractions 2 and 3; bovine serum albumin (67,000), fraction 4; alcohol dehydrogenase (150,000), fraction 6; β-amylase (200,000), fractions 8 and 9; apoferritin (443,000), fractions 14 and 15. In the second type of experiment, the receptor was fractionated on sucrose gradients of the relative distributions of α-latrotoxin binding and of the different polypeptides across the gradient were investigated. For this purpose, sucrose gradient centrifugations were carried out essentially as described above. Fractions were then analyzed for 125I-α-latrotoxin binding (0.5 nM) in the presence and absence of unlabeled α-latrotoxin competitor (200–300 nM) used for the preparation of the M, 20,000 and the M, 180,000-220,000 proteins by immunoblotting.

*N-terminal Sequence Analysis—*N-terminal sequence analysis was attempted for all purified polypeptides after separation of the proteins by SDS-PAGE and blotting to polyvinylidene difluoride membranes (17). Automated Edman degradation was performed with an Applied Biosystems (Foster City, CA) model 470A sequenator 470A (peptides and amino acids) was produced by the manufacturer's programming and chemicals. Only the M, 79,000 polypeptide showed an unequivocal N-terminal sequence (Table I), whereas the high molecular weight α-latrotoxin binding components exhibited a mixed N-terminal sequence and the other polypeptides were N-terminally blocked.

Amino Acid Sequencing—The major proteins eluted from the immobilized α-latrotoxin column were purified from each other by gel filtration in the presence of SDS. Fractions containing electrophoretically pure individual polypeptides were pooled, concentrated, and extensively dialyzed against 0.1 M Tris-HCl, pH 8.3. Fractions (0.5-3 ml of purified protein) were then subjected to proteinase K digestion and fragments were purified by one- and two-dimensional reverse phase high performance liquid chromatography essentially as described (18, 19). Peptides were subjected to automated Edman degradation, and sequences were searched for homologies using the Wisconsin package on a VAX computer (GenBank release 70, PIR release 30).

Antibody Production and Characterization—Antibodies were raised against synthetic peptides coupled to keyhole limpet hemocyanin (Calbiochem) as described (20). All peptides were synthesized with an N-terminal cysteine residue not present in the original sequence (Table III). Peptides were used to raise antibodies. For peptide IV, the sequence was obtained from the 13 amino acid codon: Peptide I: CVEFEVSQSTLET. This peptide corresponds to the peptide sequence obtained from purified M, 29,000 protein (Table II). Peptide II: CIATVSKRPEKV. This peptide was designed from sequences determined from the purified M, 79,000 protein (Table I). Peptide III: CERREATLYSDSPFMK. This sequence corresponds to sequences obtained from the purified high molecular weight receptor preparations (Table II). Peptide IV: CAPKTPSKAKKNDKEYY. This peptide corresponds to the C terminus of neurexin II, a protein related to but distinct from the high molecular weight components of the α-latrotoxin receptor (21). The peptide sequence largely overlaps with a sequence obtained from the purified α-latrotoxin receptor (Table II), and the resulting antibody reacted with both the bovine and rat receptor M, 160,000-220,000 proteins. Antibodies against peptides III and IV gave the same results in the experiments reported here. Antiserum obtained was tested by immunoblotting. Peptide blocking (100 μg/ml) was used to determine specificity.

Protein Assays, SDS-PAGE, and Immunoblotting—Protein concentrations were determined using the Pierce BCA protein assay. SDS-PAGE analysis of protein fractions was performed according to (18, 19) using prestained protein standards (Bio-Rad) as molecular weight standards. After electrophoresis, gels were stained either with silver (23) or with Coomassie Blue or were analyzed by immunoblotting using peroxidase labeled secondary antibodies (20). Immunoreactive bands were visualized either by color development or enhanced chemiluminescence according to the manufacturer's specifications (Amersham Corp.).

1 The abbreviations used are: CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
**RESULTS**

α-Latrotoxin was purified from the venom glands of black widow spiders and immobilized on a Sepharose matrix. Proteins solubilized from bovine or rat brain membranes were loaded onto the immobilized α-latrotoxin column at low ionic strength in the presence of Ca²⁺. After extensive washing, bound proteins were eluted first with a KCl gradient in the presence of Ca²⁺ and then with an EDTA-KCl gradient. Three major polypeptides of M, 79,000, 65,000, and 43,000 were purified by the first gradient as described earlier (15), whereas a set of high molecular weight polypeptides (M, 160,000–220,000) and a M, 29,000 protein were co-eluted by the second gradient (Fig. 1). In binding assays with ^125^I-α-latrotoxin, only the proteins eluted with the second gradient showed high affinity binding activity, in agreement with previous reports (14, 15). We have now systematically investigated the identity of the different polypeptides and their relationship to each other.

**M, 79,000, 65,000, and 43,000 Polypeptides**—These three proteins are the major components of the eluate obtained in the presence of Ca²⁺ by increasing concentrations of KCl (fractions 4–10, Fig. 1). Partial amino acid sequences were obtained from tryptic peptides from all three proteins. No significant homology to proteins in the current data banks was discovered for the sequences obtained from the M, 79,000 and 43,000 polypeptides (Table 1). The sequences from the M, 65,000 protein were identical to synaptotagmin, a synaptic vesicle protein that was recently shown to specifically interact with the α-latrotoxin receptor (Ref. 15 and data not shown).

Synaptotagmin elutes slightly earlier from the loaded α-latrotoxin column than the M, 79,000 and 43,000 polypeptides, suggesting that the latter proteins are bound more tightly to the affinity matrix (Fig. 1). In order to investigate if the M, 79,000 and 43,000 proteins, like synaptotagmin, form a complex with the α-latrotoxin receptor, an antibody was raised against a synthetic peptide derived from the sequence of the M, 79,000 protein. In brain, the antibody raised was specific for a M, 79,000 protein and its reactivity could be blocked with its cognate peptide (data not shown). When this antibody was used on immunoblots of membranes from different bovine tissues, the protein was found to be ubiquitously distributed. Highest levels of immunoreactive M, 79,000 protein were observed in tissues such as heart and kidney but not in brain (Fig. 2).

The action of α-latrotoxin and the presence of high affinity binding sites for α-latrotoxin is specific for the nervous system (5, 7, 23). In agreement with this finding and with the ubiquitous tissue distribution of this protein, the M, 79,000 protein shows no specific ^125^I-α-latrotoxin binding in vitro (data not shown). It follows that the M, 79,000 protein either belongs to a ubiquitous set of proteins in a general signaling pathway with which the α-latrotoxin receptor interacts, or is purified by a low affinity, nonphysiologic interaction with immobilized α-latrotoxin. In the first case, the protein should form a complex with the α-latrotoxin receptor; in the second case, it should interact with immobilized α-latrotoxin in the absence of the receptor. To test these hypotheses, solubilized proteins from liver membranes were subjected to affinity chromatography on immobilized α-latrotoxin. Liver membranes contain no detectable binding activity for ^125^I-α-latrotoxin and presumably no α-latrotoxin receptors. Nevertheless, proteins of M, 79,000 and 43,000 proteins were purified from liver membranes on immobilized α-latrotoxin in a manner identical to that observed in brain membranes (Fig. 3). Direct comparisons of fractions purified from liver and brain membranes demonstrate that the M, 79,000 and 43,000 polypeptides are similarly purified from both tissues whereas synaptotagmin is only purified from brain (Fig. 4).

Together these results suggest that in contrast to synaptotagmin, the M, 79,000 and 43,000 proteins nonspecifically interact with α-latrotoxin rather than the receptor, and that their purification does not reflect a physiologically relevant interaction. This conclusion also agrees well with the immunocytochemical localization of the M, 79,000 protein, suggesting that this protein may be mitochondrial (data not shown).

**M, 29,000 and 160,000–220,000 Polypeptides**—^125^I-α-Latrotoxin binding activity is eluted from the immobilized α-latrotoxin column by application of EDTA at high ionic strength. Elution of the binding activity correlated with a set

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**Fig. 1. Purification of α-latrotoxin-binding proteins from bovine brain membranes.** Solubilized membrane proteins were chromatographed on immobilized α-latrotoxin as described under “Experimental Procedures.” Fractions 4–10 were collected while eluting the column with the 0.13–0.6 M KCl gradient, and fractions 11–14 corresponded to the step elution with 1 M KCl, 10 mM EDTA. Proteins in equal portions of each fraction were separated on 9% SDS-polyacrylamide gels. Gels were either stained with Coomassie Blue (top panel) or transferred onto nitrocellulose and reacted with the following antibodies: an antibody against the α-latrotoxin receptor (Ref. 15 and data not shown). The M, 160,000–220,000 proteins (second panel, labeled anti-α-latrotoxin receptor) or with an antibody against the M, 29,000 protein (bottom two panels, anti-29K). For the blot shown in the bottom panel, the peptide used for the immunization was added to determine the specificity of the reaction. Numbers on the right show migration of molecular weight markers. The long arrow in the top panel points to synaptotagmin, which copurifies with the α-latrotoxin receptor but stains poorly with Coomassie (15). The short arrows point to the positions of the high and low molecular weight components of the α-latrotoxin binding component that are visualized with the antibodies in the second and third panels. The M, 120,000 protein that is eluting in the second half of the affinity chromatography (top panel) represents α-latrotoxin bleeding from the column.
Polypeptide Composition of the α-Latrotoxin Receptor

TABLE I
Peptide sequences from purified M, 79,000 and 43,000 polypeptides

Peptide sequences from the M, 79,000 and 43,000 polypeptides that were purified on immobilized α-latrotoxin followed by gel filtration in the presence of SDS. Sequences were determined from intact proteins (N-terminal sequence) or from purified tryptic fragments (internal peptides). The unfragmented M, 43,000 polypeptide gave no readable sequence and is presumed to be blocked. Sequences are shown in single-letter amino acid code with X denoting unidentifiable amino acid residues.

M, 79,000 polypeptide

| N-terminal sequence | Internal peptides |
|---------------------|------------------|
| b-1-2-4             | FGAPQK           |
| b-2-5-1             | TGIQEQSAG        |
| b-3-1-1             | GFKEY            |
| b-3-1-2-2-3         | MPSQPRDSS        |
| b-3-1-7-1           | THINY            |
| b-3-1-9-3           | GDAVK            |
| b-3-2-5-2           | TTLPALG          |
| b-4-3-1             | IATVSKRPEKVI     |
| b-4-21              | KLDSLTVFGFPGVAAATLVDGKX|

M, 43,000 polypeptide

| Internal peptides |
|-------------------|
| g-1-5             | PYGTTVAAAD       |
| g-1-7-1           | GATPK            |
| g-2-1-4-9-1       | DAQDEGLLSD       |
| g-2-6-2           | AYLR             |
| g-2-7-2           | SAVPSQTSNK       |
| g-3-9-1           | GGEVQTS          |
| g-4-1-1-1         | DFMVYDQS         |
| g-4-1-3           | QGLLGGP          |
| g-4-6-1           | TPFILSN          |
| g-4-9-1           | RAQDEGLLSD       |
| g-5-10            | VPPNTVMPG        |
| g-6               | TLNPVNEQFTFK     |
| g-9               | AQDEGLI          |

Fig. 2. Tissue distribution of the M, 79,000 protein.
Membrane proteins from the indicated bovine tissues (100 µg of protein/lane) were analyzed by SDS-PAGE followed by immunoblotting with an antibody against peptide II, a peptide derived from the partial sequence of the M, 79,000 protein (Table I). Purified M, 79,000 and 43,000 proteins were loaded on the right lane (fraction 5 in Fig. 1). Numbers on the right indicate positions of molecular weight markers.

of proteins of approximately 160,000–220,000 daltons. In addition, a M, 29,000 protein and several minor proteins were observed (Fig. 1). The M, 29,000 stains only weakly with Coomassie Blue but can be well visualized by silver staining after gel filtration of the α-latrotoxin receptor preparation (Fig. 5). This experiment also reveals that the M, 79,000 and 160,000–220,000 proteins are present in comparable amounts, whereas no other polypeptides appear to be similarly enriched. These results pose the following questions: What is the relationship between the high and low molecular weight components of the α-latrotoxin-binding proteins, and are they or one of the minor proteins present in the preparation responsible for the α-latrotoxin binding activity?

To address these questions, it was essential to obtain protein sequences from and specific antibodies against the major proteins in this preparation. The M, 29,000 protein and the M, 160,000–220,000 proteins could be purified from each other by gel filtration in the presence of SDS (Fig. 5). This purification enabled us to obtain peptide sequences from tryptic fragments from the M, 29,000 protein as well as from the M, 160,000–220,000 proteins (Table II). The peptide sequences determined did not show any significant homology to protein sequences contained in the current data banks (GenBank...
an estimation of the sizes of the free and complexed α-latrotoxin receptor.

Fig. 6 demonstrates that the high molecular weight components of the receptor migrate on sucrose gradients at approximately 150,000–250,000 daltons in the absence of toxin (top panel), whereas free α-latrotoxin exhibits an apparent size of M<sub>r</sub> 200,000–350,000 in the absence of receptor (bottom panel). This result suggests that the high molecular weight components of the receptor do not form high molecular weight complexes with each other but migrate close to their monomer molecular weights. The apparent size of the toxin on sucrose gradients is 2–3 times its subunit molecular weight and agrees well with the previously observed dimerization of α-latrotoxin (16).

Sucrose gradients of mixtures of α-latrotoxin with an excess of receptor produce an almost complete shift of the migration of the toxin to very high molecular weights. Under these conditions, no shift in the apparent size of the receptor protein is observed, because only a very small fraction of the total receptor is complexed to the toxin. Conversely, if sucrose gradients of mixtures of the receptor with an excess of α-latrotoxin are performed, a complete shift of the migration of the M<sub>r</sub> 160,000–220,000 receptor proteins to very high apparent molecular weights is revealed (Fig. 6, middle panels). These results demonstrate that in the presence of an excess of α-latrotoxin, all of the M<sub>r</sub> 160,000–220,000 proteins are stably complexed with α-latrotoxin. They must therefore be part of the binding site. In the presence of excess receptor, on the other hand, all of the toxin is complexed. The observed high molecular weight complexes between receptor and toxin are far larger than would be expected for one-to-one complexes. This suggests that the toxin dimers and trimers can cross-link receptor molecules.

What is the protein composition of the α-latrotoxin binding site? As mentioned earlier, a M<sub>r</sub> 29,000 polypeptide copurified with the M<sub>r</sub> 160,000–220,000 polypeptides. The sucrose gradient data and immunoblotting experiments demonstrated that the high molecular weight components consist of several related proteins, all of which participate in α-latrotoxin binding. The question now arises if the M<sub>r</sub> 29,000 protein forms a complex with the high molecular weight proteins, and if so, if it forms a complex with all of them. To address these questions, anion exchange chromatography of the α-latrotoxin receptor proteins was performed. The major M<sub>r</sub> 160,000 and 200,000 proteins of the receptor could be separated from each other by chromatography on a Mono Q column (Fig. 7). Immunoblotting of the anion exchange chromatography fractions demonstrated that the M<sub>r</sub> 29,000 protein completely co-migrated with both of the major high molecular weight components. This result suggests that the M<sub>r</sub> 29,000 protein forms a complex with both of the two major high molecular weight polypeptides, and that this complex is stable at the salt concentrations used for the column elution. Furthermore, the proteins present in both of the chromatography peaks were shown to bind 125I-α-latrotoxin (Ref. 14 and data not shown), indicating in agreement with the sucrose gradient centrifugation data (Fig. 6) that both major high molecular weight components are part of an α-latrotoxin binding site.

The results up to now suggest that the high molecular weight components of the α-latrotoxin receptor purified on immobilized α-latrotoxin represent a family of related proteins that are part of a specific α-latrotoxin binding site and are not complexed to each other but are complexed to a low molecular weight component of M<sub>r</sub> 29,000. To address the questions if all of the M<sub>r</sub> 160,000–220,000 proteins are complexed to the M<sub>r</sub> 29,000 protein, and how the distributions of

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**Fig. 4.** Comparison of the proteins purified on immobilized α-latrotoxin from bovine brain or liver. Peak fractions of the elution with the first gradient (fraction 5 in Figs. 1 and 3) were analyzed by SDS-PAGE followed by Coomassie Blue staining (left panel) or by immunoblotting with the antibodies against the M<sub>r</sub> 79,000 protein (middle panel) or against synaptotagmin (right panel). Numbers on the right indicate positions of molecular weight markers.

**Fig. 5.** Separation of the proteins in the α-latrotoxin receptor fraction (fractions 12–14, Fig. 1) by gel filtration in the presence of SDS. Proteins denatured by SDS were chromatographed on a Superose 12 column (see "Experimental Procedures"). Fractions were analyzed by SDS-PAGE followed by silver staining, with fraction 1 corresponding to the first fraction after the void volume. In order to reproduce the approximate molar stoichiometry of chromatographed proteins, the volume of the applied sample was increased proportionally to the decrease of molecular mass of the eluted protein: 2 μl of fractions 1–6, 4 μl of fractions 7–10, and 10 μl of fractions 11–14 were loaded on the gel. Molecular weight standards were electrophoresed on the right (apparent molecular weights, from top to bottom: 106,000, 80,000, 50,000, 32,500, and 27,500).

release 70; PIR release 30), suggesting that the proteins constituting the α-latrotoxin receptor do not represent previously sequenced polypeptides.

The generation of peptide sequences allowed the production of anti-peptide antibodies. Two different peptide antibodies that reacted with the high molecular weight components and one that reacted with the M<sub>r</sub> 29,000 protein were raised. Immunoblotting with these antibodies demonstrated that the M<sub>r</sub> 29,000 protein is not a breakdown product of the larger polypeptides since it only reacted with the M<sub>r</sub> 29,000 protein.
Polyepide Composition of the α-Latrotoxin Receptor

TABLE II

Peptide sequences from purified M, 160,000–220,000 and 29,000 proteins

Peptide sequences from the high and low molecular weight components of the α-latrotoxin receptor. Amino acids are shown in single-letter code with "X" denoting unidentified residues. Both the high and low molecular weight components of the receptor gave N-terminal sequence signals, which, however, were mixed in the case of the high molecular weight components and of unproportionately low yield in the case of the low molecular weight component.

| Internal peptides | a-1-3-3-1-2 | EYYV |
|-------------------|-------------|------|
|                   | a-4-2-26    | GGGQIT |
|                   | a-4-3-1-1a  | EAVW |
|                   | a-4-3-2-2   | VDDSDGDYELH1HQGS GLY |
|                   | a-4-3-8-2   | ETAKPNLIPSK |
|                   | a-4-3-14-1  | AGXENPYSQASAEVIA |
|                   | a-4-3-16-2  | SDLYIGGVA |
|                   | a-4-3-17-3  | QQTLGLY |
|                   | a-4-3-18-3  | GSSNLPLNQANNVMI |
|                   | a-4-4-1     | NDKQKYYV |
|                   | a-4-4-7-1   | FNIDHATELTXVEWQ |
|                   | a-4-4-8-2   | YYYDSTSGTGLR |
|                   | a-4-4-11    | VNQGEWYHFDQ |
|                   | a-4-4-15    | EAVTLSYDGSFMFK |
|                   | a-4-4-21    | FXYGTDAAEIESNAILN |
|                   | a-4-4-25    | TPYTAPEGEILDLDDEYLGLPENK |
|                   | a-4-4-27    | YLVSVPFPNFLGMQLSL |

| a-4-2-24 | DGWRNR |
| a-4-3-1-1 | NDLDK |
| a-4-3-1-2 | LTVDDQXAMTQMGDHT |
| a-4-3-8-1 | DYSNLHPTV |
| a-4-3-9-3 | WNGVPSMST |
| a-4-3-16-1 | FNIMAKAIHYDEVK |
| a-4-3-17-2 | KTGSIFS |
| a-4-3-17-5 | FGFR |
| a-4-3-19 | SADQYN |
| a-4-3-24-1 | EATVLSYDGSFMFK |
| a-4-4-5 | NLNDEWHTVR |
| a-4-4-8-1 | AGYLNDEWQTV |
| a-4-4-10-2 | KVNDXWYHADFQ |
| a-4-4-14-1 | LEPIFNETGITE |
| a-4-4-20 | XPETTLFAGYX |
| a-4-4-24 | GYLYVFEALGNGA NLK |
| a-4-4-26 | XENVATLDPITFQEPSF |

| Peptide sequences from purified M, 160,000–220,000 and 29,000 proteins. Amino acids are shown in single-letter code with "X" denoting unidentified residues. Both the high and low molecular weight components of the receptor gave N-terminal sequence signals, which, however, were mixed in the case of the high molecular weight components and of unproportionately low yield in the case of the low molecular weight component. |

| Internal peptides | a-1-3-3-1-2 | EYYV |
|-------------------|-------------|------|
|                   | a-4-2-26    | GGGQIT |
|                   | a-4-3-1-1a  | EAVW |
|                   | a-4-3-2-2   | VDDSDGDYELH1HQGS GLY |
|                   | a-4-3-8-2   | ETAKPNLIPSK |
|                   | a-4-3-14-1  | AGXENPYSQASAEVIA |
|                   | a-4-3-16-2  | SDLYIGGVA |
|                   | a-4-3-17-3  | QQTLGLY |
|                   | a-4-3-18-3  | GSSNLPLNQANNVMI |
|                   | a-4-4-1     | NDKQKYYV |
|                   | a-4-4-7-1   | FNIDHATELTXVEWQ |
|                   | a-4-4-8-2   | YYYDSTSGTGLR |
|                   | a-4-4-11    | VNQGEWYHFDQ |
|                   | a-4-4-15    | EAVTLSYDGSFMFK |
|                   | a-4-4-21    | FXYGTDAAEIESNAILN |
|                   | a-4-4-25    | TPYTAPEGEILDLDDEYLGLPENK |
|                   | a-4-4-27    | YLVSVPFPNFLGMQLSL |

| a-4-2-24 | DGWRNR |
| a-4-3-1-1 | NDLDK |
| a-4-3-1-2 | LTVDDQXAMTQMGDHT |
| a-4-3-8-1 | DYSNLHPTV |
| a-4-3-9-3 | WNGVPSMST |
| a-4-3-16-1 | FNIMAKAIHYDEVK |
| a-4-3-17-2 | KTGSIFS |
| a-4-3-17-5 | FGFR |
| a-4-3-19 | SADQYN |
| a-4-3-24-1 | EATVLSYDGSFMFK |
| a-4-4-5 | NLNDEWHTVR |
| a-4-4-8-1 | AGYLNDEWQTV |
| a-4-4-10-2 | KVNDXWYHADFQ |
| a-4-4-14-1 | LEPIFNETGITE |
| a-4-4-20 | XPETTLFAGYX |
| a-4-4-24 | GYLYVFEALGNGA NLK |
| a-4-4-26 | XENVATLDPITFQEPSF |

**Fig. 6.** Analysis by sucrose density gradient sedimentation of the interaction of 125I-α-latrotoxin with the high molecular weight α-latrotoxin receptor proteins. Sucrose gradients were run either with α-latrotoxin receptor alone (LTR, top panel, 10 µg of receptor protein) or with 125I-α-latrotoxin alone (LTX, bottom panel, 1 µCi of 125I in less than 10 ng of α-latrotoxin) or with a mixture of α-latrotoxin receptor and 125I-α-latrotoxin containing either an excess of receptor (LTR>LTX, second and third panels from top, 10 µg of receptor and 1 µCi of 125I in less than 1 ng of α-latrotoxin) or an excess of α-latrotoxin (LTR<LTX, fourth and fifth panels, 10 µg of receptor and 1 µCi of 125I in 15 µg of α-latrotoxin). Fractions were analyzed by SDS-PAGE and immunoblotting with antibodies against the M, 160,000–220,000 and the 29,000 protein (anti-peptide I, bottom panel). These results would be related better with the presence of the high molecular weight the different proteins relate to α-latrotoxin binding, additional sucrose gradient separations were carried out. Sucrose gradients were loaded with the purified receptor preparation, and fractions were analyzed in parallel by immunoblotting for the M, 160,000–220,000 receptor proteins and the M, 29,000 polypeptide. At the same time, 125I-α-latrotoxin binding was measured in the presence and absence of unlabeled α-latrotoxin to assure specificity of binding (Fig. 8).

The M, 160,000–220,000 receptor proteins and the M, 29,000 protein largely co-localized on the gradient but were slightly shifted with respect to each other in that the M, 29,000 protein peaked at a slightly higher apparent molecular weight than the high molecular weight subunits (Fig. 8, compare fraction 6 with fraction 10). These results would be compatible with the interpretation that only part of the high molecular weight components are complexed with the M, 29,000 protein, thereby making them larger and migrate at a greater apparent size on the sucrose gradients. Comparison of the distribution of α-latrotoxin binding across the gradient with the distribution of the M, 160,000–220,000 and the M, 29,000 proteins demonstrated that the binding activity correlated better with the presence of the high molecular weight 29,000 protein, thereby making them larger and migrate at a greater apparent size on the sucrose gradients. Comparison of the distribution of α-latrotoxin binding across the gradient with the distribution of the M, 160,000–220,000 and the M, 29,000 proteins demonstrated that the binding activity correlated better with the presence of the high molecular weight...
Fractions: 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

A

B

FIG. 8. Relative distributions of α-latrotoxin binding activity and high and low molecular weight components of the α-latrotoxin receptor on sucrose density gradients. Sucrose density gradients were loaded with α-latrotoxin receptor proteins eluted from the α-latrotoxin affinity column and fractionated into 24 fractions with fraction 1 representing the top of the gradient and fraction 24 the bottom. A, immunoassays of the sucrose density gradient fractions with antibodies to the M, 160,000–220,000 α-latrotoxin receptor proteins (top panel) or for the M, 29,000 protein (bottom panel). Numbers on top of the panels denote the fraction numbers. Numbers on the right indicate position of molecular weight markers. B, 125I-α-latrotoxin binding to proteins contained in the sucrose gradient fractions. Binding assays were performed on nitrocellulose filters with 0.5 nM 125I-α-latrotoxin in the absence (squares) or presence (circles) of 20 nM unlabeled α-latrotoxin as a competitor to measure specific binding. Note the slight shift in the relative distributions of the M, 160,000–220,000 proteins and the M, 29,000 protein with respect to each other and the co-migration of α-latrotoxin binding with the presence of both receptor components.

components (Fig. 8, compare fractions 6 and 10). These results suggest that high molecular weight proteins which may be complexed to the low molecular weight protein are responsible for specific α-latrotoxin binding.

DISCUSSION

α-Latrotoxin binds to the presynaptic nerve terminals of vertebrates and causes massive neurotransmitter release. It probably acts by first binding to specific, high affinity receptors and then activating neurotransmitter release in an unknown manner (7). The present studies aim at a biochemical characterization of α-latrotoxin-binding proteins purified from brain with the long term goal of defining the α-latrotoxin receptor and identifying its mechanism of action.

Using immobilized α-latrotoxin, two sets of proteins were purified from bovine and rat brain membranes: three proteins of M, 79,000, 65,000, and 43,000 that elute with increasing KCl concentrations in the presence of Ca2+, and a family of related proteins of M, 160,000–220,000 that elute together with a M, 29,000 protein when the Ca2+ on the column is exchanged for EDTA. We have now tried to define which of the proteins purified on immobilized α-latrotoxin are part of the α-latrotoxin receptor and how they interact.

Surprisingly, of the three major proteins eluted by KCl in presence of Ca2+, the M, 79,000 and 43,000 polypeptides were found to interact with immobilized α-latrotoxin directly although they do not bind 125I-α-latrotoxin in vitro. Immuno- blotting demonstrated that the M, 79,000 polypeptide is widely distributed in tissues which have no measurable α-latrotoxin binding activity and on which α-latrotoxin has no pathophysiological effect (24), and both the M, 79,000 and 43,000 proteins could be purified from liver. These results together suggest that the purification of these proteins on immobilized α-latrotoxin results from a low affinity interaction and is not relevant for the mechanism of action of α-latrotoxin. This is in contrast to the M, 65,000 protein, which we previously identified as the synaptic vesicle protein synaptotagmin (15) and which does not interact with α-latrotoxin directly but only via the receptor.

The polypeptides eluted from immobilized α-latrotoxin after removal of Ca2+ bind α-latrotoxin and are comprised of a set of M, 160,000–220,000 proteins and an M, 29,000 protein. Silver staining revealed no other proteins in comparable quantities in the α-latrotoxin-binding fraction (Fig. 5). The M, 29,000 protein is not a breakdown product of the high molecular weight components, because an antipeptide antibody raised against it does not recognize the high molecular weight proteins (Fig. 1). The M, 160,000–220,000 proteins contain two major components of M, 160,000 and 200,000 and at least three minor components of M, 170,000, 175,000, and 220,000 that are only visualized in overloaded gels. All of the high molecular weight components seem to be related to each other since they all react with two different antipeptide antibodies. Peptide sequences were obtained from all proteins that were purified on the immobilized α-latrotoxin. The sequences demonstrate that all of these proteins except for synaptotagmin (the M, 65,000 protein) represent new proteins not in the current data banks. Using these peptide sequences, we have recently been able to isolate cDNA's encoding proteins that are related to the high and low molecular weight subunit of the α-latrotoxin receptor (21). These cDNA sequences confirm the hypothesis that the α-latrotoxin receptor is not identical with a previously characterized protein but represents the prototype of a novel class of neuronal cell surface proteins, which were named neurexins.

The evidence that the M, 160,000–220,000 proteins are part of the α-latrotoxin receptor can be summarized as follows. 1) These proteins together with the M, 29,000 protein constitute the major components of those fractions eluted from the immobilized α-latrotoxin affinity column that bind α-latrotoxin in vitro (Figs. 1 and 5). 2) All of the high molecular

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weight proteins form a stable complex with \( \alpha \)-latrotoxin in the presence of an approximately 2-fold excess of \( \alpha \)-latrotoxin as shown on sucrose gradients (Fig. 6). The two major components of the related high molecular weight proteins can be separated from each other by anion exchange chromatography but each separate component still retains \( \alpha \)-latrotoxin binding (Fig. 7 and Ref. 14). 4) Sucrose-gradient separation of the proteins contained in the \( \alpha \)-latrotoxin preparation demonstrates that \( \alpha \)-latrotoxin binding and the presence of the high molecular weight components co-localize (Fig. 8).

What is the relationship between the \( M_0 \), 160,000-220,000 proteins and the \( M_0 \), 29,000 protein? Since they co-purify with each other on all separation procedures applied in this study, it seems likely that they form a complex with each other. The anion exchange chromatography provides the strongest evidence for this conclusion (Fig. 7). This experiment allowed separation of the major high molecular weight components of the receptor from each other, but each separate component was still associated with the \( M_0 \), 29,000 protein. The sucrose gradient centrifugations on the other hand demonstrated that a small percentage of the high molecular weight components may not be associated with an \( M_0 \), 29,000 protein. \( \alpha \)-Latrotoxin binding correlated best with the presence of high molecular weight components, but the possibility that both high and low molecular weight proteins are required cannot be excluded.

What is the mechanism of action of \( \alpha \)-latrotoxin? Although this question cannot be answered definitely at this point, the large size of the complexes formed by the binding of the receptor to \( \alpha \)-latrotoxin (Fig. 8) is intriguing. It has been shown previously (16) that \( \alpha \)-latrotoxin may form dimers, and our sucrose gradient results (Fig. 6) are consistent with this idea. Interaction of \( \alpha \)-latrotoxin with the receptor may cause clustering of the receptor similar to the aggregation of the receptor by the toxin observed on sucrose gradients. It is possible that such clustering, similar for example to the dimerization of epidermal growth factor receptors by epidermal growth factor (25), may cause signal transduction in the nerve terminal.

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