High Affinity Binding of α-Latrotoxin to Recombinant Neurexin Iα*

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α-Latrotoxin is a potent neurotoxin from black widow spider venom that stimulates neurotransmitter release. α-Latrotoxin is thought to act by binding to a high affinity receptor on presynaptic nerve terminals. In previous studies, high affinity α-latrotoxin binding proteins were isolated and demonstrated to contain neurexin Iα as a major component. Neurexin Iα is a cell surface protein that exists in multiple differentially spliced isoforms and belongs to a large family of neuron-specific proteins. Using a series of neurexin I-αG fusion proteins, we now show that recombinant neurexin Iα binds α-latrotoxin directly with high affinity (Kd ~ 4 nm). Binding of α-latrotoxin to recombinant neurexin Iα is dependent on Ca2+ (EC50 ~ 30 μM). Our data suggest that neurexin Iα is a Ca2+-dependent high affinity receptor for α-latrotoxin.

α-Latrotoxin, a component of black widow spider venom, is one of the most potent excitatory neurotoxins known. α-Latrotoxin stimulates neurotransmitter release from vertebrate nerve terminals by triggering massive exocytosis of small synaptic vesicles (1, 2). α-Latrotoxin-stimulated neurotransmitter release is accompanied by presynaptic membrane depolarization and the influx of Ca2+ through ion channels induced by the toxin (3, 4). Purified α-latrotoxin forms cation channels in black lipid membranes, leading to the hypothesis that the toxin may act as an ionophore although the channel characteristics differ from those observed in intoxicated PC12 cells (5–7). α-Latrotoxin binds to specific membrane receptors that are found only in the nervous system (8, 9). Immunofluorescence localization of bound α-latrotoxin at the neuromuscular junction suggested that the binding sites are localized to the presynaptic plasma membrane (10). Together, these studies suggest that α-latrotoxin acts by binding to presynaptic receptors, which it either activates directly or which serves to target its insertion into the presynaptic plasma membrane.

The binding sites for α-latrotoxin in brain membranes are of low abundance (~0.3 pmol/g of protein) and of high affinity (~10⁻⁹ M). Affinity purification of α-latrotoxin-binding proteins from brain resulted in the isolation of a protein fraction that bound α-latrotoxin with high affinity (11) and contained two classes of proteins (12–14): a family of high molecular mass proteins (180–220 kDa) that were shown by molecular cloning to be composed of variants of neurexin Iα, and a distinct low molecular mass protein (29 kDa) named neurexophilin. The purification of these proteins suggested that they represent components of the α-latrotoxin receptor. However, it was impossible to define the exact binding partner because direct binding of recombinant proteins to α-latrotoxin was not achieved.

Neurexin Iα and its isoforms, Iα and IIIα, structurally resemble cell surface proteins (13, 15). The neurexins are highly polymorphic due to extensive alternative splicing (16). Each neurexin gene is polymorphic due to extensive alternative splicing (16). Each neurexin gene not only generates α-neurexins but also β-neurexins that have a distinct N terminus but share the C-terminal sequences with α-neurexin (13, 17). The discovery of neurexin Iα as a component of the protein complex that binds α-latrotoxin with high affinity raised the question of whether neurexin Iα represents an α-latrotoxin receptor or is only purified indirectly. We have now studied the interaction of α-latrotoxin with recombinant neurexins and determined the requirements for high affinity binding. Our data demonstrate that neurexin Iα represents a high affinity, Ca2+-dependent cell surface binding molecule for α-latrotoxin.

EXPERIMENTAL PROCEDURES

Construction and Transfection of Expression Vectors—Vectors directing expression of extracellular domains of neurexins fused to the Fc domain of human IgG were obtained by an adaptation of the method of Aruffo et al. (18) utilizing pCD5-IgG as the starting vector as described (17, 19) and the rat and bovine neurexin CDAs (13, 15, 16). The vectors used in the current study encode the following residues and splice variants of neurexins (all numbers correspond to the numbering of the rat proteins in Refs. 13 and 15; splice variants are given in parentheses for splice sites 1 to 4 in the terminology of Ref. 16): pCMVIGNIα-1 (B/C/A/B), -2 (B/A/A/B), -3 (B/A/B/A), -4 (G/A/C/A), and -7 (G/A/A/B) encode residues 1 to 1339 or bovine neurexin Iα; pCMVIGNIβ-1 (G/C/A/A) of rat neurexin Iα; pCMVIGNIβ-15 and -17 encode residues 1–588 and 1–655 (splice variants for splice sites 1 and 2 for both: B/B); pCMVIGNIβ-1 and -3 encode residues 1 to 300 from rat neurexin Iβ without or with an insert in splice site 4, respectively; and pCMVIGNIIIα-2 encodes residues 1–1499 of rat neurexin IIIα with no insert in splice sites 1 and 5 and full inserts in 3 and 4. The proteins encoded by the vectors are depicted schematically in Fig. 1. Plasmid DNA was transfected into COS cells using DEAE-dextran (20), and expressed proteins were purified from the medium as described (17, 19). As controls, media from COS cells transfected with salmon sperm DNA or with control IgG vector were used.

α-Latrotoxin Binding to Recombinant Neurexins—α-Latrotoxin was purified from the glands of Latrodectus mactans as described (12). Activity was assayed using release of labeled noradrenaline from synaptic vesicles. For some experiments, α-latrotoxin was iodinated using chloramine T (12). α-Latrotoxin was incubated in buffer A (50 mM Tris-HCl, pH 7.7, 2 g/liter bovine serum albumin, and 150 mM NaCl) containing the indicated concentrations of EGTa, Ca2⁺, and Mg2⁺ with protein A-Sepharose beads to which the recombinant proteins had been attached. Incubations were for 15 min under vigorous shaking at room temperature. Beads were then washed with buffer A containing the

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1 A. G. Petrenko and T. C. Südhof, unpublished observation.
additions described in the figure legends, and bound proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining or immunoblotting and determination of radioactivity. For determinations of binding affinities, aliquots of the COS cell medium were spotted onto nitrocellulose, and binding of 125I-labeled α-latrotoxin to the immobilized neurexins was analyzed as described (12).

Materials and Procedures—SDS-PAGE, Coomassie staining, and immunoblotting were performed as described (14). Immunoreactive bands were detected by enhanced chemiluminescence (Amersham). The antibody against α-latrotoxin (X751) was raised against purified protein in rabbits.

RESULTS AND DISCUSSION

We constructed a series of fusion proteins of bovine neurexin Iα with IgG in order to take advantage of the recent cloning of a large number of independent neurexin Iα cDNAs (16). The neurexin Iα IgG fusion proteins are depicted schematically in Fig. 1 together with the other IgG fusion proteins used for the current study. Incubation of neurexin Iα-IgG fusion protein immobilized on protein A-Sepharose with purified α-latrotoxin demonstrated stoichiometric and specific binding of α-latrotoxin only in the presence of Ca2+ (Fig. 2, lanes 1–4). Binding was reversible since α-latrotoxin that was bound to neurexin Iα-IgG in the presence of Ca2+ could be readily dissociated by EGTA (Fig. 2, lane 6). Thus, α-latrotoxin binds to the extracellular domains of recombinant neurexin Iα in a Ca2+-dependent manner.

Previous studies using recombinant rat neurexin Iα were unsuccessful in detecting binding. Therefore, we studied the potential dependence of binding on splice variants by analyzing a series of independent cDNAs. Four different neurexin Iα-IgG fusion proteins containing a variety of inserts in the first three splice sites of α-neurexins bound α-latrotoxin, whereas the recombinant proteins corresponding to neurexin IIIα and the previously studied rat neurexin Iα did not (lanes 1–8 versus 13–16, Fig. 3). Furthermore, C-terminal truncations of cDNAs that bound α-latrotoxin as full-length protein abolished binding (lanes 9–12), and the two splice variants of neurexin Iβ were also unable to bind (lanes 17–20, Fig. 3; see Fig. 1 for an overview of the structures of the neurexin-IgG fusion proteins). Thus, several recombinant neurexin Iα proteins with different splice site variants bind α-latrotoxin. Both N-terminal α-specific sequences of neurexin Iα and its C-terminal half are required for binding.

The nearly stoichiometric binding of α-latrotoxin to neurexin Iα suggests a stable interaction of high affinity. To test this, the binding of radiolabeled α-latrotoxin to recombinant neurexin Iα was measured (Fig. 4). A binding affinity of approximately 4 nM was determined, suggesting that neurexin Iα is indeed a high affinity α-latrotoxin-binding protein. The affinity of recombinant neurexin Iα was compared with that of the high affinity binding proteins that were purified by affinity chromatography on immobilized α-latrotoxin (11, 12, 14). Recombinant neurexin Iα had an almost identical affinity as the purified protein, confirming that the α-latrotoxin binding observed in the purified receptor corresponds to neurexin Iα (Fig. 4).

The experiment in Fig. 2 suggested that α-latrotoxin binding to neurexin Iα may be Ca2+-dependent. To investigate this further, we studied the effect of different Ca2+ concentrations on binding in the presence of a saturating concentration of Mg2+ (Fig. 5). Mg2+ alone was unable to trigger binding. Ca2+ acted in a concentration-dependent manner with an EC50 of ~35 μM and with a single apparent binding site. This result suggests that α-latrotoxin and/or neurexins contain a structural Ca2+ binding site which has to be occupied in order for the two proteins to interact. Since previous studies demonstrated
extracellular domains of neurexins contain structural Ca\(^{2+}\) conformation.

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massie staining (shown in the

is interesting because binding to it may mediate the ability of

quantified by determining the radioactivity of the bound

rotoxin in the presence of 2 mM Mg\(^{2+}\)

Ca\(^{2+}\) dependence of

- latrotoxin was analyzed by SDS-PAGE and Coo-

FIG. 4. **Affinity of α-latrotoxin for neurexin Iα-IgG.** Immobilized

neurexin Iα-IgG (squares, IG-Nx) or α-latrotoxin binding proteins pu-

rified by affinity chromatography on α-latrotoxin (diamonds, LTR) (11, 12, 14) were incubated with radiolabeled α-latrotoxin at the indicated

concentrations. The amount of bound and free α-latrotoxin was deter-

mined and analyzed in a Scatchard plot as shown. Note that the affinity of

recombinant neurexin is virtually identical with that of the purified

protein complex.

FIG. 5. **Ca\(^{2+}\) dependence of α-latrotoxin binding to neurexin Iα.** Protein A-beads coated with neurexin Iα-IgG (closed circles) or with

neurexin Iβ-IgG (open circles) were incubated with radiolabeled α-lat-

rotoxin in the presence of 2 mM Mg\(^{2+}\) and the indicated Ca\(^{2+}\) con-

centrations. Binding of α-latrotoxin was analyzed by SDS-PAGE and Coo-

massie staining (shown in the top panel for neurexin Iα-IgG) and quantified by determining the radioactivity of the bound α-latrotoxin (bottom panel). The curve drawn in the bottom panel was generated with the GraphPad program and corresponds to a single binding site with an EC\(_{50}\) of 35 \(\mu M\) and a Hill coefficient of 0.9.

that neurexin 1, the ligand for β-neurexins, also requires Ca\(^{2+}\) for binding (19), it is tempting to speculate that the extracellular domains of neurexins contain structural Ca\(^{2+}\) binding sites that are required to keep the molecule in an active conformation.

The goal of the current study was to investigate the candi-

dacy of neurexin Iα as the α-latrotoxin receptor. This receptor is interesting because binding to it may mediate the ability of α-latrotoxin to trigger massive neurotransmitter release. Syn-

aptotagmin, a nerve terminal Ca\(^{2+}\) sensor (21), co-purifies with

this receptor on an α-latrotoxin column, suggesting a possible role of the α-latrotoxin receptor in regulating synaptic vesicle fusion with the plasma membrane (22). The current study demonstrates that the extracellular domains of neurexin Iα bind α-latrotoxin with high affinity in a Ca\(^{2+}\)-dependent manner. This binding is specific since it was observed with only a subset of neurexin Iα-IgG fusion proteins and not with control proteins or other IgG fusion proteins.

The affinity of the interaction between neurexin Iα and α-lat-

rotoxin agrees well with the α-latrotoxin concentrations re-

quired for toxic actions (1–4). However, the Ca\(^{2+}\) dependence of

the interaction is puzzling, even though the Ca\(^{2+}\) concentration required for binding is low. Although the α-latrotoxin receptor purified by affinity chromatography also requires Ca\(^{2+}\) for binding, α-latrotoxin binding to brain membranes is decreased but not abolished in the absence of Ca\(^{2+}\) (23). Furthermore, α-latrotoxin is capable of triggering neurotransmitter release in the absence of extracellular Ca\(^{2+}\) if Mg\(^{2+}\) is present. Thus, it is possible that a second high affinity binding protein for α-latrotoxin exists that is distinct from neurexin Iα and binds the toxin in the absence of Ca\(^{2+}\). Since Scatchard plots of α-latrotoxin binding demonstrated only a single class of binding sites, any putative receptor would have to bind α-latrotoxin with the same affinity as neurexin Iα. Alternatively, a neurexin isoform may exist that does not require Ca\(^{2+}\) for α-latrotoxin binding. Future experiments will have to address these possibilities.

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