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Isolation and properties of the α-latrotoxin receptor

A.G.Petrenko, V.A.Kovalenko, O.G.Shamotienko, I.N.Surkova, T.A.Tarasyuk, Yu.A.Ushkaryov and E.V.Grishin

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 GSP, Moscow, USSR

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The receptor protein of α-latrotoxin (αLTx, a neurotoxin with 'pure' presynaptic action isolated from black widow spider venom), was solubilized by Triton X-100 from bovine brain membranes and purified by affinity chromatography on αLTx–Sepharose. The purified receptor preparation contained four major polypeptides of molecular masses 200 (α), 160 (α'), 79 (β) and 43 (γ) kd according to SDS electrophoresis with molecular ratio $\alpha_1\alpha'_1\beta_2\gamma_2$. The α- and α'-subunits are glycoproteins binding to wheat germ lectin and can be separated under non-denaturing conditions by anion exchange chromatography. Purified to homogeneity, both of them, though differing in the carbohydrate composition, retain the αLTx-binding activity and give closely related peptide maps. Anti-α antibodies recognize the α'-subunit as well. These results suggest that αLTx receptor is present in purified preparations in two very close forms containing the α- or α'-subunit. β and γ proteins do not specifically bind αLTx and their physiological role is unclear. They form a complex with solubilized α- and α'-subunits independently of αLTx presence. The receptor proteins were purified to homogeneity by high performance gel filtration in the presence of SDS, their amino acid composition was determined.

Key words: affinity purification/α-latrotoxin/receptor/neurosecretion/presynaptic membrane

Introduction

Secretion of neurotransmitters is one of the least studied stages of nerve signal transduction. Although several protein components of synaptic vesicles have been identified and isolated during the last decade, the general picture of the molecular mechanism of neurosecretion still remains incomplete (for a recent review, see Kelly, 1988). Specific neurotoxins are a tool that can be used to identify presynaptic membrane proteins. α-Latrotoxin (αLTx), the principal toxic constituent of black widow spider venom, induces massive exocytotic release from many (possibly all) types of synaptic junctions of vertebrates (Frontali *et al.*, 1976; Tzeng *et al.*, 1978; Hurlbut and Ceccarelli, 1979; Meldolesi, 1982). Specific high-affinity ($K_D < 10^{-9}$ M) αLTx receptors were detected in different nerve membrane preparations (Tzeng and Siekevitz, 1979; Meldolesi *et al.*, 1983; Ushkaryov and Grishin, 1986). Immunocytochemical studies demonstrated that at the neuromuscular junction αLTx binds only to the exoplasmic side of the nerve ending (Valtorta *et al.*, 1984). The αLTx receptor is therefore a protein marker of the presynaptic membrane, where it appears to act by controlling neurotransmitter release.

Previous affinity chromatography studies carried out using αLTx–Sepharose columns have demonstrated that the αLTx receptor can be isolated in an active binding form and reconstituted into liposomes and planar lipid membranes (Scheer and Meldolesi, 1985; Scheer *et al.*, 1986). Because of its low concentration within the membranes (<0.4 pmol/mg protein), the amounts of purified receptors were, however, very low. As a consequence the degree of purification could not be established precisely, the receptor could not be characterized biochemically and no anti-receptor antibodies could be generated. The work has therefore been pursued at a large scale.

Here we report the purification of the αLTx receptor in milligram quantities, its toxin-binding properties and the characterization of its protein components. Four individual polypeptides were isolated. Among these, two closely related proteins with apparent molecular masses of 200 and 160 kd were identified as the αLTx-binding subunits of the receptor.

Results

Crude membranes from bovine brain cortices were used as a source of the αLTx receptor. The density of binding sites varied in the 0.3–0.5 pmol/mg protein. The membrane receptor was solubilized with non-ionic detergents Triton X-100 and Lubrol PX, as described (Scheer and Meldolesi, 1985; Ushkaryov and Grishin, 1986). To perform the solubilized receptor assay, a method was developed based on adsorption of solubilized membrane protein onto nitrocellulose followed by bovine serum albumin (BSA) saturation and incubation with [^{125}I]αLTx. The αLTx binding activity of the solubilized receptor ($K_D = 8 \times 10^{-10}$ M; 0.3 pmol binding sites/mg protein) did not differ greatly from that of intact membranes ($K_D = 1.6 \times 10^{-10}$ M, 0.3 pmol/mg).

Receptor proteins were purified by affinity chromatography using a matrix prepared by coupling αLTx to BrCN-activated Sepharose 4B and characterized by a lower level of non-specific binding compared to the matrix used by Scheer and Meldolesi (1985). Extensive washing of the loaded column with 0.2 M KCl buffer was a critical step as the necessary purification factor was of the order of 10 000.

The absence of specific αLTx receptor antagonists hinders αLTx receptor elution. We found that in the absence of Ca^{2+} the stability of the solubilized toxin–receptor complex depends on the salt concentration in the buffer (Figure 1). It is noteworthy that the complex stability differs greatly for the membrane-bound and solubilized receptor, the membrane complex being stable even in 1–3 M buffers and the soluble receptor starting to dissociate in >0.2 M salt (but only in the absence of calcium). The buffer

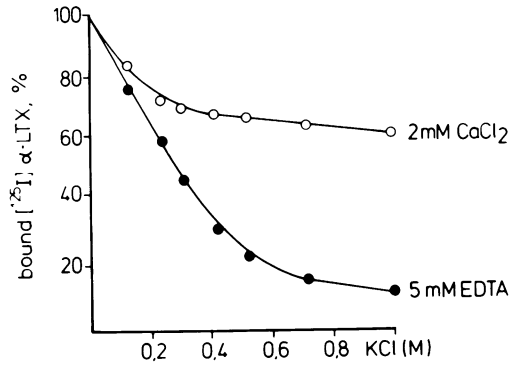

Fig. 1. Salt concentration dependence of the [^{125}I]αLTx–receptor complex dissociation in the presence or absence of Ca^{2+}.

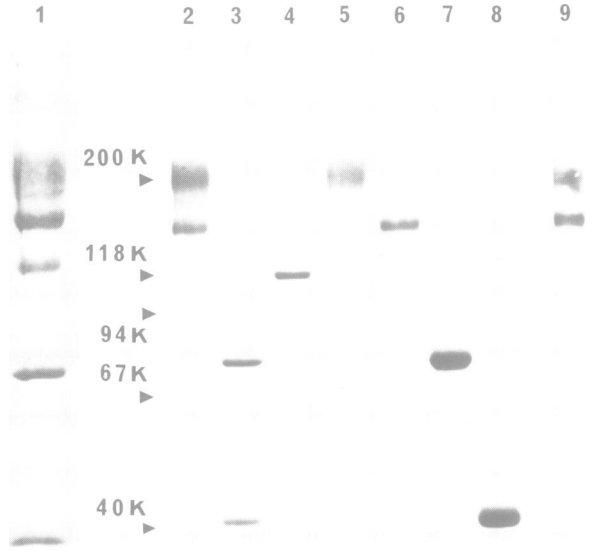

Fig. 2. SDS–PAGE of purified receptor and individual subunits (Coomassie stained). **Lane 1**, one-step eluate of the affinity column; **lanes 2** and **3**, EDTA eluate and Ca^{2+} eluate, respectively (two-step elution); **lane 4**, αLTx; **lanes 5, 6, 7** and **8**, individual α-, α′-, β and γ-subunits purified as described in Materials and methods; **lane 9**, immunostaining of the blotted receptor complex (as in lane 1) with anti-α-antibodies.

containing 1 M KCl and 10 mM EDTA elutes the bound receptor from affinity columns almost completely after 15 min. Further elution with SDS containing Laemmli sample buffer gives only insignificant traces of the protein. Receptor elution can thus be achieved under milder conditions than those described earlier (6 M urea).

Dozens of independently purified receptor preparations were analysed by SDS–PAGE. Under both reducing (Figure 2, lane 1) and non-reducing conditions their major protein components, named α, α′, β and γ had apparent molecular masses of 200 (diffuse band), 160, 79 and 43 kd, respectively. None of these polypeptides could be detected in control experiments, when calcium salts were omitted from the protein mixture used for purification, or if solubilized membranes were applied to BrCN–Sepharose, prepared without addition of αLTx, or when αLTx–Sepharose was inactivated with an acidic buffer.

Table I. Summary of the αLTx receptor purification[a]

Preparation	Protein[b] (mg)	Total activity[c] (pmol)	Specific activity (pmol/mg)	Purification (fold)	Yield (%)
Membranes	6500	2100	0.3	1	100
Triton extract	2800	840	0.3	1	40
Ca^{2+} eluate from αLTx–Sepharose	0.15	0	–	–	–
EDTA eluate from αLTx–Sepharose	0.25	400	1600	5300	19

[a]αLTx receptor was purified by the αLTx–Sepharose affinity chromatography as described in text. Data presented are the averages of three different experiments.
[b]Protein was estimated by a modification of Lowry assay (Kovalenko *et al.*, 1981) with BSA as a standard.
[c]Receptor activity is presented in pmol of [^{125}I]αLTx maximum specific binding assayed as in Materials and methods, and calculated from Scatchard plots.

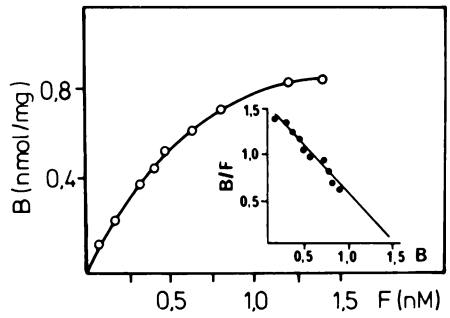

Fig. 3. Saturation analysis of α- and α′-receptor subunit preparations. Inset: Scatchard plot of the [^{125}I]αLTx binding to α- and α′-subunits.

The addition of an intermediate elution step permitted separation of the two larger from the two smaller proteins (Figure 2, lanes 2 and 3). The β- and γ-subunits were eluted with 0.6 M KCl buffer containing 2 mM $CaCl_2$. Only traces of larger subunits could be detected in this preparation. In several cases a minor component of molecular mass 38 kd was also clearly detected. We cannot exclude, however, that this protein is not the result of proteolytic degradation of another receptor component. Finally, the second step of elution with high salt buffer and EDTA gave an essentially pure mixture of α- and α′-subunits. Blot staining with biotinylated wheat germ lectin revealed that the α- and α′-subunits were glycoproteins.

αLTx-binding activity of the purified receptor

The αLTx-binding activity of the purified receptor (in solution or after spotting to nitrocellulose membrane) was restored by Ca^{2+} re-introduction into the buffer, but only in the fraction containing the α- and α′-subunits (Table I). In this preparation maximum specific binding was 1.6 nmol/mg protein with $K_D = 9.5 \times 10^{-10}$ M. The Scatchard plot was linear, suggesting a binding site of one type (Figure 3). No significant αLTx-binding activity was found in the β- and γ-subunit fractions.

The binding activity of the purified receptor was very stable (no changes for at least 2 weeks at 4°C or for 10 h at room temperature). Three freeze–thaw cycles did not

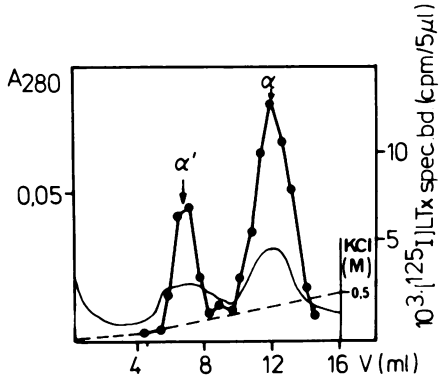

Fig. 4. Separation of α- and α′-subunits by anion exchange chromatography: (——) eluate absorbance at 280 nm; (- - - -) KCl concentration. (—○—) bound [^{125}I]αLTx. Arrows indicate the maximum of α- and α′-subunit concentration determined by SDS–PAGE.

Table II. Amino acid and carbohydrate composition of the α, α′, β and γ αLTx receptor proteins[a]

Amino acid carbohydrate[b]	α	α′	β	γ
Asx	12.54	12.55	9.02	9.83
Thr	7.38	7.54	5.59	5.95
Ser	9.87	10.25	7.11	7.50
Glx	8.98	8.26	12.26	9.69
Pro	4.31	3.84	6.48	6.12
Gly	9.88	10.56	9.30	9.59
Ala	5.68	5.99	7.83	9.15
Val	4.35	4.44	6.87	5.40
Met	1.58	1.58	1.83	2.50
Ile	4.17	4.03	4.32	3.62
Leu	7.82	8.00	8.98	9.85
Tyr	3.04	2.69	2.14	1.74
Phe	4.69	4.15	3.88	3.73
His	1.98	2.52	1.73	2.30
Lys	7.29	6.89	5.91	6.61
Arg	4.86	4.73	4.62	4.58
Trp	1.57	2.00	2.14	1.83
Fuc	13.65	9.84		
Man	26.05	29.10		
Gal	26.05	46.72		
GlcNAc	10.47	5.74		
GalNAc	23.77	8.61		
Protein[c]	78.97	71.22		
Carbohydrate[c]	21.03	28.78		

[a]Amino acid composition was determined in the Durum D-500 analyser after hydrolysis of electrophoretically pure proteins in 5.7 N HCl at 105°C for 24 h. Trp was established spectrophotometrically, Cys was not determined. Carbohydrate composition of α and α′ (excluding sialic acids) was investigated by the fluorescent detection of carbohydrate coumarinyl derivatives (Khorlin *et al.*, 1986).
[b]Presented in molar percent.
[c]Protein and sugar moiety of glycoproteins is estimated in weight percent.

affect the receptor activity. However, elevated temperatures (>60°C) caused irreversible inhibition of the receptor. The same occurred with denaturing agents, i.e. 1% SDS or 4 M guanidinium chloride. Wheat germ lectin inhibited αLTx binding of the purified receptor with $K_i = 3 \times 10^{-6}$ M, as with the membrane bound receptor.

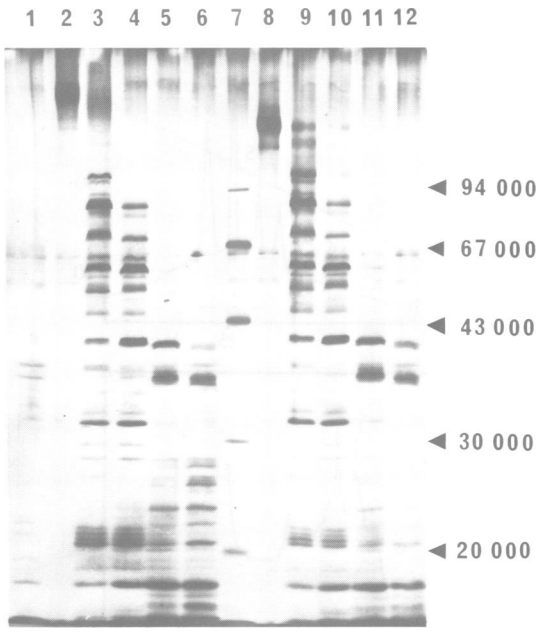

Fig. 5. Peptide maps of α- and α′-subunits, digested with *Staphylococcus aureus* V8 protease. **Lane 1**, peptides derived from enzyme autoproteolysis; α-subunit without (**lane 2**) and with the enzyme (0.1, 0.3, 1 and 3 μg; **lanes 3–6**); **lane 7**, molecular weight standards; α′-subunit without (**lane 8**) and with the enzyme (**lanes 9–12**, concentrations as in lanes 3–6). Aliquots of 40 μl of the samples (containing 3 μg protein) were digested at 37°C for 30 min by addition of 5 μl solution with the given amount of the protease.

The purified receptor binding activity was also sensitive to proteases: thermolysin, staphylococcal protease (40% inhibition after 1 h incubation at 37°C, enzyme:receptor weight ratio 1:20) and, especially, to trypsin (>80% inhibition). The treatment of the purified receptor with neuraminidase did not influence its activity but resulted in a loss of the receptor sensitivity to the inhibiting action of wheat germ lectin.

Isolation of the individual receptor proteins and identification of αLTx binding subunits

The fraction containing the α- and α′-subunits was separated by anion exchange chromatography on DEAE–Sepharose in the presence of 0.1% Lubrol PX (Figure 4). The profile of specific αLTx-binding activity correlated with the presence of the α- and α′-bands in the electrophoresis pattern of the fractions. The binding activity of receptor proteins decreased rapidly after separation, even at 4°C. The inactivation is possibly induced by delipidation during chromatography.

According to SDS electrophoresis the purity of the toxin binding subunit preparations is ~95%. The preparations were further purified to homogeneity (Figure 2, lanes 5–8) by high performance gel filtration, then used for antibody preparation and structural analysis.

Both α- and α′-subunits contain the αLTx-binding center. Some of their biochemical characteristics were compared. Monospecific anti-α antibody cross-reacted with the α′-subunit (Figure 2, lane 9), but did not stain the β- and γ-subunits. Peptide mapping (Cleveland, 1983) indicated a high degree of homology between the α- and α′-subunits (Figure 5). Both subunits appear to be moderately hydrophilic proteins according to the amino acid composition

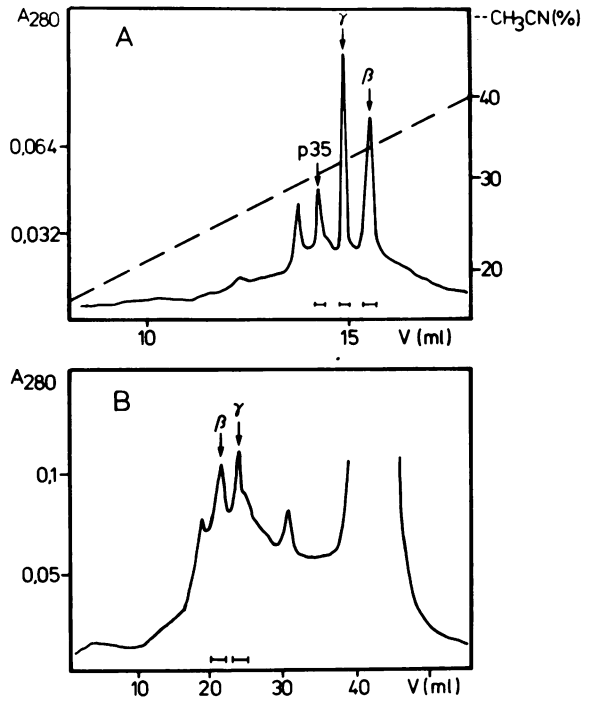

Fig. 6. Purification of β- and γ-subunits by reversed-phase HPLC on Ultrapore RPSC (**A**) or by gel filtration in the presence of 0.1% SDS on TSK 4000 SW and TSK 3000 SW columns (**B**).

(Table II). Data on carbohydrate analysis (Table II), as well as the lectin binding property mentioned above, support the glycoprotein nature of the receptor subunits.

As far as the β- and γ-subunits are concerned, they were found to be adsorbed neither on αLTx−Sepharose nor on wheat germ lectin−Sepharose. However, β- and γ-subunits are adsorbed and can be further eluted from these sorbents bearing previously immobilized α- and α′-toxin-binding subunits.

The β- and γ-subunits could not be separated from each other under non-denaturing conditions by gel filtration or ion exchange chromatography. This may be caused by their tendency to aggregate. Individual β- and γ-subunits were obtained by high performance gel filtration in SDS or by reversed-phase HPLC (Figure 6). Their amino acid composition is given in Table II.

The stoichiometry of the affinity-purified receptor complex was evaluated when scanning the gel of the electrophoresed preparation, obtained by one-step elution of the affinity matrix with high-salt buffer containing EDTA. The molar ratio of α, α′, β and γ proteins was approximately estimated as 1:1:2:2.

Discussion

The αLTx receptor is a membrane protein of considerable interest in neurobiology. In fact this receptor is the only presynaptic marker for which a function has been identified and it is the mediator of the effects of αLTx, the major component of black widow spider venom. The neurotransmitter release stimulatory effect of the toxin is known to be supported by two parallel mechanisms: a non-inactivating cation channel (Wanke *et al.*, 1986), whose activation induces an increase of the cytosolic Ca^{2+} concentration (Meldolesi *et al.*, 1984) and the stimulation of Ca^{2+}-dependent exocytosis of synaptic vesicles; and a Ca^{2+}-independent mechanism of vesicle release entirely responsible for neurotransmitter secretion observed in the absence of extracellular Ca^{2+} (Hurlbut and Ceccarelli, 1979; Meldolesi *et al.*, 1988). At present neither of these mechanisms is adequately understood. It is not clear yet whether a cationic channel is established by αLTx molecules redistributed across the membrane after binding to the receptor or by the receptor itself activated by toxin binding. Moreover, nothing is known about the Ca^{2+}-independent mechanism. αLTx and its receptor appear therefore to be potentially important tools for developing presynaptic physiology. Through their use the investigation of both a purely presynaptic cationic transmembrane transport system and a new mechanism of neurotransmitter release control seems possible. The results reported here represent the initial step in a series of studies aimed at establishing the structure and the function of the αLTx receptor.

Affinity chromatography made possible purification of the αLTx receptor with high specific binding activity. The features of the purified preparations (αLTx binding affinity, calcium dependence, thermolability, sensitivity to proteases and denaturing agents, and inhibition by lectins) correlate with the properties of the receptor while membrane bound (Meldolesi, 1982; Meldolesi *et al.*, 1983; Ushkaryov and Grishin, 1986) or solubilized from crude membranes. The presence of two polypeptides, 200 and 160 kd, correlated with the binding activity in the fractions of the receptor proteins separated by anion exchange chromatography. Specific binding could account for >30% of the theoretically maximal activity of proteins of such molecular mass. The apparently incomplete binding could be due to partial receptor inactivation in the course of purification. Alternatively, and more likely, the detection of binding at all receptor molecules could be impossible because of steric hindrance in the solid phase assay.

According to the evidence discussed in the Results, the αLTx receptor preparations are non-homogeneous. There were identified at least two receptor forms, differing in the size and charge of the α- or α′-subunit molecules, but apparently rather homologous in their primary structure. Since these subunits primarily differ in their sugar components, they are unlikely to be produced by artifactual hydrolysis.

In addition, to the α- and α′-subunits, the purified receptor preparations were found to contain two additional non-toxin-binding proteins complexed to solubilized larger subunits presumably by ionic bonds. Their physiological significance for membrane αLTx receptor functioning is still unclear. Because of the relative content of the various proteins in purified preparations the simplest stoichiometry of affinity purified αLTx receptor complexes might be $\alpha_1\beta_1\gamma_1$ or $\alpha'_1\beta_1\gamma_1$. The molecular mass could therefore vary between 280 and 320 kd.

The amino acid composition of receptor proteins indicates that they are moderately hydrophilic proteins. The presence of transmembrane segments in their structure, particularly in the case of larger toxin binding proteins, cannot be excluded. The α′- and, especially, the α-subunits are significantly glycosylated. This suggests that part of them is probably exposed to extracellular space, as expected for the receptor of a high molecular mass protein toxin.

Materials and methods

αLTx was purified from Central Asia black widow spider venom and iodinated with ^{125}I by the chloramine-T procedure as earlier described (Ushkaryov and Grishin, 1986). [^{125}I]αLTx (sp. act. 1500–2000 Ci/mmol) was used either as such or after 3- to 10-fold dilution with unlabelled toxin. αLTx–Sepharose was prepared by αLTx immobilization on BrCN–Sepharose 4B (0.5–0.8 mg protein/ml matrix).

Purification of the αLTx receptor

Purification procedure was carried out at 4°C and all solutions contained 0.3 mM phenylmethyl sulphonylfluoride, 5 mg/l pepstatin and 0.02% NaN_3. A 200 g sample of bovine brain cortices was homogenized in a Waring blender with 2800 ml of 20 mM Tris–HCl, 5 mM EDTA buffer (pH 7.7) and centrifuged at 10 000 *g* for 1 h. The pellet was resuspended in 1200 ml of the same buffer supplemented with 80 ml of 20% Triton X-100 (Koch–Light, deionized with Bio-Rad AG-501X8 resin). After a 15 min incubation, insoluble material was pelleted at 50 000 *g* for 2 h. A 1.3 l aliquot of supernatant was supplemented with KCl to a final concentration of 200 mM and $CaCl_2$ to 2.5 mM, and loaded onto an αLTx–Sepharose column (1.5 × 6 cm) at 1 ml/min. After washing with 120 ml of buffer A (0.1% Triton X-100, 20 mM Tris–HCl, pH 7.7), containing 2 mM $CaCl_2$ and 0.2 M KCl, the proteins were desorbed by either 1 M KCl, 10 mM EDTA in buffer A or a two-step elution with 100 ml of 0.6 M KCl and 2 mM $CaCl_2$ in buffer A, followed by 100 ml of 1 M KCl, 10 mM EDTA in buffer A. Finally, the αLTx–Sepharose column was re-equilibrated with buffer A, then used for 20–30 experiments without substantial loss of binding capacity.

αLTx binding assay

The preparation (10 μl) was spotted onto a square piece (1 × 1 cm) of BA-85 nitrocellulose (Schleicher & Schüll) placed in a tissue culture 24-well plate (Flow). The pieces were washed once with 100 mM KCl, 2 mM $CaCl_2$ and 20 mM Tris buffer (pH 7.7) and incubated with 1.5% BSA in the same buffer for 1 h. The solution of [^{125}I]αLTx (together with excess αLTx in control experiments) in BSA-containing buffer was applied to the nitrocellulose pieces, followed by incubation for 30 min. The pieces were subsequently washed with 3 × 1 ml of the incubation buffer for 20 min and counted for radioactivity.

Ion exchange chromatography

An 8 × 100 mm column of DEAE–ToyoPearl 650M or DEAE–Sepharose CL 6B was equilibrated with buffer A, containing 50 mM KCl and 2 mM $CaCl_2$. The column was loaded with a mixture of α- and α′-subunits eluted from the affinity matrix and dialysed against the same buffer. After washing, the column was gradient eluted with 16 ml of 0–450 mM KCl in the equilibration buffer at 10 ml/h. Fractions of 0.7 ml were collected and assayed for receptor activity immediately.

Isolation of individual receptor subunits

The fractions from ion exchange chromatography containing α- or α′-subunits (0.5–1.5 mg of protein) were precipitated with triple volume mixture of chloroform:methanol (2:1). The pellets from interphase were washed once with water, dissolved in 300–900 μl buffer B (2% SDS, 10 mM EDTA, 50 mM dithiothreitol, 0.2 M NaPi, pH 7.0) and boiled for 2 min. After 10 min centrifugation at 13 000 r.p.m. in the Eppendorf centrifuge, supernatant aliquots (300–450 μl) were loaded on two tandem TSK 4000SW columns (0.75 × 60 cm) equilibrated with buffer B, containing, however, only 0.1% SDS and 5 mM dithiothreitol. The columns were eluted with the same buffer at 30 μl/min. Fractions of 0.21 ml were collected over 9 h after sample injection. The β- and γ-subunits were isolated by two procedures: (i) gel filtration on TSK 4000SW and TSK 3000SW tandem columns as described above for the α- and α′-subunits and (ii) reversed-phase HPLC on Ultrapore RPSC in 0.1% trifluoroacetic acid, eluted with a linear acetonitril:1-propanol (50:50) gradient; the protein mixture was applied in 100 μl of 50% formic acid.

Preparation of the anti-α-subunit antibody

An antiserum directed against the α-subunit was prepared by injecting a rabbit three times at 2 weekly intervals with 125 μg of the individual protein emulsified in complete Freund's adjuvant. The rabbit was bled 10 days after the last injection. Anti-α-subunit immunoglobulins (Igs) were purified from immune sera by affinity chromatography on the matrix, prepared by coupling the α-subunit to BrCN-activated Sepharose (density 0.5 mg/ml). Aliquots of 10 ml of the antiserum were together with 1 ml of affinity matrix incubated for 1.5 h at 25°C with gentle agitation. After thorough washing with PBS, adsorbed Igs were eluted with 100 mM glycine–HCl buffer, pH 2.4. The eluate was neutralized immediately and dialysed against phosphate buffered saline.

Gel electrophoresis and nitrocellulose blotting

SDS gel electrophoresis was performed according to Laemmli (1970) in 9% or 9–18% gradient acrylamide slab gels calibrated with Pharmacia molecular standards. Gels were silver stained (Morrissey, 1981) or stained with 0.1% Coomassie R-250. Coomassie-stained gels were analysed with Ultroscan (LKB) in order to evaluate the stoichiometry of the receptor complex. For immunostaining the separated proteins were blotted onto nitrocellulose according to Towbin *et al.* (1979). After the incubation with monospecific anti-α antibodies (10 μg/ml) the blots were stained as described by Hawkes *et al.* (1982).

Acknowledgements

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References

Cleveland,D.W. (1983) *Methods Enzymol.*, **96**, 222–229.
Frontali,N., Ceccarelli,B., Gorio,A., Mauro,A., Siekevitz,P., Tzeng,M.-C. and Hurlbut,W.P. (1976) *J. Cell Biol.*, **68**, 462–479.
Hawkes,R., Niday,E. and Gordon,J. (1982) *Anal. Biochem.*, **119**, 142–147.
Hurlbut,W.P. and Ceccarelli,B. (1979) In Ceccarelli,B. and Clementi,F. (eds), *Neurotoxins, Tools in Neurobiology, Volume 3*, Raven Press, New York, pp. 87–115.
Kelly,R.B. (1988) *Neuron*, **1**, 431–438.
Khorlin,A.Ya., Shiyan,S.O., Markin,V.A., Nasonov,V.V. and Mirzyanova,M.N. (1986) *Bioorg. Khim. (Russ.)*, **12**, 1203–1212.
Kovalenko,V.A., Pashkov,V.N. and Grishin,E.V. (1981) *Bioorg. Khim. (Russ.)*, **7**, 1813–1837.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Meldolesi,J. (1982) *J. Neurochem.*, **38**, 1559–1569.
Meldolesi,J., Maddedu,L., Torda,M., Gatti,G. and Niutta,E. (1983) *Neuroscience*, **10**, 997–1009.
Meldolesi,J., Huttner,W.B., Tsien,R.Y. and Pozzan,T. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 620–624.
Meldolesi,J., Scheer,H., Maddedu,L. and Wanke,E. (1986) *Trends Pharm. Sci.*, **7**, 151–155.
Meldolesi,J., Pozzan,T. and Ceccarelli,B. (1988) In Baker,P.F. (ed.), *The Handbook of Experimental Pharmacology*. Springer Verlag, Berlin, Vol. 83, pp. 339–359.
Morrissey,J.H. (1981) *Anal. Biochem.*, **117**, 307–310.
Scheer,H. and Meldolesi,J. (1985) *EMBO J.*, **4**, 323–327.
Scheer,H., Prestipino,G. and Meldolesi,J. (1986) *EMBO J.*, **5**, 2643–2648.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Tzeng,M.-C. and Siekevitz,P. (1979) *J. Neurochem.*, **33**, 263–274.
Tzeng,M.-C., Cohen,R.S. and Siekevitz,P. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4016–4120.
Ushkaryov,Y.A. and Grishin,E.V. (1986) *Bioorg. Khim. (Russ.)*, **12**, 71–80.
Valtorta,F., Maddedu,L., Meldolesi,J. and Ceccarelli,B. (1984) *J. Cell Biol.*, **99**, 124–132.
Wanke,E., Ferroni,A., Gattanini,P. and Meldolesi,J. (1986) *Biohem. Biophys. Res. Commun.*, **134**, 320–325.

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