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α-Latrotoxin Stimulates Exocytosis by the Interaction with a Neuronal G-Protein-Coupled Receptor

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Summary

α-Latrotoxin is a potent stimulator of neurosecretion. Its action requires extracellular binding to high affinity presynaptic receptors. Neurexin Iα was previously described as a high affinity α-latrotoxin receptor that binds the toxin only in the presence of calcium ions. Therefore, the interaction of α-latrotoxin with neurexin Iα cannot explain how α-latrotoxin stimulates neurotransmitter release in the absence of calcium. We describe molecular cloning and functional expression of the calcium-independent receptor of α-latrotoxin (CIRL), which is a second high affinity α-latrotoxin receptor that may be the major mediator of α-latrotoxin's effects. CIRL appears to be a novel orphan G-protein-coupled receptor, a member of the secretin receptor family. In contrast with other known serpentine receptors, CIRL has two subunits of the 120 and 85 kDa that are the result of endogenous proteolytic cleavage of a precursor polypeptide. CIRL is found in brain where it is enriched in the striatum and cortex. Expression of CIRL in chromaffin cells increases the sensitivity of the cells to the effects of α-latrotoxin, demonstrating that this protein is functional in coupling to secretion. Syntaxin, a component of the fusion complex, copurifies with CIRL on an α-latrotoxin affinity column and forms stable complexes with this receptor in vitro. Interaction of CIRL with a specific presynaptic neurotoxin and with a component of the docking-fusion machinery suggests its role in regulation of neurosecretion.

Introduction

α-Latrotoxin is a potent extracellular stimulator of secretion from neurons and neuroendocrine cells of vertebrates (Clark et al., 1970; Frontali et al., 1976; Grasso et al., 1980; Barnett et al., 1996; reviewed by Rosenthal and Meldolesi, 1989). At least some of the effects of α-latrotoxin can be explained by its ability to induce influx of calcium and other cations through nonclosing membrane channels in the membrane that are different from endogenous cation channels in neurons (Grasso et al., 1980; Nicholls et al., 1982; Meldolesi et al., 1984; Wanke et al., 1986). It is possible that these channels are formed by toxin molecules after they bind to the plasma membrane since purified α-latrotoxin can form cation channels in artificial lipid bilayers by itself (Finkelstein et al., 1976; Robello et al., 1987). Calcium fluxes through α-latrotoxin-induced channels were observed directly (Grasso et al., 1980; Rosenthal et al., 1990; Hurlbut et al., 1994); thus, the elevation of intracellular calcium concentration is undoubtedly a significant component of this toxin's action and may be primarily responsible for its cytotoxicity, which is calcium dependent (Okamoto et al., 1971; Gorio et al., 1978; Duchen et al., 1981; Robbins et al., 1990). However, α-latrotoxin can stimulate neurotransmitter release from neuronal terminals and PC 12 cells also in nominally calcium-free buffers provided that magnesium or other divalent cations are present (Misler and Hurlbut, 1979; Ceccarelli et al., 1979; Rosenthal et al., 1990; Capogna et al., 1996a). Under these conditions, no release of calcium from the intracellular stores was observed either (Meldolesi et al., 1984; Rosenthal et al., 1990). It was therefore proposed that a second signaling mechanism exists that is independent of ion fluxes and that this mechanism is the key component of the α-latrotoxin stimulatory effect (Scheer et al., 1984; Capogna et al., 1996a).

α-Latrotoxin has specific high affinity membrane receptors in neural tissues, and its activity correlates with binding to these receptors (reviewed by Rosenthal and Meldolesi, 1989; Petrenko, 1993). Thus, it is possible that α-latrotoxin action is explained by the activation of these receptors followed by a transmembrane signaling event, which eventually triggers exocytosis. In support of this hypothesis, α-latrotoxin receptors were found to be specifically localized in the presynaptic plasma membrane in the neuromuscular junction (Valtorta et al., 1984). Comparisons of the α-latrotoxin receptor and synapsin distributions in the brain also yielded data consistent with presynaptic receptor localization (Malgaroli et al., 1989). However, the experiments performed in cerebellar primary cultures (Grasso and Mercanti-Ciotti, 1993) and in the Torpedo electric organ (Linial et al., 1995) suggested that the α-latrotoxin receptors may not be restricted to the sites of neurotransmitter release.

α-Latrotoxin receptors were detected in brain (~300 fmol/mg of membrane protein) and neuronal and neuroendocrine cell lines by using radioactive derivatives of the toxin (Tzeng and Siekevitz, 1979; Meldolesi, 1982; Scheer and Meldolesi, 1985). Two types of these receptors with the same high affinity to α-latrotoxin (K_d in the range of 10^{-9}–10^{-10} M) but differing in their calcium requirement for toxin binding have been described (Rosenthal et al., 1990). The Type I calcium-dependent receptor has been identified as neurexin Iα, a member of a large family of multiply spliced neuron-specific proteins, the neurexins (Petrenko et al., 1990; Ushkaryov et al., 1992). In contrast, a brain glycoprotein of 120 kDa, which

does not belong to the neurexin family, has been purified recently and identified as a brain membrane protein involved in the calcium-independent binding of α-latrotoxin (Davletov et al., 1996; Krasnoperov et al., 1996). Since neurexin Iα does not bind α-latrotoxin in calcium-free media (Petrenko et al., 1990; Davletov et al., 1995), only the Type II, calcium-independent receptor can be involved in the stimulation of neurotransmitter release with α-latrotoxin in calcium-free media.

By cloning and sequencing the cDNA of the calcium-independent receptor of α-latrotoxin (CIRL), we now demonstrate that this protein structurally resembles a signaling transmembrane receptor. CIRL is a novel member of the G-protein-coupled receptor superfamily, with an unusual two-subunit structure. Overexpression of exogenous CIRL increased the sensitivity of chromaffin cells to the effects of α-latrotoxin, demonstrating that this protein is functional in coupling to secretion. We have also discovered that this receptor interacts with syntaxin, a component of SNARE complexes. Altogether, our findings implicate CIRL as a novel important component of the neurosecretion machinery.

Results

The Structure of CIRL

The cDNA of CIRL was cloned on the basis of partial amino acid sequences of the 120 kDa protein (Krasnoperov et al., 1996) as described in Experimental Procedures. One long open reading frame was detected, which encoded a novel protein consisting of 1471 amino acid residues (Figure 1). The predicted size of the cloned protein was significantly larger than the apparent size of the purified 120 kDa protein. We therefore tested whether the purified receptor protein contained all of the predicted CIRL sequence by peptide mapping and by analysis with region-specific antibodies. The analysis of trypsin digests of the affinity-purified receptor by high resolution mass-spectrometry revealed the presence of peptide fragments that were attributable to >80% of the deduced protein sequence and distributed in a random manner throughout the whole sequence (data not shown). However, an antibody against the 18 amino acid residues closest to the C-terminus failed to recognize the 120 kDa protein and instead produced a diffuse staining at the top of the gel, suggesting aggregate formation (Figure 2A). We therefore included 8 M urea in the Laemmli SDS–electrophoresis system and sample buffer to further denature the proteins without boiling of the sample. In this modified system, the C-terminus antibody specifically stained a fuzzy band of ~85 kDa in both purified receptor and brain membranes (Figures 2A and 2B). In contrast, an antibody against the 120 kDa protein immunostained only the band of this size in the same preparations but not the 85 kDa band (Figures 2A and 2B).

These results suggest that the protein translated from CIRL mRNA may be proteolytically cleaved into two fragments of the 120 kDa (p120) and 85 kDa (p85). To locate the cleavage site, we analyzed a highly purified preparation of CIRL by N-terminal amino acid sequencing. A mixture of sequences was obtained. Among them, the strongest signal was produced by the sequence TNFAVLMAHREYI. This sequence begins at residue T838 of the CIRL sequence as predicted by its cDNA structure. This scission would result in two pieces, one ~70 kDa, corresponding to the p85 band on Western blots, and another 93 kDa, corresponding to the p120 band. At least in the case of the p120 subunit, the smaller predicted size can be explained by its extensive glycosylation (Davletov et al., 1996, and data not shown).

Since these proteins were found together in eluates from an α-latrotoxin affinity column, we tested whether they might form a complex between themselves and α-latrotoxin. The anti-p85 antibody efficiently immunoprecipitated the p120 subunit (Figure 2C) and calcium-independent α-latrotoxin-binding activity from brain detergent extracts (Figure 2D), thus suggesting that CIRL was originally synthesized as a single polypeptide chain, which underwent proteolytic processing to yield two strongly bound subunits, the N-terminal glycosylated p120 protein and the C-terminal p85 fragment.

When the CIRL precursor sequence was compared with known sequences in the Entrez database, several homologous proteins were found. Among them were three recently discovered orphan receptors—the leukocyte activation antigen CD97 (Hamann et al., 1995), EMR1, an EGF module-containing mucin-like hormone receptor (Baud et al., 1995); F4/80, a murine macrophage-restricted cell surface glycoprotein (McKnight et al., 1996); and other members of the secretin receptor family, which is part of the G-protein-coupled receptor

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MARLAAALWSLCVTTVLVTSATQGLSRAGLPFGLMRRELACEGYPIELRC 50
PGSDVIMVENANYGRTDDKICDADPFQMENVQCYLPDAFKIMSQRCNNRT 100
QCVVVAGSDAFPDPCPGTYKYLEVQYDCVPYKVEQKVFVCPGTLQKVLEP 150
TSTHESEHQSGAWCKDPLQAGDRIYVMPWIPYRTDTLTEYASWEDYVAAR 200
HTTTYRLPNRVDGTGFVVYDGAVFYNKERTRNIVKYDLRTRIKSGETVIN 250
TANYHDTSPYRWGGKTDIDLAVDENGLWVIYATEGNNGRLVVSQLNPYTL 300
RFEGTWETGYDKRSASNAFMVCGVLYVLRSVYVDDDSEAAGNRVDYAFNT 350
NANREEPVSLAFPNPYQFVSSVDYNPRDNQLYVWNNYFVVRYSLEFGPPD 400
PSAGPATSPPLSTTTTARPTPLTSTASPAATTPLRRAPLTTHPVGAINQL 450
GPDLPPATAPAPSTRRPPAPNLHVSPELFCEPREVRRVQWPATQQGMLVE 500
RPCPKGTRGIASFQCLPALGLWNPRGPDLSNCTSPWVNQVAQKIKSGFNA 550
ANIASELARHTRGSIYAGDVSSSVKLMEQLLDILDAQLQALRPIERESAG 600
KNYNKMHKRERTCKDYIKAVVETVDNLLRPEALESWKDMNATEQVHTATM 650
LLDVLEEGAFLLADNVREPARFLAAKQNVVLEVTVLSTEGQVQELVFPQE 700
YASESSIQLSANTIKQNSRNGVVKVVFILYNNLGLFLSTENATVKLAGEA 750
GTGGPGGASLVVNSQVIAASINKESSRVFLMDPVIFTVAHLEAKNHFNAN 800
CSFWNYSERSMLGYWSTQGCRLVESNKTHTTCACSHLTNFAVLMAHREIY 850
QGRINELLLSVITWVGIVISLVCLAICISTFCFLRGLQTDRNTIHKNLCI 900
NLFLAELLFLVGIDKTQYEVACPIFAGLLHYFFLAAFSWLCLEGVHLYLL 950
LVEVFESEYSRTKYYYLGGYCFPALVVGIAAAIDYRSYGTEKACWLRVDN 1000
YFIWSFIGPVSFVIVVNLVFLMVTLHKMIRSSSVLKPDSSRLDNIKSWAL 1050
GAIALLFLLGLTWAFGLLFINKESVVMAYLFTTFNAFQGVFIFVFHCALQ 1100
KKVHKEYSKCLRHSYCCIRSPPGGAHGSLKTSAMRSNTRYYTGTQSRIRR 1150
MWNDTVRKQTESSFMAGDINSTPTLNRGTMGNHLLTNPVLQPRGGTSPYN 1200
TLIAESVGFNPSSPPVFNSPGSYREPKHPLGGREACGMDTLPLNGNFNNS 1250
YSLRSGDFPPGDGGPEPPRGRNLADAAAFEKMISELVHNNLRGASGGAK 1300
GPPPEPPVPPVPGVSEDEAGGPGGADRAEIELLYKALEEPLLLPRAQSVL 1350
YQSDLDESESCTAEDGATSRPLSSPPGRDSLYASGANLRDSPSYPDSSPE 1400
GPNEALPPPPPAPPGPPEIYYTSRPPALVARNPLQGYYQVRRPSHEGYLA 1450
APSLEGPGPDGDGQMQLVTSL 1471
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Figure 1. The Predicted Amino Acid Sequence of the CIRL Protein Precursor

The amino acid sequence is translation of the rat cDNA sequence submitted to GenBank. Amino acid residues identical with the obtained peptide sequences from the purified bovine 120 kDa protein are underlined. Eight of ten determined peptide sequences fit very well with the predicted structure of the rat protein. The differences could be either explained by conservative substitutions or failures to identify amino acid residues, which are prone to degradation (e.g., C and W). Peptides IYVMPCIPYR and SLQLYVINAEV did not show significant homology with the deduced sequence, which could be explained by species difference.

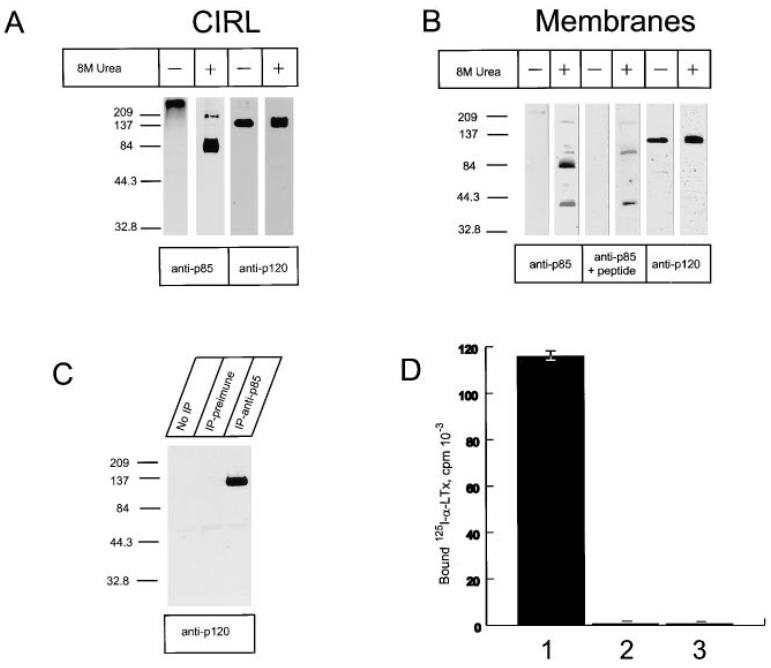


Figure 2. Two-Subunit Structure of CIRL

Purified CIRL (A) or total brain membranes (B) were analyzed by SDS–gel electrophoresis either in standard system or with addition of 8 M urea to the gel and sample buffer. Western blots are shown that were stained with either anti-p85 or anti-p120 antibodies.

(C) CIRL was immunoprecipitated from brain detergent extracts with anti-p85 antibody or the preimmune serum followed by immunostaining with anti-p120 antibody.

(D) Labeled α-latrotoxin was added to brain detergent extract followed by immunoprecipitation with anti-p85 antibody (lane 1) or the preimmune serum (lane 2). In another control, no brain extract was included (lane 3).

superfamily (Gilman, 1987; Strader et al., 1994). The hydrophobicity plot of CIRL indicates the presence of seven adjacent hydrophobic segments, potential transmembrane helices, which is a hallmark of serpentine G-protein-coupled receptors. The putative transmembrane regions of CIRL are significantly homologous (~30% identity and 50%–60% similarity) to the transmembrane regions of the orphan receptors and other members of the secretin receptor family (Segre and Goldring, 1996), e.g., secretin receptor, corticoliberin receptor, calcitonin receptor, diuretic hormone receptor, VIP receptor, etc. (Figure 3A). About 10% of amino acid residues in these regions appear to be perfectly conserved among all of the family members. Several other regions of significant homology include the predicted extracellular loops between transmembrane segments I and II, III and IV, IV and V, and a small portion of the C-terminal cytoplasmic domain. Two conserved cysteine residues are present in the extracellular loops between segments II and III, and between segments IV and V, that are typical for G-protein-coupled receptors and are thought to form a disulfide bridge on the basis of the structural studies of rhodopsin. Finally, a pair of adjacent cysteines, a potential palmitoylation site, characteristic of G-protein-coupled receptors (Dohlman et al., 1991), are found in the cytoplasmic domain close to the transmembrane segments.

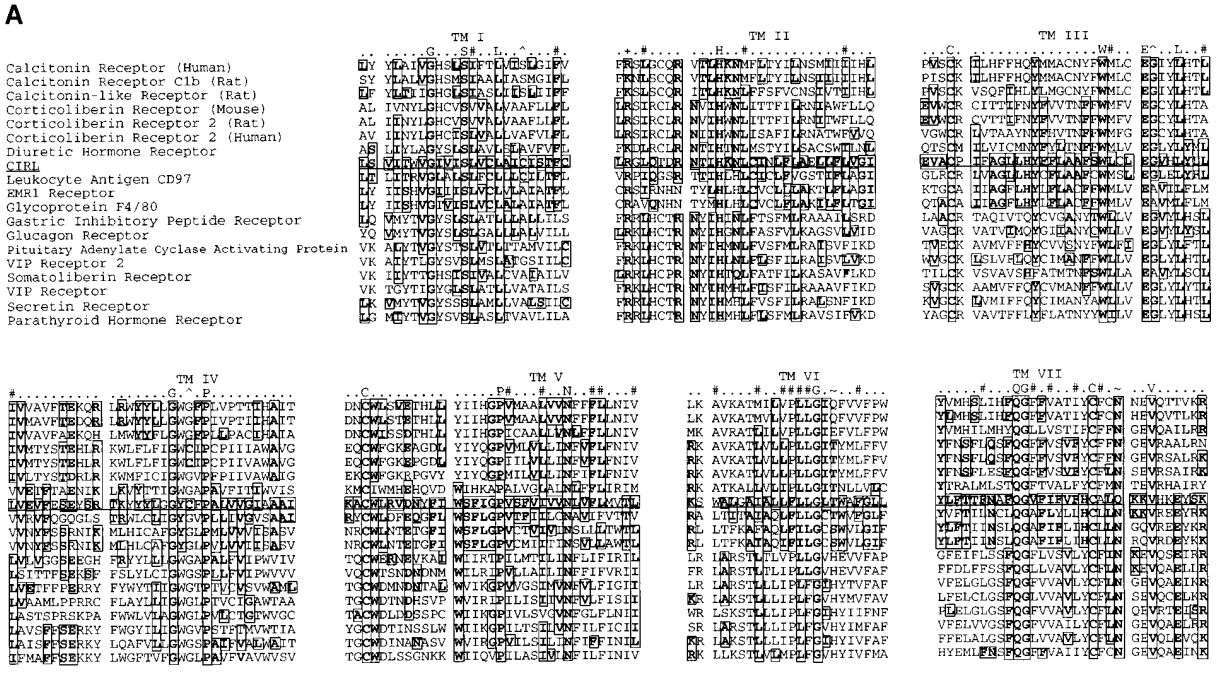
On the basis of the hydrophobicity plot and homology searches, we propose the domain model of CIRL, which consists of three major regions: the large extracellular N-terminal region, the transmembrane region including seven hydrophobic helices, and the intracellular C-terminal region (Figure 3B). The extracellular domain is proteolytically cleaved close to the transmembrane helices. This cleavage results in the formation of two tightly bound subunits. In the very amino terminus of the protein (residues 4–22), CIRL contains a hydrophobic segment, which has features typical of a secretion signal peptide sequence. This suggests that the amino terminal region of CIRL is located extracellularly, which is typical for G-protein-coupled receptors. By direct N-terminal sequencing of the p120 subunit of CIRL, the site of the signal peptide cleavage was determined as C-terminal to G24.

Several domains of the large extracellular domain of CIRL, p120, show significant homology with a galactose-binding lectin (Ozeki et al., 1991) from sea urchin eggs (35% identity and 60% similarity); with olfactomedin (Yokoe and Anholt, 1993), a major building block in the extracellular matrix of olfactory neuroepithelium (35% identity and 55% similarity); with olfactomedin-related protein (40% identity and 60% similarity) (Danielson et al., 1994); and with mucin (Gum et al., 1994). The homology of CIRL's unusually large extracellular domain to these proteins may suggest its possible interaction with glycoproteins of the extracellular matrix and/or cytoplasmic membranes of neighboring cells.

Like the extracellular domain, the carboxy-terminal intracellular domain of CIRL is also unusually large (371 residues) and enriched in proline (14% versus 7.6% in the entire sequence) due to the presence of several proline clusters. Except for proline-rich proteins (e.g., extensin, hydroxyproline-rich glycoprotein, collagen, etc.), homology searches did not show any significant similarity of this domain with known proteins.

CIRL Is an α-Latrotoxin Receptor

The identity of CIRL as a high affinity α-latrotoxin binding protein was directly confirmed by transfections of COS cells, a cell line of nonneuronal origin, with an expression plasmid containing CIRL cDNA. Western blotting of the transfected cells verified the expression of both sub-



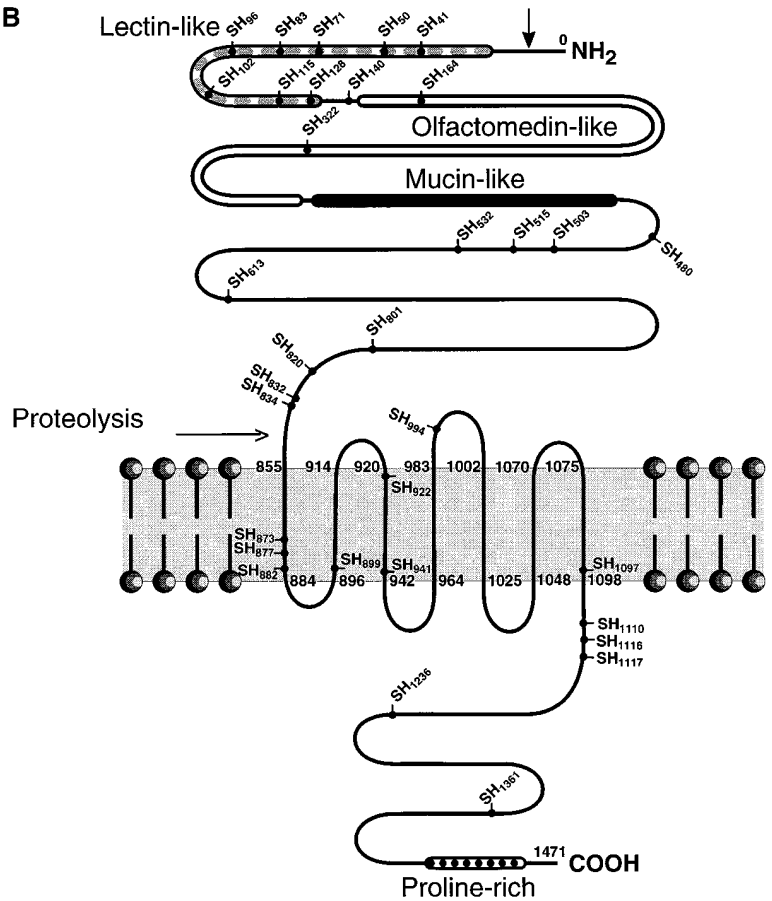


Figure 3. Domain Structure of CIRL

(A) Multiple alignment of CIRL with the members of the secretin receptor family. The proteins homologous to CIRL were identified by searching Entrez database using BLASTP program with the default BLOSUM62 matrix. The multiple sequence alignment was generated with the ICM program, which uses a tree-directed multiple sequence alignment algorithm similar to the Clustal method (Thompson et al., 1994). Seven predicted transmembrane helices are marked as TM 1–7 and separated from adjacent regions of homology. The sequence of CIRL is underlined. The residues identical or similar (K=R, E=D, L=I, Q=N) to CIRL residues are marked as bold and boxed to identify the regions of homology. In the consensus line, a character identifies amino acid conserved in all sequences; #, hydrophobic amino acid (F, I, L, M, P, V, W); ^, small amino acid (A, S, G, C); ~, polar amino acid (C,D,E,G,H,N,Q,S,T,Y); +, positively charged amino acid (R, K); −, negatively charged amino acids (E, D); and dot, the rest (no consensus, no gap).

(B) The proposed transmembrane topology of CIRL. A closed arrow in the amino-terminus indicates the site of cleavage of the signal peptide sequence. An open arrow denotes the region of endogenous proteolytic processing of CIRL. All cysteine/cystine residues are marked as SH. The domains with homology to a sea urchin lectin, olfactomedin, mucin, and a cytoplasmic proline-rich cluster are labeled as such.

units of CIRL. However, both anti-p120 and anti-p85 antibodies also produced diffuse staining on the top of the gels, suggesting that proteolytic processing of overexpressed CIRL in COS cells was not as efficient as in neurons (Figure 4A). Since the larger band was not solubilized efficiently and was virtually absent after

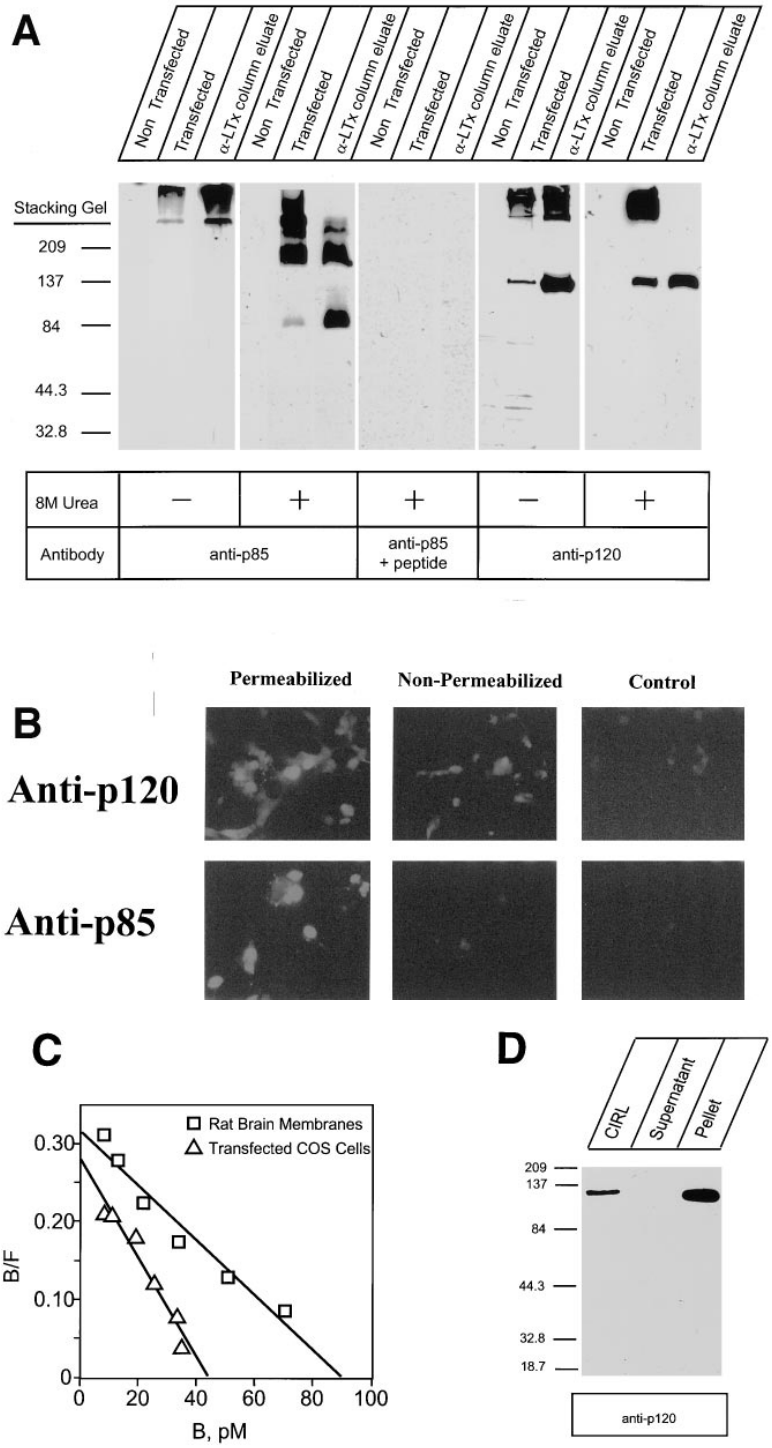


Figure 4. Expression of CIRL in COS Cells

COS cells were transfected with a CIRL expression construct as described in Experimental Procedures.

(A) The transfected cells were lysed with SDS sample buffer or solubilized with 2% Triton X-100 and precipitated with α-latrotoxin-Sepharose. These samples together with mock-transfected cells were analyzed by SDS–electrophoresis in a standard system or with added 8 M urea, blotted onto nitrocellulose and immunostained with either anti-p120 or anti-p85 antibody. The peptide used to generate anti-p85 antibody was added (50 μg/ml) to control for nonspecific staining. The band of ~170 kDa stained with anti-p85 antibody most likely represents a nondissociated dimer of p85.

(B) The CIRL-transfected and mock-transfected COS cells were immunostained with anti-p120 and C-terminal anti-p85 antibodies as described in Experimental Procedures. The left panels show the staining pattern of the cells permeabilized with 0.1% saponin, the center panels show the fixed intact cells, and the right panels show the staining of mock-transfected permeabilized cells.

(C) Approximately 20% of the cell material harvested from one 100 mm Petri dish was used for each measurement in the α-latrotoxin-binding assay. In parallel, α-latrotoxin binding activity of rat brain membranes (220 μg protein) was measured under identical conditions. The value of specific binding was calculated by subtraction of the nonspecific binding obtained in the presence of 0.1 μM α-latrotoxin from the total binding for each α-latrotoxin concentration. The results of binding assays are presented in a Scatchard plot.

(D) COS cells were transfected with an expression plasmid encoding the N-terminal 1–850 residues. Media (0.4 ml) from transfected COS cells were precipitated with 10 μl of α-latrotoxin-Sepharose, the matrix was eluted with SDS sample buffer (pellet) and together with 30 μl of the media (supernatant), analyzed by electrophoresis and Western blotting with anti-p120 subunit antibody.

precipitation of COS cell detergent extracts on α-latrotoxin-Sepharose, it is possible that the noncleaved protein does not bind the toxin.

We have tested whether the proposed transmembrane topology of CIRL (the extracellular p120 subunit and the intracellular C-terminus of p85, as shown in Figure 3B) is correct. The COS cells transiently transfected with the CIRL-encoding plasmid were stained with the anti-p120 antibody and anti-p85 antibody directed against the C-terminal epitope, with and without permeabilization (Figure 4B). Both antibodies produced uniform labeling of only a subset of cells, which reflected moderate efficiency of transfection. However, the effect of cell permeabilization on the staining pattern was

different for these two antibodies. With the anti-p85 antibody, virtually no staining was observed in nonpermeabilized cells. However, after permeabilization, a strong signal was observed in a subset of cells. In contrast, the anti-p120 antibody showed a good signal in intact cells, which was further enhanced by permeabilization. Obviously, upon overexpression, a significant part of synthesized CIRL was either transiently or permanently retained in intracellular membrane compartments and did not reach the cytoplasmic membrane.

Most importantly, the transfected COS cells exhibited high affinity specific binding of radioactive α-latrotoxin in the absence of calcium (180+/-28 fmol/mg protein in CIRL transfected cells versus 3.9+/-3.3 in mock transfections). Scatchard plot analysis (Figure 4C) demonstrated that the cells transfected with the CIRL expression construct bound α-latrotoxin with an affinity similar to that of the calcium-independent binding sites in rat brain membranes (calculated $K_d = 0.16$ nM for recombinant receptor; $K_d = 0.28$ nM for brain membranes).

Since α-latrotoxin acts extracellularly, we also tested whether the recombinant extracellular domain of CIRL by itself would bind α-latrotoxin. COS cells were transfected with a deletion construct encoding the entire extracellular sequence, which was expected to be secreted from the transfected cells into the media. The cell conditioned media were chromatographed on α-latrotoxin-Sepharose, and the retained proteins were analyzed by Western blotting with the anti-p120 antibody. The immunostaining demonstrated complete absorption from the media of the band of the expected size (~120 kDa), thus confirming interaction of α-latrotoxin with the extracellular portion of CIRL (Figure 4D).

α-Latrotoxin has been shown to stimulate inositol phosphate accumulation in PC 12 cells in a calcium-dependent manner (Vicentini and Meldolesi, 1984; Rosenthal et al., 1990). This signaling event cannot explain α-latrotoxin action in calcium-free media, however, it may be an important component of its calcium-dependent effects in secretory cells. We have tested whether α-latrotoxin produced a similar effect in COS cells transfected with CIRL. In nontransfected COS cells, no accumulation of inositol phosphates was observed as a result of α-latrotoxin (1 nM) treatment. In transfected cells, ~2.5-fold increase in inositol phosphate release was observed in calcium-containing buffers, and this effect was completely abolished by addition of EGTA (data not shown).

Overexpressed CIRL Couples to the Exocytotic Response in Chromaffin Cells

We determined that untransfected bovine chromaffin cells contain ~60 fmol/mg protein of α-latrotoxin-binding sites (~13% of the level found in brain). Two thirds of the binding was calcium independent. α-Latrotoxin is an effective secretagogue in these cells at concentrations >100 pM (Bittner and Holz, unpublished data; Surkova, 1994; Barnett et al., 1996). We predicted that expression of the CIRL protein would increase the number of α-latrotoxin binding sites on the cells, thus rendering them more sensitive to the stimulatory effects of α-latrotoxin. Chromaffin cells were cotransfected with a plasmid encoding human growth hormone (hGH), and with either a plasmid encoding the CIRL (pCDR7) or a control plasmid (pCMVneo). Transiently expressed hGH is stored in secretory granules (Wick et al., 1993), and serves as a marker for regulated secretion from the small population of transfected cells. Under our transfection conditions, at least 90%–95% of cells that express hGH also express the test plasmid of interest. Preliminary experiments demonstrated that no secretion occurs when chromaffin cells are exposed to α-latrotoxin in a calcium-free buffer containing a calcium chelator even when magnesium is present. Subsequent addition of calcium is required to support secretion induced by the toxin. In the following experiment, cells were exposed to α-latrotoxin in physiological saline (PSS) without magnesium or calcium, and with EGTA. After removal of the toxin, secretion was initiated in PSS containing both magnesium and calcium. This protocol ensured that only those receptors that bind α-latrotoxin in the absence of calcium were being studied.

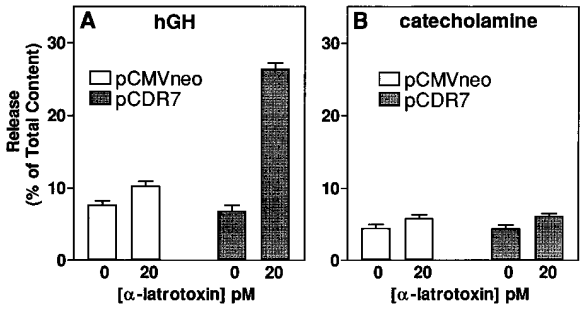


Figure 5. Expression of CIRL Increases the Sensitivity of Intact Chromaffin Cells to Stimulation by α-Latrotoxin

Chromaffin cells were transfected with plasmids for hGH (pXGH5) and either CIRL (pCDR7) or pCMVneo (a control) as described. Four days later, cells were incubated with or without 20 pM α-latrotoxin in PSS without calcium or magnesium and with 0.1 mM EGTA. After 4 min, the toxin was removed, and the cells were incubated for an additional 5 min in PSS containing 2.2 mM Ca^{2+} and 0.5 mM Mg^{2+}. The amounts of hGH (A) and catecholamine (B) released into the medium and the amounts remaining in the cells were determined as described. Each bar shows the average of four measurements. Average hGH expression was 2.57 ± 0.14 ng/well in cells with pCMVneo and 2.98 ± 0.11 ng/well in cells with pCDR7 (CIRL).

When cells transiently overexpressing CIRL were stimulated with 20 pM α-latrotoxin, hGH secretion was strongly stimulated (Figure 5A). A 10-fold higher concentration of α-latrotoxin (200 pM) was required to elicit equivalent hGH secretion in cells without CIRL (not shown). This increased sensitivity of CIRL-expressing cells to α-latrotoxin demonstrates that CIRL can function as an α-latrotoxin receptor in chromaffin cells. As expected, 20 pM α-latrotoxin stimulated little or no catecholamine secretion (which measures secretion from all cells in the culture, the bulk of which are not transfected) in cultures with or without CIRL (Figure 5B). Again, higher concentrations of α-latrotoxin (200 pM) were required to elicit catecholamine release in cultures with or without CIRL (not shown).

Distribution of CIRL in the Brain

We have previously demonstrated by Western blotting that the p120 subunit has a brain-specific distribution

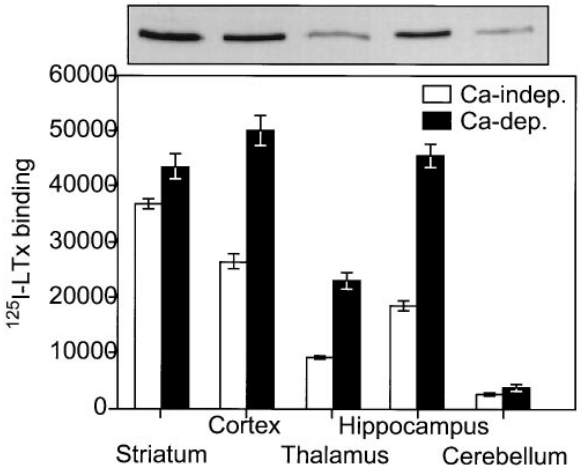


Figure 6. Distribution of CIRL in Different Brain Regions

Rat brains were dissected to separate cortex, striatum, thalamus, hippocampus, and cerebellum. The tissues were homogenized in 150 mM NaCl, 50 mM Tris-HCl, and 2 mM EDTA buffer (pH 7.9), and the crude membrane fractions were obtained by centrifugation. The specific binding of 0.5 nM ^{125}I-α-latrotoxin to the membranes was analyzed in the buffer containing either 2 mM Ca^{2+} or 2 mM EDTA in triplicate. A 100-fold excess of unlabeled α-latrotoxin was added to control for nonspecific binding. Calcium-dependent binding activity was calculated as a difference between α-latrotoxin binding measured in the presence of Ca^{2+} and in EDTA-containing buffer (lower graph). The same membrane samples were blotted and immunostained with anti-p120 antibody (upper blot).

(Krasnoperov et al., 1996). This finding was further confirmed by the Northern blot analysis of the tissue distribution of CIRL mRNA. Of seven rat tissues analyzed (brain, liver, heart, lung, kidney, spleen, skeletal muscle, and duodenum), only brain showed a specifically hybridized band of ~6 kb (data not shown). The size of this band was very close to the size of the longest clone (5391 base) isolated from the rat brain cDNA library, which was full length with respect to the coding sequence.

To compare CIRL concentrations within several regions of the rat brain, we used Western blotting with the anti-p120 antibody. Rat brains were dissected to isolate cortex, cerebellum, hippocampus, thalamus, and striatum. Crude membranes were prepared from each tissue and analyzed for both α-latrotoxin-binding activity and CIRL immunoreactivity. The highest concentrations of calcium-independent receptors were found in striatum, somewhat lower in cortex and hippocampus, and much less of these receptors were detected in cerebellum (Figure 6). When the films of ECL-developed blots with different exposures were quantitated, they showed a larger concentration of CIRL in the striatum than in the cortex by ~40%. Therefore, the distribution of CIRL immunoreactivity was in good agreement with the pattern of calcium-independent α-latrotoxin binding activity, thus supporting the idea that CIRL is the calcium-independent brain receptor of α-latrotoxin.

Interaction of CIRL with Syntaxin

It had been previously reported that synaptotagmin and syntaxin copurify with α-latrotoxin receptors in the course of affinity chromatography on α-latrotoxin-Sepharose with calcium-containing buffers when neurexin Iα is the major component of the column eluate (Petrenko et al., 1991; O'Connor et al., 1993). We tested whether these proteins were also present in the eluate of an α-latrotoxin affinity column when all stages of purification were performed in EDTA-containing buffers and therefore neurexin Iα was not retained on the column (Krasnoperov et al., 1996). Syntaxin and synaptotagmin were detected by Western blotting of the eluted receptor preparations but not other nerve terminal proteins such as synaptophysin, SNAP-25, synapsins, rab 3A, synaptobrevin I and II, and Munc 18/nSec1 (Figure 7A, and data not shown). When the α-latrotoxin affinity column was eluted with a salt gradient, synaptotagmin was found in the beginning of the gradient (0.2–0.3 M salt), whereas syntaxin coeluted with CIRL at salt concentrations >0.6 M (Petrenko et al., 1991, and data not shown).

To assure the specificity of complexing of CIRL with syntaxin, we tested whether anti-syntaxin antibody could immunoprecipitate calcium-independent α-latrotoxin binding activity from detergent extracts of total brain membranes. The syntaxin family contains several homologous differentially distributed proteins (Bennett et al., 1993). The antibody that we used recognized syntaxin 1A, which is frequently referred to as syntaxin. As a negative control, normal mouse IgGs were used. Additional controls were included to test whether these antibodies could immunoprecipitate the radiolabel by direct interaction with α-latrotoxin when brain membranes were omitted. After incubations and washes, immunomatrices with anti-p120 (included as a positive control) and anti-syntaxin antibodies retained significantly more labeled α-latrotoxin than the negative control sorbents (Figure 7B). The immunoprecipitates were also analyzed by blotting with the anti-p120 antibody. A small amount of CIRL immunoreactivity was detected consistent with much lower concentration of CIRL in brain as compared to syntaxin (data not shown). Finally, the anti-p85 antibody was tested for its ability to immunoprecipitate syntaxin in addition to CIRL from brain detergent extracts. In good agreement with previous experiments, syntaxin was found in immunoprecipitates with the immune but not preimmune sera, and this interaction was not disrupted by the washes of matrices with 0.5 M salt buffers (Figure 7C, and data not shown).

Although we found synaptotagmin in the preparations of calcium-independent receptors and it was previously reported that calcium-independent α-latrotoxin stimulation of neurosecretion but not the calcium-dependent type is impaired in synaptotagmin-deficient PC12 cells (Shoji-Kasai et al., 1994), an anti-synaptotagmin antibody that we tested failed to immunoprecipitate the complex of CIRL with α-latrotoxin in the absence of calcium (data not shown). Therefore, it remains to be seen whether the interaction of synaptotagmin with CIRL and toxin is specific or may reflect a weaker indirect complexing through syntaxin or possibly some other protein.

Discussion

We have previously identified and purified two types of high affinity α-latrotoxin receptors (Petrenko et al., 1990;

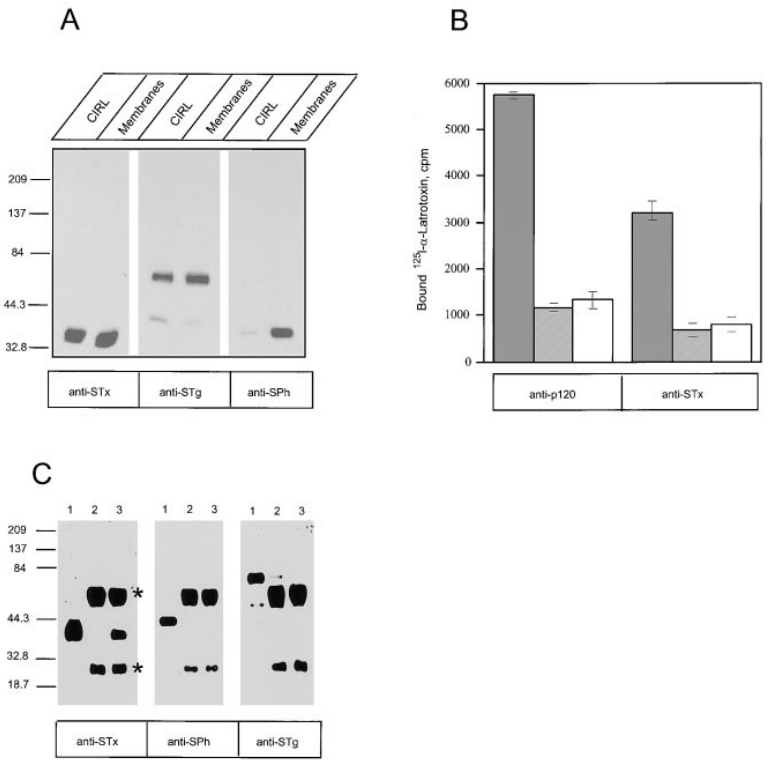


Figure 7. Interaction of CIRL with Syntaxin

(A) Copurification of syntaxin and synaptotagmin with CIRL on an α-latrotoxin affinity column. The total rat brain membranes in the amount of 35 μg or affinity-purified CIRL in the amount of 0.15 μg were separated on a 10% SDS gel, blotted, and immunostained with the antibodies against syntaxin (anti-STx), synaptotagmin (anti-STg), and synaptophysin (anti-SPh).

(B) Immunoprecipitation of α-latrotoxin-binding activity. The complex of ^{125}I-α-latrotoxin was preformed in the extracts of total brain membranes and immunoprecipitated as described in Experimental Procedures with anti-p120 or anti-syntaxin antibodies (closed bars), preimmune serum, or normal mouse IgGs (hatched bars). In control experiments, brain extracts were excluded (open bars).

(C) Coimmunoprecipitation of syntaxin with CIRL. Rat brain detergent extracts were immunoprecipitated (1:200 dilution) with the anti-p85 antibody (lane 3) or preimmune serum (lane 2). 0.25 M salt buffer was used to wash the matrices prior to elution. Solubilized brain protein (5 μg) was loaded in lane 1. The same samples were also stained with anti-synaptophysin or anti-synaptotagmin antibodies. Asterisks indicate the heavy and light chains of rabbit IgGs.

Krasnoperov et al., 1996). The type I receptor, or neurexin Iα, binds α-latrotoxin only in the presence of calcium ions (Petrenko et al., 1990; Davletov et al., 1995) and therefore cannot be involved in the α-latrotoxin-induced neurotransmitter release in calcium-free media. Also, there is no direct physiological evidence currently available that this receptor is responsible for the calcium-dependent effects of α-latrotoxin. In this paper, we describe molecular cloning and sequencing of the type II receptor (CIRL) that binds α-latrotoxin in a calcium-independent manner. This protein is likely to be an endogenous receptor because 1) it binds α-latrotoxin in a calcium-independent manner, 2) it has structural features of a membrane receptor, 3) it has an appropriate anatomical distribution, and 4) it has functional effects when expressed in COS and chromaffin cells. These issues are discussed below.

The structural features of CIRL strongly suggest that this protein is a signaling receptor, a novel member of the G-protein-coupled receptor superfamily. Seven putative transmembrane hydrophobic helices, a hallmark of serpentine receptors, are found in the amino acid sequence of CIRL. These domains are significantly homologous to the predicted membrane regions of the members of the secretin receptor family. However, in contrast with other G-protein-coupled receptors, CIRL is extraordinarily large and consists of two subunits (p120 and p85), which are products of the proteolytic cleavage of a common precursor protein and can both be detected in preparations of the purified receptor with subunit-specific antibodies. Since only these fragments but not the precursor protein were detected in either purified receptor or freshly prepared brain homogenates, we may conclude that this cleavage is not a purification artifact, but represents endogenous proteolytic processing of CIRL and may resemble the proteolytic processing of LDL receptor-related protein (Herz et al., 1990). A site of the endogenous cleavage of CIRL in its extracellular domain was detected by N-terminal amino acid sequencing of purified CIRL. Cleavage at this site would result in two fragments, a hydrophilic glycosylated protein that is located extracellularly and contains at least a part of α-latrotoxin-binding site, and a membrane polypeptide containing seven transmembrane segments and hydrophilic loops between them, the short (23 residues) hydrophilic extracellular N-terminal tail and the large intracellular C-terminal domain. The immunoprecipitation experiments performed with detergent extracts of crude brain membranes where CIRL concentration is very low (~0.2 nM) indicate that the p85 and p120 subunits are still tightly bound to each other after the proteolytic cleavage.

Our findings contrast with the proposed one-subunit structure of the putative calcium-independent α-latrotoxin receptor named latrophilin (Davletov et al., 1996), which was based on purification and sucrose density sedimentation experiments. Those results could be explained by the absence in the SDS gels of additional denaturing agent (urea) used in our experiments that would have resulted in nondetected p85 protein. Also, the molecular size estimation from sedimentation experiments is very approximate and strongly depends on the shape of the protein. In addition, it was proposed that p120/latrophilin is an integral membrane protein because it could not be extracted from membranes with salt buffers and because it went into the detergent phase in a Triton X-114 phase separation experiment (Davletov et al., 1996). Our sequencing and expression (Figure 4D) data strongly suggest that the p120 subunit is a secreted soluble protein. However, since it forms a tight complex

with the p85 subunit (Figures 2C and 2D), the results of the salt extraction and phase separation experiments may be explained by the properties of the p85 subunit, which is an integral membrane protein with a highly hydrophobic core consisting of seven membrane-spanning helices.

The molecular cloning of CIRL was confirmed by the similarity of obtained peptide sequences, production of an anti-peptide antibody on the basis of the deduced sequence and, most importantly, by expression experiments. Transfection of COS cells with a plasmid encoding CIRL resulted in the expression of high affinity calcium-independent α-latrotoxin binding sites. In good agreement with the known extracellular mode of action of α-latrotoxin, the recombinant extracellular portion of CIRL, produced as a soluble secreted protein, was sufficient for α-latrotoxin binding. In COS cells expressing CIRL, α-latrotoxin stimulated inositol phosphate accumulation as was earlier reported for PC 12 cells (Vicentini and Meldolesi, 1984; Rosenthal et al., 1990), which contain endogenous calcium-independent receptors. Finally, the analysis of secretion from the chromaffin cells, transfected with the CIRL expression construct, demonstrated a 10-fold increase in their sensitivity to stimulation with α-latrotoxin. Hence, the cloned cDNA encoded not only an α-latrotoxin-binding protein, but also a functional α-latrotoxin receptor that was coupled to secretion.

Characteristics of CIRL are in good agreement with other features typical for the putative calcium-independent α-latrotoxin receptor. First, like α-latrotoxin-binding receptors, CIRL is detected in brain tissue, but not in a number of other tissues, by Northern and Western blotting. Second, the distribution of CIRL in brain regions closely correlates with the distribution of the calcium-independent α-latrotoxin binding activity. Interestingly, unlike calcium-dependent receptors, calcium-independent receptors are more concentrated in the striatum than cortex, which coincides with the distribution of CIRL immunoreactivity. As compared with total brain homogenates, CIRL is enriched about seven times in purified synaptic plasma membranes (Davletov et al., 1995). Finally, CIRL is a glycoprotein (Davletov et al., 1996, and data not shown) in agreement with the known lectin inhibition of both α-latrotoxin activity and receptor binding (Grasso et al., 1978; Rubin et al., 1978; Tzeng and Siekevitz, 1979). The trypsin digests of the receptor preparations, obtained by affinity chromatography on α-latrotoxin-Sepharose, did not contain significant amounts of peptides other than from CIRL according to mapping by high resolution mass spectrometry. Although we cannot exclude the possibility that α-latrotoxin-binding homologs or isoforms of CIRL exist, these data suggest that the cloned cDNA encodes the major if not the only calcium-independent α-latrotoxin receptor in the brain.

Several independent in vitro binding experiments indicate that CIRL interacts with syntaxin, a synaptic protein. Syntaxin is a key component of the neurosecretion machinery possibly involved in membrane fusion (Bennett et al., 1992; Blasi et al., 1993; Sollner et al., 1993; Chapman et al., 1995; Li et al., 1995; Schulze et al., 1995). Syntaxin copurifies with CIRL on an α-latrotoxin affinity column. A monoclonal antibody against syntaxin coimmunoprecipitates CIRL. In a reciprocal experiment, syntaxin is found together with CIRL in immunoprecipitates with the anti-p85 antibody. Also, calcium-independent α-latrotoxin-binding activity can be precipitated with anti-syntaxin antibody from detergent extracts of brain membranes. Since the concentration of calcium-independent α-latrotoxin binding sites in brain membranes is very low (~200 fmol/mg protein), this assay reflects a highly specific interaction between the members of this complex. These data suggest that CIRL and syntaxin can form a complex both in the absence or presence of α-latrotoxin.

α-Latrotoxin exerts its stimulatory action in neurons in calcium-free media. It is also fully active after being prebound to calcium-independent receptors in secretory cells if calcium is added later (Grasso et al., 1980; Meldolesi et al., 1983; Bittner et al., unpublished data). Since at this time no direct data is available on the role of neurexin Iα in secretion, the simplest explanation of the α-latrotoxin effect would be that its binding to CIRL is sufficient for further stimulation of neurosecretion either in calcium-free or calcium-containing media.

Our data suggest that α-latrotoxin may produce multiple effects by binding to CIRL. In the presence of extracellular calcium, α-latrotoxin causes calcium influx. The increase in calcium permeability could result from the channel-forming ability of the toxin while it is tethered to the receptor or because of activation of the receptor. This elevation of intracellular calcium may explain the accumulation of inositolphosphates observed in our experiments. The seven membrane-spanning domains of CIRL, which are homologous to those of the members of the secretin receptor family, suggest that α-latrotoxin may work as an agonist or antagonist of CIRL initiating intracellular signaling via a heterotrimeric G protein in a calcium-independent manner. In support of this hypothesis, α-latrotoxin effects were found to be inhibited by agonists of $GABA_B$ and μ-opioid receptors, which are G-protein-linked receptors that may be coupled to the same G protein(s) as CIRL (Capogna et al., 1996b). Alternatively, α-latrotoxin bound to CIRL may activate secretion through its interaction with syntaxin and, possibly, synaptotagmin.

Further understanding of CIRL function will require identification of an endogenous ligand(s) of this receptor. Since the unusually large extracellular subunit of CIRL has domains homologous to olfactomedin, a major component of olfactory extracellular matrix, and to a sea urchin egg lectin, we may speculate that an endogenous ligand of CIRL exists that is a component of the extracellular matrix or a membrane protein. If this is true, then a possible function of CIRL would be regulation of neurosecretion in response to extracellular cell–cell interactions.

Experimental Procedures

α-Latrotoxin was purified from lyophilized black widow spider venom glands and radioactively labeled with ^{125}I by chloramine T procedure as previously described (Petrenko et al., 1990). The α-latrotoxin binding activity was analyzed by the rapid centrifugation assay (Davletov et al., 1995). SDS–PAGE and Western blotting with ECL detection were performed according to Bio-Rad and Amersham

protocols, respectively. In some experiments, 8 M urea was included in the gel and the sample buffer, and in this case, the samples were not boiled. Purification of CIRL from brain membranes, proteolytic digests, and peptide sequencing were performed as described (Krasnoperov et al., 1996). Northern blotting was done with a premade multiple tissue blot with 20 μg of each tissue total RNA (Bios Laboratories) according to the manufacturer's protocol with a randomly labeled probe (Boehringer-Mannheim) obtained from a full-length CIRL cDNA fragment.

Antibodies

Chickens (egg-laying hens) were immunized with p120, which had been isolated by preparative gel electrophoresis from affinity-purified CIRL as described (Krasnoperov et al., 1996). The titer and specificity of antibodies was tested in Western blotting with purified CIRL and total brain membranes. The purified antibody stained one protein band of 120 kDa in total brain membranes. However, it did not inhibit α-latrotoxin binding to CIRL, indicating that the site of α-latrotoxin binding was not a strong immunogen probably because it is highly conserved in vertebrates.

Anti-p85 antibody was raised in a rabbit by injecting it with the synthetic peptide CEGPGPDGDGQMQLVTSL coupled to KLH using Imject Activated Immunogen Conjugation Kit (Pierce). The rabbit was injected three times with 2-week intervals and bled 2 weeks later. For some experiments, monospecific IgGs were purified by chromatography on a column with immobilized BSA, which had been covalently modified with the synthetic peptide. The titer and specificity of the anti-p85 antibody were analyzed by dot blotting with the peptide coupled to BSA and Western blotting with purified CIRL and total brain membranes. Preimmune sera or peptide inhibition (5.0 μg/ml) were used in control experiments.

Monoclonal antibodies against syntaxin and synaptophysin were purchased from Sigma. Other antibodies against synaptic proteins were kindly provided by Drs. R. Jahn, T. C. Südhof, and P. DeCamilli.

Cloning and Sequencing of CIRL

Molecular cloning experiments were performed according to established procedures and protocols (Petrenko et al., 1996). The sequence of a 17 residue peptide VFLMDPVIFTVAHLEAK, confirmed by mass spectrometry (1930.3), was used to design two degenerate PCR primers. PCR reactions on a rat cDNA random-primed library (kindly provided by Dr. James Boulter, Salk Institute) resulted in the isolation of a cDNA fragment of the necessary size. This fragment was used as a template in a PCR reaction with ^{32}P-dCTP to generate a probe for the library screening. About 10 overlapping clones were isolated that encoded most of the protein structure. The most 5′-extended clone was randomly labeled and used to screen a size-selected oligo-dT-primed rat brain cDNA (also supplied by Dr. James Boulter), which resulted in the isolation of a number of clones; one of them was full length with respect to the coding cDNA. Several overlapping clones were sequenced completely on both strands by dideoxy automated method using synthetic primers.

Cell Transfection Assays

The insert of the longest clone (p87–7) encoding full-length protein was subcloned into pcDNA 3.1, a eukaryotic expression vector (Invitrogen). The resulting plasmid was purified by a midi-prep kit (Promega) and used in transfections of COS-7 cells by the calcium phosphate precipitate method or with lipofectamine (Life Technology). After a 2 day incubation, the cells were harvested in a cold buffer containing 0.1 M Tris-HCl and 2 mM EDTA (pH 7.9). The cells were lysed by a freeze-thaw procedure, and the membranes were pelleted by centrifugation and used for α-latrotoxin-binding assay in a calcium-deficient buffer.

For the analysis of secondary messenger signaling, COS-7 cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum under 5% CO_2 at 37°C. For transfection, the cells were seeded into 24-well plates at a density of 1×10^5 cells/well the day before transfection. The media were removed the next day, and 0.25 ml of Opti-MEM (Life Technology) containing 2 μl of lipofectamine and 0.5 μg of plasmid DNA were added to each well. Five hours later, the transfection media were replaced with the culture media. The cells were further labeled with 10 μCi/ml of myo-[2–^{3}H]inositol on the following day, and the levels of inositol phosphates were determined 1 day later as previously described (Wu et al., 1992; Wu et al., 1993a, 1993b). All of the cDNAs used in these studies were constructed in expression vectors driven by the CMV promoter.

Immunofluorescence Staining

Immunofluorescence staining of transfected COS cells was performed as previously described (Schweizer et al., 1988; Linstedt and Kelly, 1991) with modifications. All steps were done at room temperature. After growing on coverslips and transfection with lipofectamine, the cells were fixed with 4% methanol-free formaldehyde in phosphate-buffered solution (PBS) for 15 min, washed three times with PBS, twice with a blocking buffer (PBS, 100 mM glycine), and permeabilized for 20 min in the blocking buffer containing 0.1% saponin. The cells were further washed twice with a washing buffer (PBS, 0.1% saponin) and incubated for 30 min in Ab-buffer (PBS, 0.1% saponin, and 1% BSA) containing either 1% normal rabbit serum (anti-p85 set) or 1% normal donkey serum (anti-p120 set). After incubation for 24 hr at 4°C with anti-p85 (1:5000) or anti-p120 (1:1000) antibodies, the coverslips with the cells were washed three times with the washing buffer and once with the Ab-buffer and incubated for 1 hr at room temperature with either donkey anti-rabbit (anti-p85 set) or rabbit anti-chicken (anti-p120 set) biotinylated secondary antibodies (1:100) in the Ab-buffer. The coverslips were again washed as above and further incubated for 1 hr with streptavidin–Texas Red (1:200) in the Ab-buffer. After three washes with the washing buffer, the coverslips were mounted in Gel/Mount-medium (Biomeda) and visualized and photographed with Zeiss Axiophot fluorescence microscope. Nonpermeabilized cells were immunostained by the same procedure in the buffers without saponin.

Transfection of Chromaffin Cells

Bovine adrenal chromaffin cells were prepared and maintained in culture as previously described (Bittner and Holz, 1992), except that the medium used was DMEM/F-12 (Dulbecco's modified Eagle's medium/Ham's F-12). Cells for transfection were grown in 12-well plates (Costar Corp., Cambridge, MA), and were transfected by calcium phosphate precipitate 14–18 hr after plating (Wilson et al., 1996). hGH was expressed with pXGH5, which is under control of the mouse metallothionein I promoter (Selden et al., 1986). Incubation with a heavy metal was not necessary to obtain adequate hGH expression. Experimental (pCDR7, 3 μg/well) and control (pCMVneo, 3 μg/well) plasmids were each mixed with pXGH5 (2 μg/well) prior to generating the precipitates. Previous work in the laboratory has demonstrated that this ensures that virtually all transfected cells express both hGH and the protein of interest (Ma et al., 1992; Wick et al., 1993; Holz et al., 1994; Chung et al., 1995; Bittner et al., 1996). hGH is stored in chromaffin granules and is released concomitantly with endogenous catecholamine by various secretagogues. Thus, hGH serves as a selective marker for secretion from transfected cells.

Neurotransmitter and Hormone Release Assays

Physiological salt solution (PSS) contained 145 mM NaCl, 5.6 mM KCl, 5.6 mM glucose, 0.5 mM ascorbate, 15 mM HEPES (pH 7.4), and 2.2 mM $CaCl_2$ and 0.5 mM $MgCl_2$ unless otherwise indicated. The potassium glutamate solution (KGENP) used in permeabilized cell experiments contained 139 mM potassium glutamate, 20 mM PIPES (pH 6.6), 2 mM MgATP, and either 5 mM EGTA and 5 mM nitrilotriacetic acid without Ca^{2+}, or 5 mM EGTA/5 mM nitrilotriacetic acid with various amounts of $CaCl_2$ to yield buffered Ca^{2+} concentrations of 1–1000 μM (Bittner and Holz, 1992). KGEP solution lacked nitrilotriacetic acid but was otherwise identical to KGENP. Human GH was measured with a luminescent assay kit from Corning Nichols Institute Diagnostics (San Juan Capistrano, CA) as described previously (Bittner et al., 1996). Endogenous catecholamines were measured by spectrofluorometric assay (Dunn and Holz, 1983). Data are expressed as mean ± standard error of the mean unless otherwise indicated. Significance was determined by Student's *t* test. Error bars smaller than symbols were omitted from figures.

Immunoprecipitation Reactions

About 3 g of rat brain were homogenized in 30 ml of 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1 mM PMSF (pH 7.9) and centrifuged at 50,000 g for 20 min. The pellet was resuspended in 30 ml of 20 mM Tris-HCl, 2 mM EDTA, 0.1 mM PMSF, and 2% Triton X-100. After 40 min incubation at 4°C, the mixture was centrifuged at 100,000 × g for 1 hr, and the supernatant was collected and supplemented with 5 M NaCl to final concentration, 130 mM. In some experiments, ^{125}I-α-latrotoxin was added to 1.4 ml samples 30 min prior to the addition of antibody. The mixtures were incubated for 2 hr at 4°C and further absorbed on Protein A- or Rabbit anti-Chicken IgG-Sepharose overnight with gentle rotation. The immunoprecipitation suspensions were pelleted, and the matrices were washed, eluted with SDS sample buffer, and analyzed by gel electrophoresis followed by Western blotting. The samples with ^{125}I-α-latrotoxin were also counted in the gamma-counter.

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GenBank Accession Number

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