A Novel Ubiquitously Expressed α-Latrotoxin Receptor Is a Member of the CIRL Family of G-protein-coupled Receptors*

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Poisoning with α-latrotoxin, a neurotoxic protein from black widow spider venom, results in a robust increase of spontaneous synaptic transmission and subsequent degeneration of affected nerve terminals. The neurotoxic action of α-latrotoxin involves extracellular binding to its high affinity receptors as a first step. One of these proteins, CIRL, is a neuronal G-protein-coupled receptor implicated in the regulation of secretion. We now demonstrate that CIRL has two close homologs with a similar domain structure and high degree of overall identity. These novel receptors, which we propose to name CIRL-2 and CIRL-3, together with CIRL (CIRL-1) belong to a recently identified subfamily of large orphan receptors with structural features typical of both G-protein-coupled receptors and cell adhesion proteins. Northern blotting experiments indicate that CIRL-2 is expressed ubiquitously with highest concentrations found in placenta, kidney, spleen, ovary, heart, and lung, whereas CIRL-3 is expressed predominantly in brain similarly to CIRL-1. It appears that CIRL-2 can also bind α-latrotoxin, although its affinity to the toxin is about 14 times less than that of CIRL-1. When overexpressed in chromaffin cells, CIRL-2 increases their sensitivity to α-latrotoxin stimulation but also inhibits Ca2+-regulated secretion. Thus, CIRL-2 is a functionally competent receptor of α-latrotoxin. Our findings suggest that although the nervous system is the primary target of low doses of α-latrotoxin, cells of other tissues are also susceptible to the toxic effects of α-latrotoxin because of the presence of CIRL-2, a low affinity receptor of the toxin.

Poisoning with black widow spider venom results in the activation of spontaneous synaptic activity in the peripheral nervous system (1). The major component of the venom that is responsible for the toxic effects in vertebrates is α-latrotoxin, a large protein with multiple ankyrin repeats (2–4). Purified α-latrotoxin causes spontaneous neurotransmitter release at neuromuscular junctions and in preparations of central neurons such as synaptosomes, brain slices, and primary neuronal cell cultures (5). It has been recently shown that the toxic effects of α-latrotoxin are not restricted to the nervous system and that it can also augment secretion from chromaffin cells and β-pancreatic cells (6–8). Spontaneous α-latrotoxin-stimulated secretion is paralleled by an increase in transmembrane cation fluxes through induced large conductance channels of unknown nature (9). It has been thus suggested that ionophoric properties of α-latrotoxin are at the basis of its toxic effects. However, at least part of the action of α-latrotoxin may be independent of cation fluxes both in neurons and secretory cells (7, 8, 10–12).

Stimulation of neurotransmitter release by α-latrotoxin correlates with its extracellular binding to high affinity membrane receptors (5, 13). The toxin-binding sites in membrane preparations were originally identified in ligand binding assays with radiolabeled toxin (14). In those experiments, the α-latrotoxin receptors were detected only in the preparations of neural tissues. By immunofluorescence with anti-α-latrotoxin antibodies, it was shown that α-latrotoxin-binding sites are localized presynaptically in neuro-muscular synapses and therefore might be directly involved in the regulation of neuronal exocytosis (15).

Two types of high affinity α-latrotoxin receptors were identified by purification and molecular cloning: neurexin Iα, a calcium-dependent receptor (16, 17), and a calcium-independent receptor, CIRL, also called latrophilin (18, 19). The interaction of neurexin Iα with α-latrotoxin significantly contributes to the effects of the toxin only in physiological high-Ca2+ media, whereas CIRL is important for the toxic effects either in physiological or in nominally Ca2+-free media (20).

CIRL is a G-protein-coupled receptor (GPCR)1 with an unusually large N-terminal extracellular region that contains characteristics of cell adhesion proteins. CIRL is an interesting example of a two-subunit GPCR (18). Its two non-covalently bound subunits (p120 and p85) result from the expression of one gene followed by endogenous proteolytic cleavage of the precursor protein in its extracellular region close to the first transmembrane segment. Similar posttranslational modification was reported for leukocyte antigen CD97, a large orphan GPCR homologous to CIRL (21).

The functional properties of CIRL as an α-latrotoxin receptor were confirmed by the analysis of secretion in transfected chromaffin cells and β-pancreatic cells (7, 8, 18). When overexpressed, CIRL renders them supersensitive to the toxin. Interestingly, overexpression of CIRL in chromaffin cells also results in the modulation of physiologically evoked secretion in

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF063102 and AF063103.

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The CIRL Family of G-protein-coupled Receptors

Fig. 1. The structure of the CIRL receptors. A, the amino acid sequences of CIRL-2 and CIRL-3 (GenBank™ accession numbers AF063102, AF063103) are shown aligned with the sequence of CIRL-1 (U72487). The alignment was produced by Clustal algorithm. B, the domain model of the CIRLs. The N-terminal domain shown after the signal peptide sequence is present only in CIRL-3. SR, the secretin receptor family; PM, plasma membrane.
the absence of any stimulation with α-latrotoxin. The ATP-dependent stage of secretion is specifically inhibited by CIRL expression, suggesting that this receptor couples to exocytosis (7).

We now show that in addition to neuronal receptors neurexin Iα and CIRL, another α-latrotoxin-binding protein exists that is a ubiquitously expressed homolog of CIRL. This membrane protein, which we named CIRL-2, binds α-latrotoxin with lower affinity than CIRL (CIRL-1). Overexpression of CIRL-2 in chromaffin cells indicates that it is a functional receptor of α-latrotoxin and that, similarly to CIRL-1, it couples to exocytosis.

Our results suggest that α-latrotoxin can produce toxic effects not only in neurons but also in other tissues.

**EXPERIMENTAL PROCEDURES**

α-Latrotoxin was purified from lyophilized black widow spider glands and radioactively labeled with 125I by chloramine T procedure. The toxin was immobilized on BrCN-Sepharose as described (16). Chromaffin cell and HEK293 cell transfections and functional analysis of CIRLs were performed as described previously (7, 18).

**Cloning and Sequencing of CIRL-2 and CIRL-3**—Molecular cloning experiments were performed according to established procedures and protocols (18, 22). Screening of a directionally cloned rat brain cDNA library in AzAP1 (kindly provided by Dr. James Boulter, Salk Institute) has resulted in the isolation of clones highly homologous to the 5′-region of CIRL-1 cDNA (18) but yet significantly different according to restriction endonuclease mapping and partial sequencing. Two sets of overlapping clones were identified encoding CIRL-2 cDNA (15–1, 15–7, 15–11, 15–12, 15–14, 15–16, 15–19, and 15–20) and CIRL-3 cDNA (17–1, 17–2, 17–9, 17–14, 17–15, 17–17, 17–20, and 17–21). All plasmids were sequenced with T3 and T7 primers. The potentially full-length clones 15–19, 15–12, 17–9, and 17–20 were sequenced on both strands using synthetic primers.

In addition, all clones were sequenced in the regions of alternative splicing, identified by restriction endonuclease mapping. **Expression Constructs**—The eukaryotic expression constructs of CIRLs were designed so that they contained minimal noncoding sequence in the 5′-region. The CIRL-2 expression plasmid pCDCIRL-2 was prepared by triple ligation of the fragment BamHI/MfeI of the clone 15–12 and the fragment MfeI/XhoI of the clone 15–19 into expression vector pCDNA3 Zeo (+) (Invitrogen), predigested with restriction endonucleases BamHI and XhoI. The CIRL-3 expression plasmid pCDCIRL-3 was prepared by ligation of the fragment NotI/XhoI of the clone 17–20 into the same expression vector, predigested with NotI/XhoI.

**Northern Blotting**—Commercially available blots (CLONTECH) containing ~2 μg of poly(A)+ RNA from human tissues were prehybridized for 24 h at 42 °C in the buffer H (50% formamide, 5× saline/sodium phosphate buffer, 5 mM EDTA, 5× Denhardt solution, 1.7% SDS, 200 μg/ml sonicated salmon sperm DNA, 2.5 mM sodium pyrophosphate) followed by hybridization in the same buffer under stringent conditions (50 °C, 16 h) with α-32PdCTP uniformly labeled probes of full-length CIRL-2 or CIRL-3 (Random Primed DNA labeling kit, Boehringer Mannheim). The unincorporated label was removed with ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech). For hybridization, the probes were diluted with buffer H to final concentrations of 5.7 × 10⁷ cpm/ml. After the hybridization, the blots were washed 3–4 times for 25 min each in 2× SSC (0.15 M NaCl and 0.015 M sodium citrate) with 1% SDS at ascending temperatures starting from 60 °C, with 2 °C increment. The blots were dried and autoradiographed with an x-ray film and intensifying screen at −70 °C for 3–7 days.

**Antibody Preparation**—A DNA fragment encoding the extracellular region of CIRL-2 was prepared by a PCR reaction on pCDR7 plasmid as a template with primers CGAGGATCCTTCAGCAGAGCAGCCTTGCCA and GTGCTCGAGGTGGCTGCATGCACACGTCGT. The 2500-base pair polymerase chain reaction product was digested with restriction endonucleases BamHI and XhoI and subcloned into predigested vectors pet22a and pET21a to yield plasmid pet22CIRL-2. The plasmid was transformed into the Escherichia coli BL21(DE3) strain, and individual colonies were isolated and propagated in 1 liter of LB media at room temperature until bacterial cultures reached mid-log phase (−8 h). At this point, isopropyl-1-thio-b-D-galactopyranoside solution was added to 50 μM final concentration. 4 h post-induction, the cells were harvested, washed, and lysed by ultrasound sonication in 50 ml of hypotonic buffer A (10 mM NaCl, 25 mM Tris-HCl, pH 8.0) with the addition of protease inhibitors mixture (Boehringer Mannheim). The lysate was centrifuged for 30 min at 40,000 rpm, and the insoluble pellet was resuspended in 50 ml of buffer B (450 mM NaCl, 8 mM urea, 25 mM Tris-HCl, pH 8.0) and centrifuged for 30 min at 40,000 rpm. The supernatant was chromatographed on 2 ml of Ni²⁺-matrix (Novagen) pre-equilibrated with the buffer B supplemented with 50 μM imidazole. The extracellular CIRL-2 fragment was eluted from buffer B containing 200 mM imidazole. The eluate was sequentially dialyzed against buffer B with gradually decreased concentration of urea from 6 M to zero. The recombinant proteins were used for custom production of antibodies in rabbits (Alpha Diagnostics).

**Expression of CIRLs in COS Cells**—Expression plasmids encoding CIRLs (pCDR7, pCDCIRL-2, and pCDCIRL-3) were transfected into COS-7 cells by LipofectAMINE procedure with Opti-MEM I serum-free medium according to the manufacturer’s protocol (Life Technologies, Inc.). The transfected cells were harvested 48–72 h after transfection. For Western blotting analysis, approximately 30% of the COS cells harvested from one 100-mm dish were solubilized with 2% Triton X-100 low salt buffer (10 mM NaCl, 20 mM Tris-HCl, pH 7.5). After a 15-min centrifugation at 80,000 × g, the supernatant was incubated with 20 μl of α-latrotoxin-agarose for 16 h with rotation. After 2 washes with phosphate-buffered saline, the beads were eluted with 50 μl of 2× SDS sample buffer. 15 μl of the eluate was analyzed by separation in 10% SDS gel electrophoresis followed by Western blotting with anti-CIRL-1 and anti-CIRL-2 antibodies.

**Purification of α-Latrotoxin Receptors from Multiple Tissues**—Approximately 5 g of each fresh rat tissue (brain, heart, lung, kidney, spleen) and 5 g of ovaries (Pel-Freez Biologicals) were homogenized (Polytron) in 25 ml of a low salt buffer (10 mM NaCl, 20 mM Tris-HCl, final pH 7.5) with protease inhibitors mixture (Boehringer Mannheim) followed by 2% Triton X-100 solubilization for 3 h, and 30 min of centrifugation at 80,000 × g. The 15-ml supernatant fractions were incubated with 50 μl of α-latrotoxin-agarose and washed twice with a high salt buffer (750 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, pH 7.5). Protein from the beads was eluted with SDS sample buffer followed by SDS electrophoresis and Western blotting with the appropriate polyclonal antibodies.

**RESULTS**

CIRL and Its Homologs Define a Novel Family of GPCRs—In the course of molecular cloning of CIRL (CIRL-1), two sets of cDNA clones were isolated that were homologous and yet significantly different from CIRL-1 cDNA (18). Significant homology of these novel sequences with CIRL cDNA was found in the region encoding the N-terminal region of CIRL. These clones...
The CIRL Family of G-protein-coupled Receptors

were characterized by mapping with restriction endonucleases and partial DNA sequencing. Several clones were identified that contained a large open reading frame with a Kozak consensus sequence and upstream stop codons in all frames in the 5'-end and a poly(A) sequence in the 3'-end, suggesting that they contained full-length cDNAs with respect to the coding sequence. These cDNA clones were sequenced completely on both strands.

The protein sequences of CIRL-2 and CIRL-3 were deduced from the cDNA sequences. Multiple alignment of three CIRL sequences revealed significant homology of these proteins (Fig. 1A). A major difference among them was that CIRL-3 contained an additional small domain in the N-terminal region right after the signal peptide sequence (Fig. 1B). The cytoplasmic C-terminal region of CIRLs was significantly less conserved in these proteins than their extracellular and membrane domains. Also, the Ser, Thr, and Pro-rich domain (STP domain) located in the center of the N-terminal extracellular region of CIRLs showed very little degree of conservation.

We analyzed the sequences of the newly cloned proteins by BLAST searches of protein data bases. The searches revealed that the CIRLs is a subfamily of the secretin receptor family of GPCRs. CIRLs have seven transmembrane hydrophobic segments that are homologous to other members of the secretin receptor family. Several large orphan GPCRs were identified by searches that are significantly homologous to CIRLs not only in their transmembrane hydrophobic segments but also in a relatively short (about 80 residues) region that is adjacent to the transmembrane core N-terminus and therefore, should be exposed extracellularly (Fig. 1B). Because this domain is involved in the endogenous proteolytic processing of CIRL-1 and possibly other receptors, we propose to name it GPS which stands for GPCR proteolytic site.2

Interestingly, the N-terminal tails of other members of the secretin receptor family, in addition to the large orphan GPCRs, are also homologous to CIRLs. However, this region of homology is separated by about 350 residues from the transmembrane core of CIRLs (Fig. 1B). The same Cys-rich motif is also found in other large orphan receptors of the secretin receptor family.

In the N termini, after the domain found only in CIRL-3, the CIRLs have domains homologous to a sea urchin lectin and to olfactomedin (Fig. 1B). The variable between homologs the STP domain is followed by a region that is homologous between the CIRL homologs and three recently discovered genes of the BAI domain is followed by a region that is homologous between the secretin receptor family, in addition to the large orphan GPCRs. CIRLs have seven transmembrane hydrophobic segments that are homologous to other members of the secretin receptor family. Several large orphan GPCRs were identified by searches that are significantly homologous to CIRLs not only in their transmembrane hydrophobic segments but also in a relatively short (about 80 residues) region that is adjacent to the transmembrane core N-terminus and therefore, should be exposed extracellularly (Fig. 1B). Because this domain is involved in the endogenous proteolytic processing of CIRL-1 and possibly other receptors, we propose to name it GPS which stands for GPCR proteolytic site.2

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Tissue Distribution of CIRLs—To further characterize CIRL-2 and CIRL-3, we analyzed their tissue distribution by Northern blotting (Fig. 2). In contrast with CIRL-1, which is predominantly expressed in brain, CIRL-2 messages were found almost in all tissues tested although in variable concentrations. The highest levels of CIRL-2 were found in placenta, heart, lung, kidney, pancreas, spleen, and ovary. Brain, liver, and testis showed intermediate amounts of CIRL-2. The lowest quantities of this mRNA were detected in skeletal muscle, whereas in thymus and peripheral blood leukocytes, no signal could be detected at this level of sensitivity. The CIRL-3 mRNA was expressed predominantly in brain with significantly lower levels in heart, placenta, pancreas, kidney, and testis. Expression of CIRLs in COS Cells—Structural similarity between CIRL-1 and its newly cloned homologs CIRL-2 and CIRL-3 suggested that the latter two may also serve as receptors of a-latrotoxin. To test this hypothesis, the expression plasmids encoding CIRL-2 and CIRL-3 were prepared on the basis of pcDNA3.1 eukaryotic vector and transfected into COS cells. The transfected cells were analyzed for their a-latrotoxin binding activity using radiolabeled a-latrotoxin. CIRL-1- and CIRL-2-expressing cells bound iodinated a-latrotoxin specifically, whereas no binding was observed in CIRL-3 and mock-transfected cells (data not shown). The binding activity of CIRL-2 transfected cells was reproducibly lower than that of the cells expressing CIRL-3. This could be explained by either lower affinity of CIRL-2 or by different expression efficiency of the receptors or by both. To discriminate between these possibilities, we analyzed a-latrotoxin binding activity of CIRL-1 and CIRL-2-expressing cells by Scatchard plots (Fig. 3). The cells that were transfected with CIRL-2 plasmid showed higher concentration of a-latrotoxin-binding sites. However, the affinity of CIRL-2 to a-latrotoxin was about 14 times lower than the affinity of CIRL-1.

The results of the binding experiments were confirmed by precipitation of solubilized transfected cells with an immobilized a-latrotoxin matrix. The adsorbed proteins were eluted from a-latrotoxin-agarose and analyzed by Western blotting with anti-CIRL-1 and anti-CIRL-2 antibodies directed against their N-terminal extracellular regions (Fig. 4). Both CIRL-1 and CIRL-2 were specifically detected by the antibodies as M, 120,000 bands. This result indicates that CIRL-2 is an a-latrotoxin-binding protein and that it is proteolytically processed in the same manner as CIRL-1. With a long exposure shown, some cross-reactivity of the antibodies could be noted. No CIRLs were detected in mock-transfected COS cells, although

V. Krasnoperov, K. Ichchenko, and A. G. Petrenko, manuscript in preparation.
Northern blotting indicated that CIRL-2 is ubiquitously expressed protein. We may thus assume that either this cell line does not contain CIRL-2 at all or the simian protein is not recognized by our antibody raised against the rat protein or that the levels of this receptor in COS cells are beyond the sensitivity of this assay. Neither anti-CIRL-1 nor anti-CIRL-2 antibody could produce any staining of CIRL-3 transfected cells.

Functional Expression of CIRL-2 in Chromaffin Cells and HEK293 Cells—The functional properties of CIRL-2 were examined in HEK293 cells and bovine chromaffin cells. When transfected HEK293 cells were exposed to 50 μM α-latrotoxin in PSS containing both Ca²⁺ and Mg²⁺, CIRL-2 supported a substantial ⁴⁵Ca uptake as did CIRL-1 (Fig. 5). A small amount of α-latrotoxin-stimulated uptake occurred in cells expressing pCMVneo and in untransfected cells (not shown), suggesting the presence of an endogenous α-latrotoxin receptor.

We have previously shown that overexpression of CIRL-1 in chromaffin cells results in at least 10-fold increase in their sensitivity to the toxin (7, 18). The ability of CIRL-2 to support α-latrotoxin-induced Ca²⁺ influx was also coupled to an increase in secretion. Chromaffin cells were cotransfected with a plasmid encoding human growth hormone (hGH) and with either a plasmid encoding the CIRL-2 or a control plasmid. Transiently expressed hGH is stored in secretory granules and serves as a marker for regulated secretion from the small population of transfected cells. Chromaffin cells expressing CIRL-2 and hGH were incubated for 4 min with various concentrations of α-latrotoxin in PSS without Ca²⁺ or Mg²⁺ and with 0.2 mM EGTA. The incubation with toxin in the absence of Ca²⁺ (during which no secretion occurs (7) was followed by an incubation with PSS containing Ca²⁺ and Mg²⁺. Expression of CIRL-2 enhanced the sensitivity of cells to α-latrotoxin, as evidenced by the shift to the left in the dose response curve for hGH secretion (Fig. 6). Thus, CIRL-2 functions as an α-latrotoxin receptor. Furthermore, as is the case with CIRL itself, CIRL-2 binds latrotoxin in the absence of Ca²⁺.

We previously demonstrated that expression of CIRL-1 inhibits Ca²⁺-dependent secretion in permeabilized chromaffin cells in the absence of α-latrotoxin (7). The data suggested that CIRL-1 regulates secretion by slowing a specific step in the ATP-dependent priming pathway. We thus determined whether CIRL-2 was similarly able to regulate secretion in permeabilized cells. Chromaffin cells transfected with or without CIRL-2 were permeabilized with 20 μM digitonin for 4 min in KGE buffer 139 mM potassium glutamate, 20 mM PIPES, pH 6.6, 2 mM MgATP, 5 mM EGTA) containing 2 mM MgATP but without Ca²⁺ followed by a 2-min stimulation with 30 μM Ca²⁺ in the continuing presence of MgATP. Expression of CIRL-2 inhibited Ca²⁺-dependent secretion by 42% (Fig. 7), which is comparable with the inhibition seen with CIRL-1.
Thus, we conclude that the CIRL-2 protein is a functional α-latrotoxin receptor with properties (e.g. Ca\(^2+\)-independence of α-latrotoxin binding, inhibition of secretion in permeabilized cells) that resemble those of CIRL-1.

**Low Affinity α-Latrotoxin Receptors in Nonneural Tissues**—Our experiments on the expression of CIRL-2 indicated that CIRL-2 is an α-latrotoxin-binding protein and that it can serve as a functional receptor of the toxin. The mRNA of CIRL-2 was detected in various tissues not belonging to the nervous system, in some of them (e.g. kidney) at significantly higher concentrations than in brain. However, the presence of α-latrotoxin receptors in other than neural or neuroendocrine tissues has never been reported. We prepared crude kidney membranes and analyzed them for binding of radiolabeled α-latrotoxin in the same manner as we did with brain membranes. No statistically significant specific binding could be detected in kidney membranes in this assay (data not shown).

To reconcile these apparently contradictory data, we tested various tissues for the presence of α-latrotoxin receptors using a more sensitive assay (Fig. 8). The tissues were homogenized and extracted with a Triton X-100-containing buffer, and the extracts were chromatographed on α-latrotoxin-agarose. The adsorbed proteins were eluted with the SDS sample buffer and analyzed by Western blotting with anti-CIRL-1 and anti-CIRL-2 antibody. The staining with anti-CIRL-1 antibody verified the presence of CIRL-1 exclusively in neural tissues. In contrast, CIRL-2 was detected in almost all tissues tested, although the relative amount of CIRL-2 in brain extracts was noticeably higher than could be predicted from the results of Northern blotting. A possible explanation would be cross-reactivity of the anti-CIRL-2 antibody with significant amounts of CIRL-1 purified from the brain along with CIRL-2.

**DISCUSSION**

The action of α-latrotoxin in neurons requires its extracellular binding to high affinity membrane receptors of two structurally and functionally different types. Type I or calcium-dependent receptors are represented by neurexin Iα and,
possibly, other neurexinas that are neuron-specific cell membrane proteins with one transmembrane domain and the extracellular region typical for cell adhesion molecules. Neurexin Iα is responsible for part of α-latrotoxin effects in physiological Ca^{2+}-containing media. Type II receptors can bind α-latrotoxin independently of Ca^{2+} presence in the extracellular media. CIRL, a recently discovered representative of the second class of α-latrotoxin receptors, is a G-protein-coupled receptor with unusual two-subunit structure. We now demonstrate that CIRL (CIRL-1) belongs to a novel family of large orphan GPCRs that is encoded by at least three different genes. One of these proteins, CIRL-2, is a low affinity calcium-independent receptor of α-latrotoxin. Our current data suggest that the toxic effects of α-latrotoxin have an even more complex mechanism than was thought earlier because, in addition to two high affinity neuronal receptors, a third, low affinity receptor exists that is expressed ubiquitously.

Several independent experiments indicate that CIRL-2 is an α-latrotoxin receptor. When expressed in COS cells, it binds radiolabeled α-latrotoxin with affinity in the nM range, which is approximately 14 times lower than the affinity of CIRL-1. In HEK293 cells with overexpressed CIRL-2, α-latrotoxin stimulates robust Ca^{2+} fluxes, an effect that follows the interaction of the toxin with its receptors. Finally, in a chromaffin cell system that allows analysis of secretion quantitatively, CIRL-2 overexpression results in a significant increase in their sensitivity to α-latrotoxin. This effect is less pronounced than in the cells with overexpressed CIRL-1 (7), which correlates with a lower affinity of CIRL-2 to the toxin.

Unexpectedly, Northern blotting experiments revealed that, in contrast to CIRL-1 and CIRL-3, CIRL-2 is a ubiquitously expressed protein. α-Latrotoxin has been thought to be a specific presynaptic neurotoxin because the primary site of its physiologic toxicity is the neuromuscular junctions (5).

A highly sensitive assay was developed to detect CIRL-2 in tissues. We raised anti-CIRL-2 antibody, which was directed against the extracellular domain of the receptor. A similar antibody against CIRL-1 showed very little cross-reactivity with CIRL-2 and vice versa. Using these antibodies, we were able to detect CIRL-1 and CIRL-2 in tissue extracts enriched by a chromatography on immobilized α-latrotoxin (Fig. 8). In close agreement with the data from Northern blotting, CIRL-2 was detected in brain, heart, lung, kidney, and spleen tissues, whereas CIRL-1 could be found only in brain. CIRL-2, detected in this experiment, must represent an α-latrotoxin-binding protein, because it was precipitated with α-latrotoxin-agarose. We may therefore conclude that α-latrotoxin receptors are present in nonneural tissues. CIRL-2 concentrations in various tissues are significantly lower than the concentration of CIRL-1 in brain, which hampered their direct detection by the 125I-latrotoxin binding assay. Another possible explanation would be that CIRL-2 in nonneuronal cells is not transported to the plasma membrane. However, this seems unlikely because when CIRL-2 is overexpressed in nonneuronal COS cells, α-latrotoxin receptors can be reliably detected on the cell surface.

What might be the consequences of α-latrotoxin interaction with CIRL-2 in nonneural tissues? α-Latrotoxin treatment in neurons produces two major effects, spontaneous neurotransmitter release and degeneration of nerve terminals accompanied by general cytotoxicity. In nonneuronal cells, secretory granules, if present, can undergo exocytosis in response to α-latrotoxin. This has been demonstrated for chromaffin and β-pancreatic cells and may be also true for secretory cells of other types.

α-Latrotoxin also induces formation of cation-selective pores of high conductance after the binding to its receptors. The fact that these pores are permeable to Ca^{2+} may explain cytotoxic effects as well as the secretagogue function of the toxin. In CIRL-1-transfected HEK293 cells, robust Ca^{2+} transmembrane fluxes were observed (7). A similar effect was noted in nontransfected cells, however it was weaker and required higher concentrations of the toxin (25). One of the explanations of this effect is that α-latrotoxin interacts with endogenous CIRL-2. If α-latrotoxin could induce similar cation channels in various nonneuronal cells, they would cause cellular toxicity as a result of Ca^{2+} entry.

CIRL-1, CIRL-2, and CIRL-3 define a novel family of GPCRs. The homology of their protein sequence is quite strong, and their domain structure is almost identical. At least two of them, CIRL-1 and CIRL-2, are α-latrotoxin-binding proteins. In our experiments, no α-latrotoxin binding of CIRL-3 could be detected. However, because the expression of CIRL-3 could not be verified with available antibodies, we cannot make any definite conclusion about α-latrotoxin binding properties of CIRL-3 at this time.

Homology searches indicate that CIRLs are part of a novel growing family of large orphan GPCRs with unusual structural features. These receptors include leukocyte antigen CD97, EMR1 hormone receptor (F4/80), the BAI family, an orphan receptor from brain similar to Drosophila melanogaster cadherin-related tumor suppressor, MEGF2 protein, and two putative GPCRs identified by sequencing the Caenorhabditis elegans genome (GenBank™ accession numbers Z54306 and U39848). The members of this family share highest homology in their transmembrane domains and in a short adjacent extracellular Cys-rich GPS domain. CIRL-1, CIRL-2, and CD97 are proteolytically processed endogenously (18, 21), and the integrity of the GPS domain is required for the proteolytic processing of CIRL-1.2 We may therefore hypothesize that all orphan GPCRs with the GPS motif are endogenously cleaved and, thus, consist of two heterologous subunits similarly to CIRL-1.

Another common structural feature of this family of large orphan GPCRs is that the extracellular regions of these receptors contain structural modules typical for cell adhesion or extracellular matrix proteins. These modules include EGF motifs, thrombospondin repeats, STP or mucin-like domains, and lectin- and fibronectin-related domains. These "chimeric" receptors may therefore have a dual function as signaling receptors and cell adhesion proteins. It is also possible that intercellular contacts may serve as agonists to activate these receptors.

α-Latrotoxin produces strong intracellular response as a result of its interaction with CIRL-1 and CIRL-2. However, it remains unclear whether α-latrotoxin can work as an agonist or antagonist of CIRL and produce any receptor-mediated signaling. Our recent experiments with C-terminal deletion mutants of CIRL-1 suggest that G-protein-signaling is not critically important for α-latrotoxin-stimulated secretion in chromaffin cells (25). The endogenous ligands of CIRLs are not known yet. Chromaffin cell transfection experiments suggest that CIRLs may act as physiological receptors and regulators of secretion because overexpression of CIRL-1 and CIRL-2 results in the inhibition of calcium-evoked secretion (Ref. 7 and Fig. 7). Identification of endogenous ligands and intracellular effectors of CIRLs in the future experiments will be a next important step to better understand the physiological importance of the CIRLs.

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