α-Latrotoxin Receptor CIRL/Latrophilin 1 (CL1) Defines an Unusual Family of Ubiquitous G-protein-linked Receptors

G-PROTEIN COUPLING NOT REQUIRED FOR TRIGGERING EXOCYTOSIS

(Received for publication, July 13, 1998, and in revised form, September 2, 1998)

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α-Latrotoxin, a potent excitatory neurotoxin, binds to two receptors: a G-protein-coupled receptor called CIRL/latrophilin 1 (CL1) and a cell-surface protein called neurexin Iα. We now show that CL1 belongs to a family of closely related receptors called CL1, CL2, and CL3. CLs exhibit an unusual multidomain structure with similar alternative splicing and large extra- and intracellular sequences. CLs share domains with other G-protein-coupled receptors, lectins, and olfactomedins/myocilin. In addition, CLs contain a novel, widespread cysteine-rich domain that may direct endoproteolytic processing of CLs during transport to the cell surface. Although the mRNAs for CLs are enriched in brain, CLs are ubiquitously expressed in all tissues. To examine how binding of α-latrotoxin to CL1 triggers exocytosis, we used PC12 cells transfected with human growth hormone. Ca2+-dependent secretion of human growth hormone from transfected PC12 cells was triggered by KCl depolarization or α-latrotoxin and was inhibited by tetanus toxin and by phenylarsine oxide, a phosphoinositide kinase inhibitor. When CL1 was transfected into PC12 cells, their response to α-latrotoxin was sensitized dramatically. A similar sensitization to α-latrotoxin was observed with different splice variants of CL1, whereas CL2 and CL3 were inactive in this assay. A truncated form of CL1 that contains only a single transmembrane region and presumably is unable to mediate G-protein-signaling was as active as wild type CL1 in α-latrotoxin-triggered exocytosis. Our data show that CL1, CL2, and CL3 perform a general and ubiquitous function as G-protein-coupled receptors in cellular signaling. In addition, CL1 serves a specialized role as an α-latrotoxin receptor that does not require G-protein-signaling for triggering exocytosis. This suggests that as an α-latrotoxin receptor, CL1 recruits α-latrotoxin to target membranes without participating in exocytosis directly.

α-Latrotoxin is a component of black widow spider venom that triggers exocytosis from neurons and neuroendocrine cells (reviewed in Ref. 1). α-Latrotoxin is thought to act by binding to specific cell surface receptors (2). Two hypotheses have been advanced to explain how α-latrotoxin induces exocytosis. 1) α-Latrotoxin activates exocytosis by a Ca2+-independent mechanism that involves the secretory apparatus directly (3). 2) α-Latrotoxin acts indirectly in exocytosis as a Ca2+-ionophore, allowing Ca2+ influx into the cell (4). The two proposed mechanisms are not mutually exclusive. Each mechanism is supported by a large amount of data, suggesting that both mechanisms are being used (5–11). In nerve terminals, α-latrotoxin induces synaptic vesicle exocytosis equally well with or without Ca2+ (3, 5). In neuroendocrine cells such as chromaffin cells and PC12 cells, α-latrotoxin stimulates exocytosis only in the presence of Ca2+ (11–14). These data suggest that in neuroendocrine cells, α-latrotoxin primarily uses the second mechanism of action and stimulates exocytosis by Ca2+ influx. Although a direct receptor-based α-latrotoxin action in chromaffin cells has also been proposed (14), the inability of neuroendocrine cells to respond to α-latrotoxin in the absence of Ca2+ suggests that these cells lack synaptic components required for a Ca2+-independent mechanism.

Two receptors for α-latrotoxin have been cloned. A neuron-specific cell surface protein called neurexin Iα was initially characterized as an α-latrotoxin receptor (15). More recently, a G-protein-linked receptor for α-latrotoxin was independently identified by two laboratories and named CIRL or latrophilin 1 (abbreviated here as CL1) (16, 17). Neurexin Iα and CL1 bind α-latrotoxin with similarly high affinities but exhibit strikingly different properties. Neurexin Iα binds α-latrotoxin only in the presence of Ca2+, suggesting that α-latrotoxin does not mediate the Ca2+-independent effect of α-latrotoxin (18). CL1, in contrast, binds α-latrotoxin Ca2+-independently. This led to the proposal that CL1 may be responsible for most or all of the actions of α-latrotoxin (16, 17). Experiments with knockout mice revealed that in the absence of neurexin Iα, the Ca2+-dependent activities of α-latrotoxin were impaired, whereas Ca2+-independent release triggered by α-latrotoxin was unaffected (19). Thus neurexin Iα functions either as a genuine α-latrotoxin receptor or as an essential Ca2+-dependent coreceptor for α-latrotoxin.

The identification of CL1 as a G-protein-coupled receptor for α-latrotoxin raised the exciting possibility that CL1 constitutes a novel synapse-specific G-protein-linked receptor with a function related to exocytosis. This possibility was supported by the apparent brain-specific expression of CL1 (16, 17) and by the observation that transfection of CL1 into chromaffin cells sensitized these cells to α-latrotoxin action (14, 16). A synapse-specific G-protein-coupled receptor that directly modulates synaptic vesicle exocytosis would have major implications for.
without mediating its ability to trigger exocytosis. Recently we showed that recombinant α-latrotoxin was fully active in triggering neurotransmitter release. Mutant α-latrotoxin with substitutions in conserved cysteine residues or with an insertion of four amino acids lacked this activity. Surprisingly, the mutant toxin with a four-amino acid insertion still bound to CL1 and to neurexin I with the same affinity as wild type toxin and stimulated phospholipase C similar to wild type toxin. This result was confirmed in experiments with $\text{La}^{3+}$. $\text{La}^{3+}$ blocked the ability of α-latrotoxin to induce exocytosis without inhibiting its ability to stimulate phospholipase C.

These data suggest that binding of α-latrotoxin to its receptors and stimulation of phospholipase C are not sufficient for α-latrotoxin to induce exocytosis. However, these findings did not reveal if CL1 is a novel type of G-protein-linked receptor with a synapse-specific function. We have addressed this question in the current study. Our data define CL1 as a member of a novel gene family of related G-protein-linked receptors of unusual size and structure. We have investigated the mechanism by which α-latrotoxin acts in PC12 cells and demonstrate that CL1 acts in a manner that does not require coupling to G-proteins. Our findings support a model whereby α-latrotoxin evolved to bind to multiple receptors that primarily serve to recruit the toxin to its site of action without mediating its ability to trigger exocytosis.

**EXPERIMENTAL PROCEDURES**

Cloning of CL1, CL2, and CL3—Data bank searches identified multiple human and mouse EST clones encoding homologs of CL1. We used restriction enzyme fragments from three human EST clones (CL2, 0.8-kb EcoRI-XhoI fragment from clone 71509, GenBank accession number T47920; CL3, 0.5-kb EcoRI-HindIII fragment from clone 33529, accession number R19057, and 0.37-kb EcoRI-XhoI fragment from clone 285779, accession number N69329) and a PCR product of the 5′ end of the CL1 cDNA to isolate cDNA clones for CL1, CL2, and CL3 from a rat brain cDNA library as described (20, 21). Nine independent CL1 clones were isolated and sequenced. The largest clone, pBSCL1OR4, lacked the first 23 amino acids of CL1 (16, 17). Of four clones extending to the N-terminal site of alternative splicing (residues 132–136, see Fig. 1), two clones (pBSCL1OR4, CL1OR12) lacked and two clones (pBSCL1OR10, CL1OR11) contained an insert. For the alternatively spliced cytoplasmic sequence of CL1 (residues 1146–1189, see Fig. 1), two cDNA clones (pBSCL1OR6 and CL1OR9) contained and two clones (pBSCL1OR1 and CL1OR4) lacked an insert. Of five independent CL2 cDNA clones, pBSCL2–2 contained the entire coding region. The sequence of this clone revealed a possible mutation between residues 1146 and 1189, suggesting that CL1 is a novel type of G-protein-linked receptor with a synapse-specific function. We have addressed this question in the current study. Our data define CL1 as a member of a novel gene family of related G-protein-linked receptors of unusual size and structure. We have investigated the mechanism by which α-latrotoxin acts in PC12 cells and demonstrate that CL1 acts in a manner that does not require coupling to G-proteins. Our findings support a model whereby α-latrotoxin evolved to bind to multiple receptors that primarily serve to recruit the toxin to its site of action without mediating its ability to trigger exocytosis.

**Construction of Expression Vectors and Transfection Experiments**—The initial CL1 expression vector (pCMVCL1–1) was constructed in the EcoRI-HindIII sites of pCMV5 by fusing a 5.2-kb Nehl-HindIII fragment of pBSCL1OR10 to a 0.2-kb EcoRI-NheI PCR fragment from the 5′ end of the mRNA. pCMV1–1 lacks inserted inserts in both of the alternatively spliced sites and was used to derive pCMVCL1–4, containing an insert in the C-terminal site, and pCMVCL1–5, containing an insert in the N-terminal site of alternative splicing. pCMVCL1–4 was generated by replacing the C-terminal BamHI-XhoI fragment in pCMV1–1 with the 4.1-kb BamHI-AvrII fragment of pBSCL1OR9. pCMVCL1–5 was obtained exchanging the N-terminal BglII-BamHI fragment of pCMVCL1–1 with the 0.3-kb BglII-BamHI fragment of pBSCL1OR10 and shortening the 3′-untranslated region to the AvrII site. The truncated CL1 expression plasmid with a stop codon after the first TMR (pCMVCL1–1STOP997) was constructed in pCMV5 by PCR. The CL2 expression vector pCMVCL2–1 was made by fusing a 4.2-kb BglII-AvrII fragment of pBSCL2–2 to a 0.2-kb BSSCI–PCR fragment from the 5′ end of the mRNA. pCMVCL1–2 was inserted at the BglII-XhoI site of pCMV5. When it became apparent that the 5′ end of pBSCL2–2 carries a mutation, we replaced it with a 0.25-kb BglII-MfeI PCR fragment to create pCMVCL2–3. Furthermore, we generated a hybrid CL1/CL2 expression vector (pCMVCL2–4) in which a 0.2-kb EcoRI-BglII fragment of pCMVCL1–1 was exchanged for the corresponding fragment in pCMVCL2–3. The resultant construct contained N-terminal 70 residues of CL1 followed by the 1407-residue of CL2. For CL2, we obtained a chimeric construct (pCMVCL3/CL1) with the N-terminal 1026 residues of CL2 followed by C-terminal 530 residues of CL1–1 (see Fig. 1). For expression of human growth hormone (hGH) in PC12 cells, a 2.8-kb BamHI-EcoRI fragment encoding hGH was subcloned into the blunt AvrII site of the pCMV5 vector to produce pHGMV5 in which hGH expression is driven by the SV40 promoter. To test the effect of tetanus toxin light chain on hGH secretion, the coding region of tetanus toxin light chain was subcloned into the polylinker of pHGMV5 to create pHGMC-VeToTx (22). To examine the effects of CL expression on hGH secretion triggered by KCl or α-latrotoxin, pHGMV5 was co-transfected with pCMVCLs. As control, pHGMV3 was co-transfected with the empty pCMV5 vector. Expression of the different constructs of CL4 were confirmed by COS cell transfections (20). COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum under 5% CO2 at 37 °C and transfected using DEAE-dextran with chloroquin and a 2-mglycerol shock with 6.6 μg of DNA for 90,000 cells in a 10-cm dish. Cells were washed with PBS h 24 h after transfections and harvested in 0.4 ml of sample buffer. Samples were processed through a 25-gauge needle (10 times). Aliquots (5–20 μl) were analyzed by standard SDS-polyacrylamide gel electrophoresis and immunoblotting using ECL detection.
and retained in the cells were measured by radioimmunoassay (Nichols Institute, CA).

Miscellaneous Procedures—RNA blotting experiments were carried out as described (20) using commercially available human multitissue RNA blots (CLONTECH). SDS-polyacrylamide gel electrophoresis and immunoblotting analyses were performed as described (20–22). The antibodies used were reported previously except for the polyclonal CL1 antibodies, which were raised against an N-terminal 17-residue peptide (LSRAGLPGFLMRRELAC) (antibody U552) and a C-terminal 9-residue peptide (GQMQLVTSIL) (U554) coupled to keyhole limpet hemocyanin as described (20, 22).

RESULTS

Molecular Cloning of CL1, CL2, and CL3—We used the BLAST programs to search the EST data banks for potential homologs of CL1. Multiple candidate EST clones were identified and employed in cDNA library screens to isolate full-length rat clones. In this manner, we obtained cDNA clones encoding three different CLs: CL1 that was described previously and two new homologs that we named CL2 and CL3. We determined the nucleotide sequences of overlapping cDNA clones for all three CLs and aligned their deduced amino acid sequences for maximal homology (Fig. 1). The following findings suggest that our sequences are full-length with respect to the coding region. 1) All three sequences contain an N-terminal sequence resembling a signal peptide; 2) the putative starting methionine codon conforms well to the consensus of initiator methionines; and 3) the previously determined N-terminal sequence of CL1 (16, 17) aligns well with corresponding sequences in CL2 and CL3.

The three CL isoforms are closely related proteins of approximately 165,000–175,000 Daltons that are homologous to each other over their entire lengths. Hydropathy plots suggest that all three proteins are serpentine receptors with seven transmembrane domains or TMRs. There are several blocks of highly homologous regions: 1) a signal peptide; 2) the putative starting methionine codon conforms well to the consensus of initiator methionines; and 3) the previously determined N-terminal sequence of CL1 (16, 17) aligns well with corresponding sequences in CL2 and CL3.

Among the extracellular domains and the transmembrane regions exhibit the highest degree of identity, whereas the intracellular sequences are more variable. Data bank searches identified human and mouse ESTs encoding the CLs whose sequences are highly homologous to the rat sequences, suggesting that all three isoforms are evolutionarily conserved (data not shown; see also Fig. 2).

Comparisons between the sequences show that they are similarly related to each other, with an overall sequence identity of 54–63%. The extracellular domains and the transmembrane regions exhibit the highest degree of identity, whereas the intracellular sequences are more variable. Data bank searches identified human and mouse ESTs encoding the CLs whose sequences are highly homologous to the rat sequences, suggesting that all three isoforms are evolutionarily conserved (data not shown; see also Fig. 2).

Alternative Splicing of CL1, CL2, and CL3—Comparisons between the sequences of multiple cDNA clones for CL1, CL2, and CL3 revealed several variations between clones (underlined in Fig. 1). These variations were probably caused by alternative splicing because they were present in independent cDNA clones and/or EST sequences. It is possible that the alternatively spliced long sequences at the C terminus of CL1 and at the N terminus of CL3 represent unspliced introns. However, this is improbable because these inserts were observed in multiple independent cDNA clones in the context of full-length sequences with open reading frames. Several of the alternatively spliced sequences include conserved regions. In two cases, different CLs appear to be alternatively spliced at the same position.

CL1 and CL3 are alternatively spliced in the N-terminal extracellular domains, whereas CL2 is not. In CL1, a short sequence was either present or absent in the N terminus (KVEQK; residues 131–135). The insert-plus variant was reported in the CL1 sequence by Krasnapo0000 (16), and the insert-minus variant was reported by Lelianova et al. (17). Although the alternatively spliced sequence is conserved among CLs, it does not appear to be alternatively spliced in CL2 or CL3. The CL3 sequence contains a large insert (residues 19–86 of CL3) at the N terminus close to the site of signal peptide cleavage. This alternatively spliced sequence is proline- and glycine-rich but otherwise unremarkable.

The intracellular sequences of all three CLs are subject to alternative splicing. CL1 contains a long variable sequence at residues 1146–1190 that is not present in CL2 or CL3. In CL2, the third intracellular loop that connects the fifth and sixth TMR includes an alternatively spliced region (residues 1138 to 1153). Although we isolated no CL1 or CL3 cDNA clones with a similar variation, data bank searches revealed that human EST clones encoding CL3 exhibit similar alternative splicing. It is possible that alternative splicing of the third cytoplasmic loop is a general phenomenon of CLs. This could be functionally important because of the critical role of this cytoplasmic loop for G-protein coupling in other G-protein-linked receptors (23–25).

In addition to the third cytoplasmic loop, CL2 and CL3 are also alternatively spliced at the same site in a relatively well conserved region of the cytoplasmic tail (residue 1185 to 1227 in CL2; Figs. 1 and 2). At this position CL3 contains two alternatively spliced regions referred to as A and B. Region A (residues 1271 to 1313 in Fig. 1) represents an in-frame sequence that precisely corresponds to the alternatively spliced sequence in CL2. Region B, however, results in a frameshift with a stop codon in the cytoplasmic tail of CL3 (Fig. 2). This out-of-frame alternative splicing of CL3 was observed in multiple independent cDNA clones and is also found in human ESTs with 100% sequence conservation, indicating that it is not a cloning artifact. Thus CL3 can be expressed in two major forms that differ in the size of the cytoplasmic tail. The fact that CL2 also exhibits alternative splicing of region A raises the possibility that region B may be alternatively spliced in other CLs as well and that all CLs may be expressed with short and long cytoplasmic tails.

Domain Structures of CL1, 2, and 3—CL1, CL2, and CL3 are multidomain proteins with interesting patterns of homology (Fig. 3). As G-protein-linked receptor, CLs are unusually large; few G-protein-coupled receptors are larger (e.g. the orphan receptors called BAI 1, 2, and 3 (26, 27)). The central part of CLs is occupied by seven TMRs and their connecting loops. The TMRs of CLs are homologous to TMRs of other serpentine receptors, in particular those of the secretin family of peptide hormone receptors (28). On the N-terminal extracellular side, the TMRs are preceded by at least six domains: 1) a signal peptide; 2) a short cysteine-rich sequence (~92 residues) distantly related to lectins (29, 30) and long domain (~280 amino acids) that exhibits homology only to BAI 1, 2, and 3. BAI 1, 2, and 3 are large serpentine receptors of unknown function that were characterized as potential inhibitors of brain angiogenesis (26, 27). 6) a short (90 residues) cysteine-rich domain that is located immediately N-terminal of the first TMR and represents a novel type of widely distributed extracellular protein module (see below).

The combination of extracellular domains in CLs creates a mosaic of modules exposed on the cell surface. In contrast, the cytoplasmic sequences of CLs exhibit no similarities to known proteins in the current data banks. The intracellular tails of the CLs contain almost 400 residues and are also unusually long for G-protein-coupled receptors; even the shorter cytoplasmic tails generated by alternative splicing in CL3 and possibly other CLs (Fig. 2) measure nearly 150 amino acids. The cytoplasmic sequences are less conserved than the extracellular domains or TMRs. There are several blocks of highly homolo-
FIG. 1. Sequence alignments of CL1, 2, and 3. The amino acids sequences for rat CL1, 2, and 3 as deduced from cDNA sequences are aligned for maximal homology. Residues that are identical in at least two sequences are shown on a colored background. Domains are coded by different colors: dark yellow, signal peptide (residues 1–24 in CL1); red, lectin-like domain; magenta, olfactomedin/myocilin-like; light gray, linker sequences; dark blue, domain shared with BAI1, 2, and 3; light blue, cysteine-rich domain before transmembrane regions; green, transmembrane regions; yellow, intra- and extracellular loops connecting transmembrane regions; dark gray, cytoplasmic sequence. Cysteine residues in extracellular sequences are shown on a black background. Alternatively spliced sequences are shown underlined in red. The absence of the alternatively spliced sequence at residue 123 in CL1 results in an isoleucine residue instead of the KVEQK sequence encoded by the insert. In CL3, no alternatively spliced sequence was identified in the third intracellular region in the rat cDNA clones sequences, but such an alternatively spliced sequence/structure/function relation of CL1 α-Latrotoxin receptor.
gous sequences, especially close to the membrane (Fig. 1). At the beginning of the cytoplasmic tail, two vicinal cysteine residues are present in all CLs (residues 1116, 1117 in CL1, Fig. 1). Similar vicinal cysteines are palmitoylated in other G-protein-linked receptors, indicating that these cysteines may also be modified (24, 25). Strikingly, the C terminus of all CLs is composed of the sequence LVTSL. This sequence represents a perfect recognition motif for PDZ domains of the PSD-95 type (34, 35). In addition, the cytoplasmic tails contain several conserved phosphorylation consensus sequences, most notably a perfect site for CaM kinase II and protein kinase A in the middle of the tail (RKQS/T; residues 1201–1204 of CL1).

As G-protein-linked receptors, CL1, CL2, and CL3 are most homologous to BAI1, BAI2, and BAI3 with which they share two extracellular domains (the BAI homology region and the cysteine-rich domain) and the TMRs; next, they are most similar to CD97, Emr1 (F4/80), and related receptors that also contain the cysteine-rich sequence, and finally they are similar to the secretin family of G-protein-linked receptors in their TMRs (Fig. 3). The overall structure of CLs suggests that they function to couple cell adhesion via the olfactomedin- and lecin-like domains to cell signaling. A similar function has been proposed for serpentine receptors containing epidermal growth factor-like repeats (36). It seems likely that CLs will have two regions of alternatively spliced DNA sequences were observed in a tandem arrangement. Region A is in-frame (yellow letters on black background) and is followed by region B, which disrupts the reading frame (white letters on black background). Region A corresponds to residues 1271–1313 in Fig. 1 and is also alternatively spliced in CL2; region B is not shown in Fig. 1. Rat cDNA clones lacking regions A and B or containing region B with and without region A were sequenced. Human EST clones containing or lacking both regions were identified in the data banks (e.g. accession numbers R60519 and T78230) and exhibit almost identical sequences. It is possible that all CLs are alternatively spliced with region A and B inserts, leading to shorter (ProA) and longer (ProB) protein variants.
endogenous ligands with dual functions in activating signal transduction cascades and mediating cell adhesion.

A Novel Widely Distributed Cysteine-rich Domain in CLs—CLs contain a cysteine-rich domain immediately before the TMRs (Fig. 1). Data bank searches revealed that this domain constitutes a novel extracellular module that is also found in a number of other G-protein-linked receptors and other proteins (Fig. 4). In G-protein-coupled receptors, this domain is located immediately N-terminal to the TMRs (37–40). The cysteine-rich domain is of particular interest because CL1 may be physiologically cleaved at the end of this domain during transport to the cell surface (16). When we transfected CL1 into COS cells, we found it to be efficiently cleaved similar to brain protein, indicating that COS cells have the appropriate proteases for cleavage. Furthermore, CL2 and CL3 also appear to be cleaved, suggesting a general process (data not shown). It is striking that the amino acids surrounding the putative cleavage site are the most highly conserved residues in the domain but are not composed of basic residues, which usually represent a signature for intracellular cleavage events. These results raise the possibility that all proteins containing the cysteine-rich domain may be cleaved at this position. The cysteine-rich domain may serve as a signal for an unknown processing protease in the secretory pathway.

Tissue Distributions of CL1, CL2, and CL3—To determine which tissues express the different CLs, we performed RNA blotting analyses (Fig. 5). In agreement with earlier studies (16, 17), two mRNAs species (~9.0 and 6.5 kb) were observed for CL1. Both mRNAs were highly enriched in brain. The size difference between the two mRNAs is too large to be caused by the alternative splicing events described above. The two CL1 mRNAs may have arisen by alternative usage of polyadenylation sequences. Long exposures revealed significant mRNA levels for CL1 outside of brain in virtually all tissues tested (Fig. 5). This result shows that different from neurexins (15), CL1 is not neuron-specific. The relative levels of the two CL1 mRNAs did not vary between tissues. PCR confirmed that CL1 mRNA was expressed in fibroblasts such as COS cells (data not shown). The failure to observe CL1 mRNA outside of brain in previous studies (16, 17) was probably because of the short exposure times used.

CL2 mRNA is expressed in a dramatically different pattern than CL1 mRNA. CL2 mRNA was ubiquitously present in all tissues with little variation in levels between tissues. The highest amounts of CL2 mRNA were observed in placenta and lung, and the lowest were observed in brain and liver (Fig. 5). Again, two mRNAs were detected that were equally present in all tissues. In contrast to CL2, the expression of CL3 resembled that of CL1. CL3 mRNA was only observed in brain. Even after prolonged exposures, we detected no CL3 mRNA in peripheral tissues, indicating that CL3 is the most brain-specific CL isoform (Fig. 5).

\(\alpha\)-Latrotoxin Triggers Vesicular Exocytosis in PC12 Cells—PC12 cells and chromaffin cells transfected with hGH are used as a standard model system to study regulated exocytosis (14, 41). hGH is co-transfected with a second plasmid and serves as a reporter for exocytosis. In this manner, only exocytosis from transfected cells is monitored. We recently produced recombinant \(\alpha\)-latrotoxin that is fully active in triggering transmitter release from nerve terminals and have shown that mutations in the recombinant toxin abolish this activity. To test if recombinant \(\alpha\)-latrotoxin also induces secretion in PC12 cells, we transfected PC12 cells with hGH and measured hGH secretion as a function of \(\alpha\)-latrotoxin effectiveness at high toxin concentrations reproducible in triggering transmitter release from nerve terminals and have shown that mutations in the recombinant toxin abolish this activity. To test if recombinant \(\alpha\)-latrotoxin also induces secretion in PC12 cells, we transfected PC12 cells with hGH and measured hGH secretion as a function of \(\alpha\)-latrotoxin in the presence of Ca\(^{2+}\) or Mg\(^{2+}\). (Fig. 6A). Similar to results reported by others (12, 13), \(\alpha\)-latrotoxin also triggers hGH secretion from PC12 cells in a Ca\(^{2+}\)-dependent fashion. \(\alpha\)-Latrotoxin induced hGH release from PC12 cells starting at low nanomolar concentrations with a bell-shaped concentration dependence (Fig. 6B). The decrease in \(\alpha\)-latrotoxin effectiveness at high toxin concentrations reproduces a recent observation in chromaffin cells by Bittner et al. (14). As a further control for specificity, we also applied a mutant \(\alpha\)-latrotoxin carrying a substitution in a conserved cysteine. This mutant is unable to trigger neurotransmitter release from nerve terminals. Mutant \(\alpha\)-latrotoxin was also unable to stimulate hGH release from PC12 cells, demonstrating that the stimulation of secretion by \(\alpha\)-latrotoxin is not because of a nonspecific lytic effect (Fig. 6B).

\(\alpha\)-Latrotoxin is thought to stimulate vesicular exocytosis that is inhibited by tetanus toxin, which proteolyses the vesicular fusion proteins synaptobrevin and cellubrevin (32, 42, 43). Therefore we investigated if tetanus toxin interferes with \(\alpha\)-latrotoxin...
tetroxin action. For this purpose we co-transfected tetanus
toxin light chain with hGH into PC12 cells and stimulated hGH
release with KCl or α-latrotoxin (Fig. 7C). Compared with
controls, tetanus toxin significantly inhibited hGH release in-
duced by either KCl or α-latrotoxin, indicating that both stim-
ulate vesicular exocytosis.

Recent studies have shown that phosphatidylinositol phos-
phorylation is essential for Ca2+-dependent exocytosis from
PC12 cells (44, 45). Phosphoinositide kinases can be efficiently
inhibited by phenylarsine oxide (PAO), a membrane-permeable
compound that reacts with vicinal sulfhydryl groups. This in-
hibition blocks exocytosis of norepinephrine in chromaffin cells
and in nerve terminals (46–48). To further investigate whether
KCl depolarization and α-latrotoxin stimulate exocytosis in
PC12 cells by a similar mechanism, we studied the effect of
PAO on hGH secretion from transfected PC12 cells (Fig. 7).
Both KCl- and α-latrotoxin-induced hGH secretion from PC12
cells was severely inhibited by PAO. The addition of Me2SO,
the vehicle in which PAO was applied, had no effect. Similarly,
we tested the effect of peroxyvanadate (VOOH), a phos-
phatidylserine phosphatase inhibitor, because PAO was also re-
ported to inhibit phosphatidylserine phosphatases. Peroxyvanadate
also caused no change in hGH secretion (Fig. 7). These data docu-
ment that α-latrotoxin causes vesicular exocytosis in PC12 cells
only in the presence of Ca2++ in a fashion that is sensitive to
cleavage of synaptobrevin and/or cellubrevin by tetanus toxin
and to inhibition of phosphatidylinositol kinases by PAO.

**CL1 but Not CL2 and CL3 Sensitizes PC12 Cells to α-Latro-
toxin**—We next investigated if transfection of CL1 changes the
response of PC12 cells to α-latrotoxin. Previous studies showed
that transfection of CL1 shifts the α-latrotoxin dose-response
curve in chromaffin cells to the left, making the cells sensitive
to low α-latrotoxin concentrations (14, 16). In addition, these
studies revealed that such transfections are unable to confer
onto the chromaffin cells a Ca2+-independent response and
that they lower the overall secretory response. Our results
confirm these conclusions in PC12 cells (Fig. 8A). After CL1
transfection, PC12 cells became sensitive to picomolar concen-
trations of α-latrotoxin (Fig. 8A). In addition, the overall secre-
tory response to α-latrotoxin was diminished. Most impor-
tantly, CL1-transfected PC12 cells were still unable to respond
to α-latrotoxin in the absence of Ca2++. This suggests that even
in this para-neuronal cell line, CL1 is insufficient to confer a
synaptic phenotype onto the α-latrotoxin response.

We showed above that alternative splicing generates several
variants of CL1, with one alternatively spliced sequence in the
extracellular domain and one in the intracellular tail (Fig. 1).
To investigate if alternative splicing regulates the response of
CL1 to α-latrotoxin, we transfected three different splice vari-
ants (Fig. 8B). No significant differences in the response of the
transfected PC12 cells were observed, suggesting that the
events of alternative splicing studied are not involved in recep-
tor action.

CL2 and CL3 are highly homologous to CL1, suggesting that
the three CLs perform similar functions. We therefore tested if
CL2 and CL3 also confer onto transfected PC12 cells an in-
creased responsiveness to α-latrotoxin similar to CL1. Full-
length CL2 and a hybrid CL3 construct composed of the full-
length extracellular domains and TMRS of CL3 coupled to the
intracellular sequences of CL1 (to facilitate detection with an-
tibodies) were transfected into PC12 cells. However, we ob-
erved no major changes in the α-latrotoxin response of the
transfected PC12 cells (Fig. 9). CL2 had a small effect that was
not statistically significant. In the same experiment, CL1 was
fully active (Fig. 9). These results show that in transfected
PC12 cells, CL2 and CL3 do not shift the α-latrotoxin response
to significantly lower toxin concentrations.

**G-protein Coupling Is Not Required for the Function of CL1
as Latrotoxin Receptor**—In G-protein-coupled receptors, the
seven TMRS and their connecting loops are essential for recep-
tor function and for G-protein-mediated transduction (23–25).
To explore if CL1 mediates the effect of α-latrotoxin by a
G-protein-coupled mechanism, we expressed a truncated form
of CL1. A stop codon was introduced into the coding sequence
immediately after the first TMR at residue 891 (Figs. 1 and 10).
We then transfected PC12 cells with this construct, the full-
length CL1 cDNA, and two control plasmids. The responses of
the transfected PC12 cells to low concentrations of α-latrotoxin
demonstrated that the truncated CL1 receptor activated the
α-latrotoxin response as well as full-length CL1 (Fig. 10). Thus
the extracellular domains of CL1 coupled to a single transmem-
brane region are sufficient for full responsiveness to α-latro-
toxin, suggesting that the receptor does not mediate its effect
via a G-protein-coupled mechanism.

**Transfection of CL1 Does Not Depress the Secretory Response
of PC12 Cells**—In our transfection experiments, we observed
that expression of CL1-sensitized PC12 cells to lower concen-
trations of α-latrotoxin but attenuated the maximal secretory
response (Fig. 8). This result suggests the possibility that CL1
directly modulates the secretory apparatus. To test this possi-
bility, we studied the effect of CL1 transfections on secretion
evoked by KCl depolarization (Fig. 11). Transfections of splice
variants of CL1 or of truncated CL1 had no major effect on the
base-line secretion of hGH or on the amount of hGH release
stimulated by KCl. Under similar conditions, the maximal α-la-
троtoxin response was diminished (Fig. 8). This result indicates
that the effect of the overexpression of CL1 on the maximal
response to α-latrotoxin is not because of a general depression
of the secretory apparatus.
Produced. Data shown are means ± S.E. from multiple experiments. α-Latrotoxin (LTx, 0.3 nM) in the presence of Ca²⁺−latrotoxin (10 min, 0.3 nM). Note that secretion triggered by both agents is inhibited similarly by tetanus toxin. The assays in the presence of EGTA (E and F) were performed to monitor the background levels of secretion. Data shown are from a representative experiment repeated multiple times.

**FIG. 6.** Characterization of α-latrotoxin-induced secretion of hGH from transfected PC12 cells. A, cation dependence of secretion. PC12 cells transfected with hGH were stimulated with recombinant α-latrotoxin (α-LTx) in the presence or absence of Ca²⁺ or Mg²⁺. The 0.0 mM Ca²⁺ condition was carried out in 0.2 mM EGTA. hGH secretion into the medium was measured by radioimmunoassay as a percentage of total hGH produced. Data shown are from a representative experiment performed in duplicate. B, concentration dependence of α-latrotoxin action. PC12 cells transfected with hGH were stimulated with different concentrations of wild type α-latrotoxin (WT) or mutant recombinant α-latrotoxin (C14S). In the mutant, cysteine 14 was substituted for serine. hGH secretion was determined as percent of total hGH produced. Data shown are means ± S.E. from two experiments. C, effect of tetanus toxin light chain (TeTx) on α-latrotoxin action. PC12 cells transfected either with hGH alone or with hGH in combination with tetanus toxin light chain were stimulated by KCl depolarization (15 min, 56 mM KCl) or with α-latrotoxin (10 min, 0.3 nM). Note that secretion triggered by both agents is inhibited similarly by tetanus toxin. Data shown are means ± S.E. from multiple experiments.

**DISCUSSION**

α-Latrotoxin is a fascinating toxin that triggers massive exocytosis in neurons and neuroendocrine cells (1). In the absence of Ca²⁺, α-latrotoxin probably induces exocytosis by binding to a Ca²⁺−independent cell surface receptor called CIRL 1 or latrophilin 1 (16, 17), here referred to as CL1. CL1 is not only interesting because of its action as α-latrotoxin receptor. CL1 is also intriguing because it is a large G-protein-linked receptor that may represent a synapse-specific receptor that directly functions in synaptic signaling (16, 17). In the current study, we have explored this possibility.

Most G-protein-linked receptors are members of gene families. Therefore we first investigated if CL1 also belongs to a gene family. Our results demonstrate that CL1 is the founding member of a family of G-protein-linked receptors with at least three isoforms called CL1, 2, and 3, each of which is subject to extensive alternative splicing. The three CLs exhibit identical domain structures, similar alternative splicing, and significant sequence identity over their entire lengths. The structures of CLs reveal several unusual features. They resemble recently characterized G-protein-linked receptors which contain extracellular domains that are normally found in cell adhesion molecules, suggesting that these receptors perform hybrid functions in cell adhesion and signal transduction. In CLs, these domains are lectin- and olfactomedin-like sequences at the N terminus. More C-terminal CLs contain two novel domains not previously characterized: a large domain shared with orphan G-protein-coupled receptors called BAI 1, 2, and 3 and a shorter cysteine-rich domain immediately before the TM1s. The cysteine-rich domain was identified by data bank searches in multiple G-protein-linked receptors (Fig. 4). It is particularly interesting because CL1 is physiologically cleaved by proteolysis after synthesis at the end of this domain (16). CL2 and CL3 also appear to be cleaved, indicating that the novel cysteine-rich domain may represent a signal for proteolytic processing in the secretory pathway. It is likely that the various extracellular domains of CLs constitute ligand binding domains, suggesting that CLs interact with multiple ligands.

The high degree of homology between CL1, CL2, and CL3 suggests that the three CLs perform similar functions and interact with related endogenous ligands. Despite these similarities, however, we found that the tissue distributions of CLs differ dramatically. CL1 is primarily expressed in brain but is also present in peripheral tissues. CL2 shows no preferential expression in brain; instead, it is uniformly present in all tissues tested. Finally, CL3 is highly enriched in brain. Together these results characterize CL1, the Ca²⁺−independent receptor for α-latrotoxin, as a member of a ubiquitous family of closely related G-protein-coupled receptors. Because CLs are ubiquitously expressed, their endogenous ligands are presumably involved in general cellular functions. Based on these data, it seems likely that CL1 and other CLs do not perform...
cells because receptors are desensitized and not because the secretory apparatus is inhibited by CL1. CL2 and CL3 transfections had no effect on α-latrotoxin action in PC12 cells. These data support the notion that CL1 functions as an α-latrotoxin receptor. We analyzed a truncated form of CL1 that lacks all cytoplasmic sequences after the first transmembrane region. This truncated form cannot bind G-proteins or mediate G-protein-signal-transduction. In the CL3/CL1 hybrid plasmids, the extracellular domains and TMRS of CL3 are linked to the intracellular sequences of CL1. Data shown are from three experiments and represent means ± S.E.

FIG. 8. Transfection of CL1 sensitizes PC12 cells to low concentrations of α-latrotoxin. A, effect of transfection of CL1 into PC12 cells on the secretory response to α-latrotoxin. PC12 cells were cotransfected with hGH and a control vector (Control) or with hGH and CL1 (CL1-1), hGH secretion was stimulated by different concentrations of α-latrotoxin in the presence and absence of Ca²⁺. Note that after CL1 transfection, release is enhanced at low α-latrotoxin concentrations (<0.1 nM) but decreased at high α-latrotoxin concentrations (>0.1 nM). Data shown are means ± S.E. from three experiments. B, analysis of the activity of different splice variants of CL1 on α-latrotoxin action in PC12 cells. PC12 cells were co-transfected with hGH and a control vector (A) or with hGH and three splice variants of CL1: CL1–1 (B), which lacks inserts in the N-terminal extracellular and the C-terminal intracellular sites of alternative splicing, CL1–5 (D), which contains an insert in the N-terminal alternatively spliced position, and CL1–4 (C), which includes an insert in the C-terminal alternatively spliced position (Fig. 1). hGH secretion from transfected PC12 cells was induced at low concentrations of α-latrotoxin concentrations corresponding to the concentration range at which CL1 transfection sensitizes the secretory response. Data shown are from multiple experiments and represent means ± S.E.s.

FIG. 9. CL2 and CL3 transfections are unable to sensitize PC12 cells to α-latrotoxin. PC12 cells were co-transfected with hGH and the following plasmids: CL1 as a positive control, a CMV plasmid without insert as a negative control (Control), and full-length CL2 and CL3/CL1 hybrid plasmids to test their respective effects on α-latrotoxin action. In the CL3/CL1 hybrid plasmids, the extracellular domains and TMRS of CL3 are linked to the intracellular sequences of CL1. Data shown are from three experiments and represent means ± S.E.

FIG. 10. The cytoplasmic sequences of CL1 are not required for α-latrotoxin action. PC12 cells were co-transfected with hGH and vector without insert (control (□)) or with hGH and full-length CL1 (CL1–1 (△)), CL1 with a stop codon immediately after the first transmembrane region (CL1STOP891 (□)), or a control plasmid (●). hGH secretion from the transfected PC12 cells induced at the indicated concentrations of α-latrotoxin was analyzed. Data shown are means ± S.E. from six experiments.
specific regulator of exocytosis but a general G-protein-linked receptor of unusual structure. Our data provide further support for the hypothesis that CL1 functions as a major receptor for α-latrotoxin (16, 17) but suggest that its mechanism of action will not involve a direct coupling of the receptor to the secretory apparatus. The preferential expression of CL1 and CL3 in brain and the universal expression of CL2 in all tissues indicate that CLs perform physiological functions in all cells but are particularly important for the nervous system. It will be interesting to identify the endogenous ligands for the three CLs and to elucidate their physiological functions as opposed to their pathological roles in α-latrotoxin action.

Our results raise two questions. First, how does α-latrotoxin act in a synapse-specific manner in the absence of Ca\(^{2+}\) if its major receptor, CL1, is not functionally related to synapses? Second, why is expression of a Ca\(^{2+}\)-independent receptor for α-latrotoxin in PC12 cells not sufficient for obtaining Ca\(^{2+}\)-independent exocytosis triggered by α-latrotoxin? Although definitive answers are lacking, a working model can be proposed that addresses both questions. This model is based on the observation that CL1 does not transduce the exocytotic signal of α-latrotoxin because its coupling to G-proteins is not required for activity. We would like to propose that after binding to CL1 and/or neuroexins \(I_0\), α-latrotoxin inserts into the membrane and has the effect of a Ca\(^{2+}\) ionophore. However, according to our model this is not sufficient for Ca\(^{2+}\)-independent triggering of exocytosis. Instead, our model suggests that a downstream target of α-latrotoxin exists that binds to the toxin after it has been recruited to the membrane by CL1 or neuroexin \(I_0\). This downstream target is synapse-specific and not present in neuroendocrine cells that do not have synapses. This model would explain the confusing differences in the Ca\(^{2+}\) dependence of α-latrotoxin action between synapses and neuroendocrine cells and the lack of involvement of G-protein-signaling in exocytosis. Future studies will have to investigate this model directly.

Acknowledgments—We thank Dr. M. Okamoto for constructing phGHCVM5 and Drs. J. L. Goldstein and M. S. Brown for advice and support.
α-Latrotoxin Receptor CIRL/Latrophilin 1 (CL1) Defines an Unusual Family of Ubiquitous G-protein-linked Receptors: G-PROTEIN COUPLING NOT REQUIRED FOR TRIGGERING EXOCYTOSIS
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J. Biol. Chem. 1998, 273:32715-32724.
doi: 10.1074/jbc.273.49.32715

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