α-Latrotoxin, Latrophilin, Is a Novel Member of the Secretin Family of G Protein-coupled Receptors*

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α-Latrotoxin (LTX) stimulates massive exocytosis of synaptic vesicles and may help to elucidate the mechanism of regulation of neurosecretion. We have recently isolated latrophilin, the synaptic Ca2+-independent LTX receptor. Now we demonstrate that latrophilin is a novel member of the secretin family of G protein-coupled receptors that are involved in secretion. Northern blot analysis shows that latrophilin message is present only in neuronal tissue. Upon expression in COS cells, the cloned protein is indistinguishable from brain latrophilin and binds LTX with high affinity. Latrophilin physically interacts with a Goαi subunit of heterotrimeric G proteins, because the two proteins co-purify in a two-step affinity chromatography. Interestingly, extracellular domain of latrophilin is homologous to olfactomedin, a soluble neuronal protein thought to participate in odorant binding. Our findings suggest that latrophilin may bind unidentified endogenous ligands and transduce signals into nerve terminals, thus implicating G proteins in the control of synaptic vesicle exocytosis.

Many proteins involved in SV1 exocytosis have been recently identified, but still little is known about the signals that control this highly regulated process (1). The main release stimulus is Ca2+, which enters nerve terminals through voltage-sensitive Ca2+ channels. However, neuroexocytosis can be stimulated in the absence of extracellular Ca2+ too.

One of such Ca2+-independent activators of neurosecretion is LTX, a Black Widow spider presynaptic neurotoxin that causes massive exocytosis of SV (reviewed in Ref. 2). The toxin mode of action remains controversial. On the one hand, LTX can make massive exocytosis of SV (reviewed in Ref. 2). The toxin mode of action in the absence of extracellular Ca2+ has long been recognized (6), the latter ones being solely responsible for the toxin action in the absence of Ca2+. To elucidate the LTX mode of action and better understand the mechanism of neurosecretion, it is important to know the structure of the LTX receptor.

In an attempt to isolate the receptor, neurexins, neuronal cell surface proteins, were discovered (10). However, neurexins interact with the toxin only in the presence of Ca2+ (11) and thus may not be responsible for LTX effect in the absence of this ion. Moreover, LTX potently causes dopamine release in a PC12 cell line that completely lacks Ca2+-dependent receptors (6), thus ruling out the importance of neurexins in the toxin action. Recently, we purified latrophilin, a synaptic protein that binds LTX independently of Ca2+ (12). The isolation of the same protein was later also described by others (13). Here we report the cloning and sequencing of latrophilin, the Ca2+-independent LTX receptor, and demonstrate that it is a novel member of the secretin/calcitonin family of G protein-coupled receptors. Our findings implicate G proteins in regulation of neurotransmitter release.

EXPERIMENTAL PROCEDURES

Latrophilin Purification and Sequencing—High purity LTX was purchased from Neurogen, UK. Latrophilin from rat brain was isolated by LTX affinity chromatography (12) and further purified by SDS-PAGE. The protein was digested in gel pieces with endoproteinas Lys-C or trypsin (Wako). Peptides were recovered by sonication and applied directly to an Aquapore AX-300 column (2 × 0.5 mm, custom made) and a Relias C18 column (150 × 1 mm) in series on a Michrom Ultra Fast Protein Analyser HPLC system. The columns were developed with a linear acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 50 μl/min. Collected fractions were applied to a high sensitivity Precise system (Applied Biosystems) employing a capillary HPLC C18 column (250 × 0.8 mm). Initial yields were in the range of 0.5 to 5 pmol.

Latrophilin cDNA Isolation and Sequencing—A degenerate oligonucleotide probe, AA/AG/TACGACCT(G/C)GCCCG(G/A)AT(T/C) AA-3′, corresponding to the sequence of peptide KYDLMTRIK, was used to screen a rat brain cDNA library in XZAPII (2 × 108 recombinants, Stratagene). cDNA inserts were sequenced using a Dye Terminator Cycle Sequencing chemistry and an automated DNA sequencer, Prism 377 (Applied Biosystems). On-line data base searches were performed using FASTA and BLAST programs (Intelligenetics). Sequences were analyzed with the Lasergene software (DNA Star).

In Vivo Expression of Latrophilin—The insert from clone RBCR9–15 was recloned into a mammalian vector pCDNA3 (Invitrogen). COS-7 cells (European Cell Culture Collection) were transiently transfected with this construct or the vector alone, using SuperFect reagent (Qiagen) and 10 of μg DNA/10-cm plate. Cells were harvested after 1–4 days and washed with phosphate-buffered saline containing 20 mM EGTA,

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§ The abbreviations used are: SV, synaptic vesicles; GPCR, G protein-coupled receptor; IP3, inositol 1,4,5-trisphosphate; LPHI, latrophilin 1; LTX, α-latrotoxin; PAGE, polyacrylamide gel electrophoresis; TMS, transmembrane segment; WGA, wheat germ agglutinin; HPLC, high pressure liquid chromatography; kb, kilobase pair(s).
and aliquots (∼10^6 cells) were incubated with different concentrations of iodinated LTX for 15 min. A 100-fold excess of unlabeled LTX was included in controls. Unbound toxin was removed by filtration. For immunostaining, ∼5 × 10^6 transfected cells were solubilized in 2% Triton X-100, subjected to SDS-PAGE and Western blotting. Other Methods—Northern blots (Bios Laboratories) of total RNA from rat tissues were hybridized to a randomly radiolabeled insert from clone RBCR9–15. Latrophilin preparations enriched in heterotrimeric G proteins were made as published (12), except that 20 mM GDP was included during purification. Antibodies against G protein α-subunits and synaptotagmin were from Santa Cruz and Affiniti. Antibodies against SNAP-25, syntaxin, and synaptobrevin were kindly provided by Drs. J. O. Dolly and P. Foran. Proteins were analyzed by SDS-PAGE, transferred onto Immobilon membrane (Millipore), and visualized using respective antibodies and Chemiluminescence Substrate System (Pierce).

RESULTS AND DISCUSSION

To determine the structure of latrophilin, we purified approximately 300 μg of rat brain latrophilin. Fourteen peptides were isolated from the protease-digested protein. The peptide sequences were used to design degenerate oligonucleotide probes for the screening of a rat brain cDNA library. As a result of this screening, we isolated 20 cDNA clones, all of which overlapped and represented a piece of cDNA ∼8.3 kb long. The longest clone (RBCR9–15; 5.7 kb) contained a 4.4-kb open reading frame and encoded all peptides obtained from rat brain latrophilin. The deduced primary structure of this protein, termed rat latrophilin 1 (LPH1), is shown in Fig. 1A.

The LPH1 molecule (Fig. 1B) comprises the following putative domains: an 849-residue-long extracellular domain, seven hydrophobic transmembrane segments (TMSs) and a cytoplasmic tail of 372 amino acids. The extracellular domain begins with a hydrophobic signal peptide. This is followed by a conserved stretch homologous to galactose-binding lectin (14), with a hydrophobic signal peptide. This is followed by a cysteine-rich domain, and a glycosylated spacer fragment. The presumably cytoplasmic C-terminal portion of LPH1 contains five cysteine residues. Three of these are positioned very similarly to cysteines in the cytoplasmic tail of rhodopsin and may also be palmitoylated (17). There are several possible phosphorylation sites in the molecule (Fig. 1A) that may play a role in receptor desensitization in response to endogenous ligand(s) under physiological conditions. Proline residues, abundant in this region, could interact with SH3 domains of proteins involved in signaling.

The main attribute of LPH1 is the presence of seven TMSs that are 25–45% identical to the corresponding parts of several GPCRs. Apart from the nervous system, LTX receptors are also present in chromaffin and PC12 cells (2) and some pancreatic β-cell lines. Consistent with its function as the LTX receptor, latrophilin has only been found in those β-cell lines that are responsive to LTX.2 Also, a PC12 cell line that does not express latrophilin shows no toxin binding and cannot be stimulated by LTX.3

Although all peptides from latrophilin have been found in the structure of LPH1, we carried out experiments directed at

2 J. Lang, Y. Ushkaryov, A. Grasso, and C. Wollheim, submitted for publication.

3 Y. Ushkaryov, unpublished data.
proving that LPH1 is indeed the toxin receptor. Full-length LPH1 was expressed in COS cells using an appropriate vector. Upon transfection with this construct but not the vector alone, cells specifically bound iodinated LTX in the absence of Ca\(^{2+}\). The number of binding sites grew until day 3 (Fig. 3B). Analysis of these binding sites revealed that LTX and expressed latrophilin interact with high affinity (\(K_D\) = 2.5 nM) (Fig. 3C). This value is higher than that of LTX receptors in brain (12), possibly due to a different protein context at the surface of COS cells. The plasma membranes from transfected cells were then analyzed by WGA affinity chromatography and immunoblotting (Fig. 3D). Latrophilin expressed in COS cells was indistinguishable from the brain protein. These observations unequivocally prove that latrophilin is the Ca\(^{2+}\)-independent LTX receptor and confirm our previous conclusion that it alone is sufficient for high affinity LTX binding (12).

Our finding that full-length latrophilin has seven TMSs and provides binding sites for the toxin suggested that neurotransmitter release may be controlled through a receptor-mediated activation of heterotrimeric G proteins. Such a mechanism is well known to regulate exocytosis in secretory cells, although the precise point of the G protein action in the docking/fusion process has not been established yet. G proteins are highly enriched in synapses (31), and the basic mechanism of exocytosis regulation in neurons is likely to be similar in other secretory cells. Despite this, the idea that heterotrimeric G proteins may be implicated in SV exocytosis is relatively new (32, 33) and has not become widely accepted yet (1). Interestingly, LTX has already been shown to stimulate (in the presence of Ca\(^{2+}\)) the production of IP\(_3\) in PC12 cells (34), a function usually controlled by GPCRs. Accordingly, in LPH1-transfected cells but not in control cells, 10 nM LTX causes up to 2-fold elevation of cAMP and IP\(_3\) (Fig. 4A). Although this effect was observed only in 10 \(\mu\)M Ca\(^{2+}\), it was not mimicked by a Ca\(^{2+}\) ionophore A23187, suggesting a functional coupling between the LTX receptor and G proteins, probably enhanced by Ca\(^{2+}\) entry into the cells (see also Ref. 34). Therefore, to further confirm a putative functional link, we demonstrate that latrophilin physically interacts with a G protein \(\alpha\)-subunit. We

FIG. 2—continued

A, alignment of rat LPH1 with galactose-binding lectin from sea urchin (14) and a putative protein encoded by Caenorhabditis elegans cosmid B0457 (GenBank accession number Z54306). B, alignment of LPH1 with bullfrog olfactomedin, OLFM (15), and rat olfactomedin-related protein, OLFR (16). C, alignment of the TMS region of latrophilin 1 with corresponding parts of the secretin family GPCRs: leukocyte activation antigen (18); epidermal growth factor module-containing mucin-like receptor (EMRI) (19); vasoactive intestinal peptide receptor (VIPR) (20); pituitary adenylate cyclase-activating polypeptide receptor (PACR) (21); secretin receptor (SCRC) (22); glucagon receptor (GLR) (23); gastrin inhibitory peptide receptor (GIPR) (24); glucagon-related peptide receptor (GLPR) (25); growth hormone-releasing factor receptor (GRFR) (26); receptor for parathyroid hormone (PThrR) (27), diuretic hormone from tobacco hornworm Manduca sexta (DIHR) (28); corticotropin-releasing factor receptor (CRFR) (29); calcitonin receptor type A (CLRA) (30); and a hypothetical receptor from C. elegans, YOW3 (SwissProt P30650). Nonhomologous N- and C-terminal regions are not shown. To optimize the alignment, dashes were introduced and some short regions were omitted (dots). Residues identical in more than 30% of proteins are shaded, and those identical only in the “long receptors” are highlighted. TMS positions are identified by arrows. Asterisks mark conserved cysteines.

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found that when latrophilin purification is carried out in the presence of GDP and low micromolar Mg\(^{2+}\), a protein of 42 kDa co-purifies with latrophilin, which reacts with antibodies against G\(_{\alpha}\), but not those against G\(_{\beta\gamma}\) subunits (Fig. 4, lane 1). Such discrimination is likely to be specific because G\(_{\alpha}\) subunits are most abundant in neuronal plasma membranes, whereas G\(_{\beta\gamma}\) subunits are present mainly on synaptic vesicles (35). In addition, the preparation contained no trace of synaptotagmin, SNAP-25, synaptobrevin, or synaptotagmin, proteins implicated in vesicle docking/fusion (data not shown). The G protein band had been absent from the receptor preparations made in millimolar Mg\(^{2+}\) or in EDTA (12), pointing at physiological relevance of this interaction. To further test the specificity of latrophilin association with G\(_{\alpha}\), we subjected the preparation containing both proteins to a second affinity chromatography using a WGA column. As Fig. 4 (lanes 4–11) clearly shows, the two proteins co-elute from the second column, indicating that their interaction is stable during a two-step affinity purification. Our results support the hypothesis that LPH1 is a GPCR; however, further experiments are needed to unequivocally identify the specific functional links and determine yet unknown effectors of latrophilin.

We also examined what type of second messenger may be involved in the LTX-evoked norepinephrine secretion in neurons. However, our experiments on synaptosomes\(^4\) indicate that the main second messengers, cAMP, cGMP, and IP\(_3\), do not control SV exocytosis and do not play a major role in the toxin action in zero Ca\(^{2+}\). These results appear to be at variance with the effect of LTX in COS cells expressing LPH1 (Fig. 4A). However, functional links of a GPCR in heterologous systems may often be different and depend on the repertoire of heterotrimeric G proteins expressed in a given cell (36). What could then be the mechanism of LTX action in neurons, provided its effect is mediated by latrophilin, a GPCR? G proteins have recently been shown to control neurotransmitter release by regulating preferentially those presynaptic calcium channels that are directly associated with release sites, indicating that channel components of the release sites may be effectors for G proteins (33). Although the toxin opens Ca\(^{2+}\)-permeable channels in the plasma membrane of sensitive cells, these differ both from pores made by LTX in lipid bilayers (37) and from conventional Ca\(^{2+}\) channels (38, 39). LTX also induces fluxes of other cations in synaptosomes and neuroblastoma cells (40, 41). This implicates the involvement of nonspecific cation channels that have been found in numerous cell types (42). Such channels are also controlled by G proteins (43) and could participate in regulation of secretion. On the other hand, in some systems the toxin action may proceed without ion fluxes and membrane depolarization (44) and may be based on the activation by G proteins of components of release sites different from ion channels. It is important to stress, however, that the site of G protein action in exocytosis is still unknown both in endocrine cells and at nerve terminals.

Taken together, our findings strongly suggest that SV exocytosis may be controlled by the presynaptic machinery that links receptors to G proteins to release sites and bypasses the main second messenger. This streamlined type of coupling is already known for ligand-evoked regulation of hormone secretion (45). Consequently, like hormone release, neurotranscretion could be directly controlled by extracellular ligands. Remarkably, the N-terminal extracellular domain of LPH1 is homologous to olfactomedin, a protein of olfactory epithelium implicated in odorant binding (15), and to olfactomedin-related

\(^4\) B. A. Davletov and Y. A. Ushkaryov, in press.

**Fig. 3. Identification of latrophilin as the \(\alpha\)-latrotoxin receptor.** A, Northern blot hybridization of rat LPH1 mRNA (20 \(\mu\)g of RNA per lane). Lower panel, control hybridization of 28 S RNA. B, time course of expression of LTX receptors in COS cells transfected with pcDNA3-LPH1 (●) or the vector alone (○). C, Scatchard plot analysis of \(\alpha\)-latrotoxin binding to COS cells transfected with LPH1. D, immunoblotting of latrophilin expressed in transfected COS cells. Lane 1, latrophilin purified from rat brain, 10 ng; lanes 2, transfected with the vector and enriched by WGA affinity chromatography, whereas G\(_{\alpha}\) subunits are most abundant in neuronal plasma membranes, whereas G\(_{\beta\gamma}\) subunits are present mainly on synaptic vesicles. Lane 1, latrophilin purified from rat brain, 10 ng; lanes 2, solubilized COS cells transfected with the vector and enriched by WGA affinity chromatography, 30 \(\mu\)g of protein; lanes 3, same as lanes 2 but transfected with pcDNA3-LPH1. Latrophilin is marked by an arrowhead.

**Fig. 4. Latrophilin interacts with a G protein.** A, latrotoxin (10 nM) stimulates cAMP and IP\(_3\) production in COS cells transfected with LPH1 but not with vector in the presence of 10 \(\mu\)M Ca\(^{2+}\). B, solubilized rat brain was used for LTX affinity chromatography in 20 \(\mu\)M Mg\(^{2+}\) and 2 mM GDP. The eluate from the LTX column (lane 1) containing both latrophilin and a G\(_{\alpha}\) subunit was subsequently loaded onto a WGA column (lane 2, flow through), washed (lane 3), and eluted with 100 mM N-acetylglucosamine (lanes 4–11). Fractions were analyzed by SDS-PAGE and immunoblotting with respective antibodies. Note the absence of association between latrophilin and G\(_{\alpha}\).
proteins found throughout the brain (16) (Fig. 2B). These soluble proteins may bind some ligands and deliver them by diffusion to GPCRs in the plasma membrane. The striking feature of latrophilin is that this olfactomedin-like domain is attached to a membrane-bound GPCR, suggesting an evolutionary adaptation for a more efficient ligand search and signal transduction. The finding of endogenous latrophilin ligands should therefore reveal new ways of regulating neuroexcytosis.

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