Cloning and Expression of a Complementary DNA Encoding a Molluscan Octopamine Receptor That Couples to Chloride Channels in HEK293 Cells*

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A cDNA encoding a G-protein-coupled receptor was cloned from the central nervous system of the pond snail Lymnaea stagnalis. The predicted amino acid sequence of this cDNA most closely resembles the Drosophila tyramine/octopamine receptor, the Locusta tyramine receptor, and an octopamine receptor (Lym oa1) that we recently cloned from Lymnaea. After stable expression of the cDNA in HEK293 cells, we found that [3H]rauwolscine binds with high affinity to the receptor (KD = 6.2×10^{-9} M). Octopamine appears to be the most potent naturally occurring agonist to displace the [3H]rauwolscine binding (K_i = 3.0×10^{-7} M). Therefore, the receptor is considered to be an octopamine receptor and is consequently designated Lym oa2. The novel receptor shares little pharmacological resemblance with Lym oa1, indicating that the two receptors represent different octopamine receptor subfamilies. Octopaminergic stimulation of Lym oa2 does not induce changes in intracellular concentrations of cAMP or inositol phosphates. However, electrophysiological experiments indicate that octopamine is able to activate a voltage-independent Cl^{-} current in HEK293 cells stably expressing Lym oa2. Although opening of this chloride channel most probably does not require the activation of either protein kinase A or C, it can be blocked by inhibition of protein phosphorylation.

G-protein-coupled receptors form a large superfamily of membrane receptors that can be found in all species ranging from unicellular eukaryotes to mammals and that can interact with a large variety of signals (e.g. light, odorants, Ca^{2+}, biogenic amines, glycoprotein hormones, etc.) (1). Bioamines like dopamine, epinephrine, norepinephrine, and octopamine all interact with specific G-protein-coupled receptors. Dopamine is present in high amounts in both vertebrate and invertebrate species. Epinephrine and norepinephrine, on the other hand, are present predominantly in vertebrates, whereas octopamine is considered to act as a major neurotransmitter in invertebrate species only. Octopamine is often referred to as the invertebrate counterpart of norepinephrine because of the structural similarity between these neurotransmitters (they differ only in the presence of a single catecholic hydroxyl group) as well as their functional similarity (both serve an important role in stress adaptation) (2). Consequently, the adrenergic and octopaminergic receptors share pharmacological as well as structural properties (3, 4).

The role of octopamine as a neurotransmitter has been studied particularly well in insects, where it has been shown to interact with at least four different octopamine receptor subtypes (3). The central nervous systems of a number of snails have also been used to study octopaminergic neurotransmission. The presence of octopamine has been demonstrated in the sea slug Aplysia (5–7), the land snail Helix (8, 9), and the pond snail Lymnaea (10). In these species, the interactions of octopamine with its receptors have been described mainly at the electrophysiological level (7, 8, 11–15). In general, application of octopamine induces a hyperpolarization of susceptible snail neurons, a process that is thought to be mediated by an increase in cAMP (2, 11, 12), leading to an increased potassium (12–14) or calcium (15) conductance. In addition, a pharmacological characterization of octopamine receptors has been described for Lymnaea.2

We have recently cloned and characterized a cDNA encoding an octopamine receptor expressed in the brain of Lymnaea stagnalis (16). This receptor (Lym o2) shows moderate homology to the Drosophila tyramine/octopamine receptor (17, 18), to the Locusta tyramine receptor (19) and to the vertebrate α-adrenergic receptors. Activation of this receptor, when expressed in human embryonic kidney (HEK293) cells, leads to elevated concentrations of both intracellular inositol phosphates and cAMP. The pharmacological profile of Lym o2 suggests that this receptor represents a previously unknown octopamine receptor subtype.

This paper describes the structure of a second octopamine receptor cDNA (Lym o1) cloned from Lymnaea. The predicted amino acid sequence of this receptor only shows limited similarity to Lym o2, as well as to the insect tyramine receptors and the α-adrenergic receptors. Its pharmacological profile clearly differs from that of previously described receptors. When the novel receptor is stably expressed in HEK293 cells, application of octopamine does not lead to changes in the intracellular concentration of cAMP or inositol phosphates. Acti-

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The activation of Lymoa2 does, however, induce a long lasting opening of Cl− channels. G-protein-mediated activation of a similar Cl− current was recently described by Postma et al. (20) in rat fibroblasts. Their study also indicated that none of the known second messenger system was involved in the Cl− channel activation. Nevertheless, it was suggested that the increase in chloride conductance was closely associated with the activation of phosphoinositide hydrolysis. In contrast, our results clearly indicate that this novel signaling pathway functions independently from the activation of phospholipase C (PLC). We further show that although protein kinase A and protein kinase C are most probably not involved in the signaling pathway, protein phosphorylation is important for the opening of the Cl− channel.

![FIG. 1. Nucleotide and deduced amino acid sequence Lymoa2 cDNA. Putative transmembrane regions are boxed. Residues in filled squares represent consensus sites for N-linked glycosylation; residues in filled circles represent consensus phosphorylation sites for protein kinase C. Residues in open circles have been implicated in bioamine binding. The serine residue in an open square can be phosphorylated by protein kinase A. Restriction sites that have been used for the cloning of the expression construct are depicted in bold italics.](image)

![FIG. 2. Saturable binding of [3H]rauwolscine to Lymoa2 stably expressed in HEK293 cells. Inset, Scatchard plot of the same data. SB, specific binding in picomoles/mg of protein. SB represents the total binding minus the nonspecific binding of [3H]rauwolscine to membranes of HEK293 cells expressing Lymoa2. Nonspecific binding was determined by displacement of [3H]rauwolscine binding by mianserin (5 µM). F, free [3H]rauwolscine.](image)

**Table I**

| Compound                  | pKi or pK± S.D. Lymoa2 | Lymoa1 |
|---------------------------|------------------------|--------|
| Agonists                  |                        |        |
| (+)-p-Sympatline          | 7.01 ± 0.02            | 6.41 ± 0.24 |
| (+)-p-Octopamine          | 6.52 ± 0.14            | 5.68 ± 0.18 |
| Xylotamazoline            | 6.10 ± 0.10            | 5.63 ± 0.03 |
| B-HT 920                  | 5.93 ± 0.20            | 4.80 ± 0.11 |
| (-)-Norepinephrine        | 5.71 ± 0.05            | 4.09 ± 0.39 |
| Clonidine                 | 5.50 ± 0.01            | 6.57 ± 0.32 |
| (-)-Epinephrine           | 5.56 ± 0.18            | 4.26 ± 0.08 |
| p-Tyramine                | 5.42 ± 0.11            | 4.44 ± 0.17 |
| Phenylephrine             | 5.22 ± 0.44            | 5.60 ± 0.13 |
| Oxymetazoline             | 5.19 ± 0.05            | 5.56 ± 0.08 |
| Methoxamine               | 5.16 ± 0.05            | 4.09 ± 0.12 |
| Dopamine                  | 5.05 ± 0.14            | 4.43 ± 0.13 |
| Serotonin                 | 4.54 ± 0.21            | 4.43 ± 0.13 |
| Histamine                 | 4.03 ± 0.18            | 4.43 ± 0.13 |

Antagonists

| Rauwolscine               | 7.99 ± 0.08            | 7.46 ± 0.02 |
| Mianserin                 | 7.94 ± 0.25            | 7.67 ± 0.34 |
| Phenelamine               | 7.01 ± 0.25            | 8.07 ± 0.14 |
| Chlorpromazine            | 6.66 ± 0.28            | 8.53 ± 0.26 |
| Spiperone                 | 6.31 ± 0.05            | 8.50 ± 0.31 |
| Yohimbine                 | 6.15 ± 0.21            | 8.57 ± 0.32 |
| (-)-Propanolol            | 5.51 ± 0.14            | 4.93 ± 0.15 |
| Alprenolol                | 5.42 ± 0.12            | 5.01 ± 0.41 |
| Prazosine                 | 4.71 ± 0.19            | 7.02 ± 0.05 |
| Pindolol                  | 4.34 ± 0.46            | 4.56 ± 0.12 |
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Isolation of the cDNA Encoding Lym ao,

Central nervous system libraries of adult L. stagnalis bred in the laboratory (21) were dissected, the total RNA was isolated (22), and cDNA was transcribed using oligo(dT) primers and SuperScript™ RNase H− reverse transcriptase (Life Technologies, Inc.). This cDNA served as a template in a degenerate PCR strategy designed to isolate genes encoding G-protein-coupled bioamine receptors. The oligonucleotide primers recognize stretches of conserved amino acid residues present in transmembrane (TM) regions 6 and 7 and have been described previously (23). PCR products were cloned and sequenced (Sequenase, Life Technologies, Inc.), and one of the fragments revealed significant similarity to the TM6–TM7 region of adrenergic receptors. The presence of several large cDNA clones corresponding to this initial fragment was confirmed in a PCR-based screening of a fractionated Lymnaea central nervous system cDNA library in λ-ZAP. Isolated single plaques containing the full-length cDNA inserts were converted into pBS-SK+ phagemids by in vitro excision, and the insert was sequenced (Sequenase, Life Technologies, Inc.).

Stable Expression of Lym ao1 in HEK293 Cells—A PCR fragment covering the 5′ part of the open reading frame of pBS-Lym ao1 was generated using a sense oligonucleotide based on the DNA sequence around the start codon of the open reading frame and an antisense oligonucleotide based on the sequence 3′ from the endogenous KpnI site (located at position 563; see Fig. 1). This fragment was cloned, sequenced on both strands, and combined with the 3′-end fragment of pBS-Lym ao1 (A PCR fragment generated by amplifying the central coding region of Lym ao2 obtained as a KpnI and XbaI fragment (XbaI site located at position 1931; see Fig. 1). The total coding region was cloned into pcDNA3 (Invitrogen), yielding a construct designated pcDNA-Lym ao1.

HEK293 cells were stably transfected with pcDNA-Lym ao1 as described before (16). The level of expression of Lym ao1 was determined by measuring the binding of [3H]rauwolscine (81–85 Ci/mmol; Amer sham Corp.) to membrane preparations of resident cells. One cell line exhibiting a Bmax of 2.2 pmol/mg was selected for further study.

Radioligand Binding Assays—The preparation of membranes of HEK293 cells and the radioligand binding experiments were carried out as described previously (16).

Saturation isotherms were obtained by incubating membrane protein with increasing amounts of [3H]rauwolscine (0.1–45 nm). Nonspecific binding was determined by the addition of mianserin to a final concentration of 5 μM.

Competition curves were obtained by incubating membrane protein with 6 nM [3H]rauwolscine and increasing amounts of competitor (10−10–10−4 M). The obtained data were fitted using Kaleidagraph 3.0 (Abelbeck Software) as described previously (16).

Measurements of Inositol Phosphate Formation—HEK293 cells were grown to 50% confluency in 24-well plates, and incubated with 1 μCi of myo-[3H]inositol (18 Ci/mmol, Amersham Corp.) per ml of inositol-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) for 20–24 h. Cells were incubated with agonists in the presence of LiCl (10 mM) for 60 min at 37°C. Cells were lysed with chloroform:methanol, and total cellular inositol phosphates were extracted using Dowex AG 1-X8 anion exchange resin as described previously (16).

Whole cell voltage clamp experiments were performed using an Axoclamp 2A amplifier (Axon Instruments, Inc., Foster City, CA) in the continuous single electrode voltage clamp mode, in which case cell capacitance (~60 picofarads) was not compensated, or a Liszt EPC7 amplifier (Liszt, Darmstadt, Germany), allowing cell capacitance compensation. Pipettes (3–6 MΩ) were pulled on a Flaming/Brown P-87 (Sutter Instrument Co.) horizontal micro-electrode puller from Clark GC-150 glass (Clark Electromedical Instruments, UK; seal resistance, >2 GΩ). After disruption of the patch membrane, series resistance (~6 MΩ) was compensated for ~70%. Measurements commenced 5 min after access to the cell to allow equilibration with the pipette solution. Data acquisition was controlled by a CED 1400 AD/DA converter (Cambridge Electronics Design, Cambridge, UK) connected to an Intel 80486-based computer run with voltage clamp software developed in our laboratory. The current recordings were filtered at 1–5 kHz, sampled at 1 kHz, and stored on line. This system allowed simultaneous application of voltage steps, acquisition of current recordings, and timed application of drugs.

RESULTS

Cloning of Lym ao2 cDNA—We have applied the PCR technique to clone G-protein-coupled receptors that are expressed in the brain of L. stagnalis, using degenerated primers based on highly conserved sequences present in TM6 and TM7 of G-protein-coupled receptors. The obtained PCR products were sequenced and the sequences were conceptually translated. One derived amino acid sequence showed conceptual similarity to the sequence of the corresponding region in catecholaminergic receptors. We isolated and sequenced the corresponding full-length cDNA from a Lymnaea central nervous system library (see Fig. 1). An open reading frame that can encode a protein of 578 amino acids is present on this cDNA (see Fig. 1).
The presence of seven hydrophobic regions characteristic for G-protein-coupled receptors can clearly be recognized within the predicted amino acid sequence. Within these regions, the most prominent amino acid identity is found with the Drosophila tyramine/octopamine receptor (49%) (17, 18), the Locusta tyramine receptor (50%) (19), Lymnaea (45%), and the mammalian \(\alpha_2\)-adrenoceptors (641%), thus confirming that the open reading frame is likely to code for a bioamine receptor.

Stable Expression and Pharmacological Characterization of Lymnaea—To further delineate the nature of this receptor we stably expressed it in HEK293 cells. We then used the binding of \(^3\)Hrauwolscine to membranes of these cells as a marker to select clonal lines expressing high levels of receptor protein. One clone, exhibiting a \(B_{\text{max}}\) of 2.2 pmol/mg, was chosen for a pharmacological characterization of the receptor. Fig. 2 shows the saturation binding curve and the corresponding Scatchard plot of the binding of \(^3\)Hrauwolscine. The affinity constant \((K_D)\) of \(^3\)Hrauwolscine for the novel receptor is 6.24 nM. The ability of several (mainly adrenergic) compounds to displace \(^3\)Hrauwolscine binding, the receptor was considered to be an octopamine receptor and consequently called Lymoa2.

We recently cloned and characterized another octopamine receptor from Lymnaea (Lymoa1; Ref. 16). To allow for a comparison of the binding properties of both octopamine receptors, Table I also shows the \(pK_i\) values of the same set of ligands obtained by the displacement of \(^3\)Hrauwolscine binding from Lymoa1. As can be seen from a Pearson correlation graph (Fig. 3) the pharmacological profiles of Lymoa1 and Lymoa2 are considerably different.

Signal Transduction of Lymoa2—We tested to discover to which signal transduction pathways Lymoa2 can be coupled. In transiently transfected HEK293 cells, as well as in four independently isolated stable cell lines, octopaminergic stimulation did not induce any change in the concentration of cAMP or inositol phosphates. Also, stimulation with tyramine, epinephrine, norepinephrine, dopamine, serotonin, or histamine did not change the concentration of these second messengers as compared with non-transfected HEK293 cells. Positive control experiments, however, resulted in pronounced increases in cAMP or inositol phosphates (stimulation of endogenous \(\beta\)-adrenergic receptors increased cAMP 20-fold over basal levels, and stimulation of Lymoa1 receptors expressed in HEK293 cells increased inositol phosphates 45-fold over basal levels; data not shown).

Since activation of many neuronal receptors induces changes in membrane conductances of the cells in which they are ex-
pressed, we tested whether application of octopamine to HEK293 cells expressing Lym \( \text{o}_a \) elicited electrical responses in these cells. Application of 10 \( \mu \)M octopamine to HEK293 cells expressing Lym \( \text{oa}_a \) induced a large but slow increase in the holding current of the voltage-clamped cells (Fig. 4A). The amplitude of the octopamine-induced current decreased at more depolarized potentials. Application of synephrine (10 \( \mu \)M), an agonist with a higher potency than octopamine (see Table I), mimicked the octopamine-induced response, whereas yohimbine (10 \( \mu \)M), an antagonist, inhibited the response, thus confirming the specificity of the effect (see Fig. 4, B and C). Application of octopamine to non-transfected HEK293 cells did not show any effect (Fig. 4A).

In order to study the nature of the current (e.g., the voltage dependence and ionic selectivity), we performed voltage ramp experiments. Current responses were recorded while the voltage was continuously varied from –80 to +10 mV over a period of 8 s, both in the absence and presence of octopamine (Fig. 5A). The current voltage (IV) relation of the octopamine-induced current was obtained by subtracting the control current from the current response in the presence of octopamine. Fig. 5B shows that octopamine activates a current response over the whole voltage range tested and that the IV relation of the octopamine-induced current is almost linear, indicating that the current is voltage-independent. Comparison of the IV relations of nonstimulated and stimulated cells revealed no or only minor differences (<5 mV) in reversal potential (Fig. 5A). The IV curve of the octopamine-induced current (isolated by subtraction) showed reversal around –10 mV, which is close to the reversal potential of chloride ions (\( E_{\text{Cl}} = –2 \) mV under the present ionic conditions). To test whether the octopamine response is indeed carried by \( \text{Cl}^- \) ions, we replaced KCl in the intracellular (pipette) medium with K\(^{+}\)-aspartate, thus shifting \( E_{\text{Cl}} \) to –108 mV. This change caused a dramatic loss of the octopamine-induced inward current response (Fig. 5C and D). While under standard \( \text{Cl}^- \) conditions (measured at –90 mV) octopamine induced an increase in inward current of 149 ± 33 pA (\( n = 6 \)), this reversed to a negligible outward current of 13 ± 7 pA (\( n = 5 \)) with aspartate replacing \( \text{Cl}^- \) in the pipette. This result strongly suggests that octopamine activates chloride channels.

We then performed a limited number of initial experiments to study the signal transduction pathway underlying the activation of the \( \text{Cl}^- \) channel. The above experiments already indicated that the process is slow; the current still increased 10 min after application of octopamine. To see whether a phosphorylation step is involved, we tested the effect of the nonspecific protein kinase inhibitor H1004 on the octopamine-induced current. Fig. 6 shows that in the presence of 50 \( \mu \)M H1004, octopamine did not induce or only very slightly induced the inward \( \text{Cl}^- \) current, whereas subsequent application of only octopamine did evoke the normal response.

Interestingly, we observed that HEK293 cells stably expressing Lym \( \text{oa}_a \) showed the same current response to octopamine as cells expressing Lym \( \text{oa}_a \) (Fig. 7B). The effect, however, is not a general consequence of the activation of overexpressed, heterologous receptors, since stimulation of a \( \text{Lymnaea} \) serotonin receptor (5-HT\(_{2L}\),Ref. 23) and an octopamine receptor \( \text{(5-HT}_{2L,\text{lym}} \) stably expressed in HEK293 cells (23) did not show any effect on the \( \text{Cl}^- \) conductance (Fig. 7C). Furthermore, stimulation of \( \beta \)-adrenergic receptors that are endogenously present in HEK293 cells did not influence the inward current (Fig. 7D). Stimulation of Lym \( \text{oa}_a \), Lym \( \text{oa}_a \), 5-HT\(_{2L,\text{lym}} \), and \( \beta \)-AR all differentially influenced the outward current. These effects, however, were not studied in detail.

DISCUSSION

We have used a degenerate PCR strategy to isolate cDNAs encoding G-protein-coupled bioamine receptors that are expressed in the central nervous system of the pond snail \( \text{L. stagnalis} \). Recently, we reported the cloning and expression of a serotonin receptor (5-HT\(_{2L,\text{lym}} \)Ref. 23) and an octopamine receptor (Lym \( \text{oa}_a \); Ref. 16) using the same strategy. This paper describes the isolation of a cDNA encoding a second octopamine receptor, designated Lym \( \text{oa}_a \). The predicted amino acid sequence of Lym \( \text{oa}_a \) exhibits the highest similarity to the \( \text{Drosophila} \) tyramine/octopamine receptor, the \( \text{Locusta} \) tyramine receptor, and Lym \( \text{oa}_a \), and to the vertebrate \( \alpha \)-adrenergic receptors. Although both Lym \( \text{oa}_a \) and Lym \( \text{oa}_a \) encode \( \text{Lymnaea} \) octopamine receptors, their amino acid identity is only moderate, i.e., 45% in the TM regions. An interesting difference in the sequence of both octopamine receptors can be found in TM5. Mutagenesis studies on catecholaminergic receptors have indicated that two conserved serine residues in this domain (Ser\(^{204}\) and Ser\(^{207}\) in the \( \beta \)-adrenergic receptor) play a crucial role in ligand binding. Supposedly, the two Ser hydroxyl groups can hydrogen bond to the catechol hydroxyl groups of the ligand (see for instance Refs. 26–28). In octopamine receptors there is
no obvious need for conservation of both serine residues in TM5 because the aromatic ring of octopamine is monohydroxylated. Indeed, in Lym oa3, only a single serine is found at the relevant position in TM5. In Lym oa4, however, both serines are present. Interestingly, the catecholamines (epinephrine, norepinephrine, dopamine) show a considerably higher affinity for Lym oa4 than for Lym oa1 (see Table I). This suggests that the serine residues in TM5 play a role in agonist binding in the Lymnaea octopamine receptors similar to their role in the vertebrate catecholamine receptors.

In general, agonists exhibit higher affinities for Lym oa2 than for Lym oa1, while antagonists have higher affinities for Lym oa2 than for Lym oa1. Another interesting difference between the binding properties of both octopamine receptors is the opposite order of affinities for the isoschizomers rauwolscine and yohimbine. Lym oa1 has a higher affinity for yohimbine than for rauwolscine (pKi = 8.9 versus 7.5), while Lym oa2 has a higher affinity for rauwolscine than for yohimbine (pKi = 8.0 versus 6.2). Both the pharmacological profiles of Lym oa1 and Lym oa2 indicate a closer relationship to the α-adrenergic receptors than to the β-adrenergic receptors. In that respect it is noteworthy to mention the much higher affinity of the α1 antagonist prazosine for Lym oa1 (pKi = 7.0) than for Lym oa2 (pKi = 4.7). Whereas the pharmacological profile of Lym oa1 still shows a moderate similarity to that of the Drosophila tyramine/octopamine receptor, the Locusta tyramine receptor, and the α2-adrenergic receptors, the pharmacological profile of Lym oa2 clearly differs from that of Lym oa1, the tyramine receptors, the adrenergic receptors, and the insect octopamine receptor subtypes as described in tissue preparations.

Stimulation of Lym oa2 did not lead to the activation of the classical signal transduction pathways mediated by adenylyl cyclase and PLC. The receptor is, however, clearly able to transduce signals since we found that HEK293 cells expressing Lym oa2 showed octopamine-induced changes in their membrane conductance. More specifically, we observed a slow but large increase in the holding current of voltage-clamped HEK293 cells upon application of octopamine. At potentials below the Cl– equilibrium potential, this response was observed as an increase in inward current, most likely caused by an efflux of Cl– ions. The process underlying the opening of the Cl– channels may involve protein phosphorylation, since the (nonselective) protein kinase inhibitor HA1004 inhibited the inward current. Alternatively, the Cl– channels involved may need to be in a phosphorylated state to be able to open in response to the octopamine stimulus. Because the IV relationship of the current response proved to be linear and because no changes in inositol phosphates were observed upon octopamine application, the Cl– channels involved are suggested to be both voltage-independent and Ca2+–independent. Additional effects of octopamine on (voltage-dependent) outward currents were also observed but were not pursued in detail.

Interestingly, stimulation of HEK293 cells expressing the other Lymnaea octopamine receptor, Lym oa1, resulted in a similar outward Cl– current. In contrast to Lym oa2, Lym oa1 has previously been shown to activate both PLC and adenylyl cyclase. To examine whether the effect on the Cl– channel might be secondary to the effects on adenylyl cyclase and PLC or might represent an independent signaling route, we tested the effect of activation of two other receptors, expressed in HEK293 cells. Stimulation of these receptors, i.e. a Lymnaea serotonin receptor or an endogenous β-adrenergic receptor, led to activation of PLC and adenylyl cyclase, respectively. The subsequent rise in levels of diacylglycerol or cAMP will activate protein kinase C or protein kinase A, respectively. Stimulation of these receptors did, however, not show any effect on the inward current, suggesting that protein kinase C or protein kinase A is not involved in the phosphorylation process underlying Cl– channel activation.

It remains to be investigated whether stimulation of Lym oa2 in Lymnaea neurons will also affect the Cl– conductance. Activation of octopamine receptors on neurons of other snails has been described to lead to outward potassium currents (12–14) and inward calcium currents (15). Early studies on Aplysia
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The opening of this particular chloride channel could also be induced by activation of the thrombin receptor, the endothelin receptor, and the neurokinin A receptor. Since these receptors (as well as the lysophosphatidic acid receptor) all couple to PLC, it was suggested that the signaling pathway leading to the increased Cl⁻ conductance is closely associated with phosphoinositide hydrolysis (20).

All available data suggest that the chloride channel in HEK293 cells that we have found to be G-protein-activated is highly similar to the channel described by Postma et al. (20). Our data, however, exclude the option that phosphoinositide hydrolysis is important in the signaling pathway leading from the activated receptor to the opening of the chloride channel, since stimulation of Lym α₂ does not result in any change in intracellular concentrations of inositol phosphates. Also, activation of a serotonin receptor that has been shown to be coupled to the activation of PLC does not influence the outward Cl⁻ current. The G-protein-mediated activation of the chloride channel described by Postma et al. in Rat-1 fibroblasts (20) and by ourselves in HEK293 cells must proceed via an as yet unknown signaling pathway. Here we show that protein phosphorylation is important in this pathway but that protein kinase A and protein kinase C are not involved. The identification of the relevant kinase and the further examination of the signal transduction pathway will be the topics of future investigations.

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octopamine receptors (29) have shown that stimulation of neurons with octopamine induces the specific phosphorylation of a particular unidentified protein. In this case, however, it was suggested that the process might involve protein kinase A, since elevating the intracellular concentration of cAMP produced a similar effect.

Recently, it was shown that activation of the G-protein-coupled lysophosphatidic acid receptor present on fibroblasts leads to a long lasting depolarization of these cells due to an efflux of chloride ions (20). As was found in our studies, the signal transduction pathway responsible for opening the chloride channel was suggested to be independent of known second messengers.