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SIGNAL TRANSDUCTION IN OLFACTORY NEURONS

NINA S. LEVY, HEATHER A. BAKALYAR and RANDALL R. REED*

The Howard Hughes Medical Institute Laboratory of Genetics, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MA 21205, U.S.A.

Summary—Recent efforts in our laboratory have focused on cloning the molecular components involved in the cAMP-mediated pathway of olfactory signal transduction. These efforts have resulted in the isolation of olfactory-specific forms of a G protein, an adenylyl cyclase, and a cyclic nucleotide-gated cation channel. Functional expression of each of these proteins *in vitro* confirms their ability to carry out the function ascribed to them as part of a second-messenger cascade. Putative odorant-receptor molecules which constitute the first step in odorant signal transduction have now been cloned. We have generated oligonucleotide probes which recognize a population of olfactory receptors apparently more heterogenous than those previously reported. These probes should enable us to answer questions regarding the number of different receptors expressed per cell as well as the nature of receptor-ligand specificity.

The molecular basis of perception and discrimination of odorants by the mammalian olfactory system has long been an intriguing mystery. It is estimated that humans can perceive as many as 10,000 different odorants, some at concentrations as low as a few parts per trillion [1]. This would be analogous to tasting one teaspoon of salt dissolved in one million gallons of water. Clearly, the olfactory system has been designed to detect a wide variety of chemical compounds at exceedingly low concentrations and to propagate each of these signals in a unique manner in order for the brain to discriminate between the many different odorants. Recently, the application of molecular cloning techniques has resulted in the isolation of several components of the olfactory signal transduction pathway and has provided some insight into the biochemical processes responsible for the detection of odorant molecules.

Odorants are first encountered in the nasal lumen at the surface of the olfactory neuroepithelium. Each of the sensory neurons extends a single dendrite to the apical surface where it terminates in a knob-like structure from which several cilia project into the lumen. These cilia are thought to contain all of the machinery required for initial steps in olfactory signal transduction. The neuronal cell bodies project a single unbranched axon through the cribriform plate and synapse on target cells in the olfactory bulb. Interestingly, the olfactory neurons continuously undergo cell death and regeneration from a precursor basal-cell population present in the neuroepithelium. A rapid and extensive loss of olfactory sensory neurons can be induced by severing the olfactory nerve or by removing the target tissue, the olfactory bulb.

Signal transduction in olfactory tissue appears to follow a pathway similar to that of many neurotransmitters and peptide hormones (Fig. 1). Each of these signalling systems utilizes a cell-surface transmembrane receptor which, when ligand is bound, undergoes a conformational change facilitating interaction with a GTP-binding protein. Members of this class of guanine nucleotide-binding proteins (G proteins) are heterotrimers made up of α, β, and γ subunits [2]. Interaction of the activated receptor with the G protein results in exchange of GDP for GTP on the α subunit and the dissociation of the α subunit from the β and γ subunits. The activated Gα subunit interacts with the effector protein such as adenylyl cyclase. In the many G protein-mediated receptor pathways, there are variations on this theme with respect to the α subunit of the G protein and the second-messenger enzymes that are activated. To date, there are about ten different Gα subunits, some of which stimulate adenylyl cyclase ($G\alpha_s$) as discussed previously, while others inhibit the same enzyme ($G\alpha_i$). Other known Gα subunits stimulate cGMP

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*To whom correspondence should be addressed.

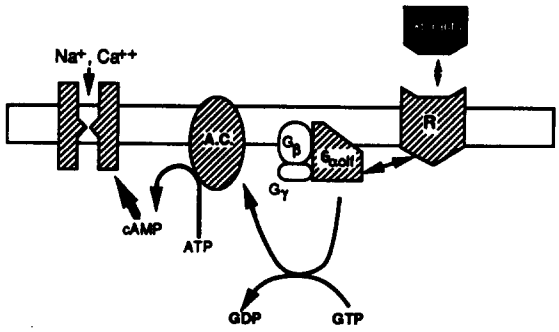

Fig. 1. The cAMP-mediated signal transduction pathway in olfactory neurons. Binding of odorant molecules to a transmembrane receptor (R) causes a conformational change in the receptor and leads to an interaction with a heterotrimeric G protein. Bound GDP is replaced by GTP on the Gα subunit, $G\alpha_{olf}$, and following dissociation from the β and γ subunits, $G\alpha_{olf}$ activates adenylate cyclase (A.C.). Increases in cAMP concentration lead to the opening of a cyclic nucleotide-responsive cation channel and results in depolarization of the cell.

phosphodiesterase ($G\alpha_T$), phospholipase C ($G\alpha_q$), open potassium channels ($G\alpha_k$), and some have uncharacterized functions ($G\alpha_o$).

Previous biochemical evidence suggested that at least some odorants could stimulate adenylyl cyclase activity in partially purified cilia preparations [3, 4]. This activation was dependent on the presence of GTP. In olfactory tissue, the activation of adenylyl cyclase leads to an increase in the concentration of cAMP and the opening of a cyclic nucleotide-responsive cation channel [5]. Subsequent depolarization of the cell initiates the generation of an action potential. These data led to studies in our laboratory attempting to identify components of the cAMP-dependent signal transduction pathway in olfactory neurons.

G_{olf}

Gα subunits share considerable homology at both the protein and nucleotide sequence level. Screening of a rat olfactory cDNA library with DNA probes homologous to well conserved regions of Gα proteins resulted in the isolation of six distinct classes of Gα subunits [6, 7]. One class was expressed exclusively and abundantly in olfactory tissue. In addition, following removal of the olfactory bulb and degeneration of the mature neurons, mRNA for this new class was greatly decreased, indicating that this Gα subunit, termed G_{olf}, was expressed primarily in the mature neurons. Antibodies prepared against G_{olf} were used to further localize this protein to the dendritic knobs and the cilia of the sensory neurons. The function of G_{olf} was investigated using a murine lymphoma cell line lacking $G\alpha_s$ (S49 cyc-cells). Recombinant retrovirus encoding G_{olf} was used to obtain stably transformed cell lines expressing this protein. In the presence of GTPγS, an activator of Gα subunits, cells expressing G_{olf} were able to stimulate adenylyl cyclase activity. G_{olf} is therefore competent to mediate the effector function thought to be responsible for olfactory signal transduction.

ADENYLYL CYCLASE

Evidence for the existence of a novel form of adenylyl cyclase in olfactory neurons was obtained using a monoclonal antibody that recognizes the calcium/calmodulin-sensitive enzyme in the brain [8]. Immunoblot analysis detected an adenylyl cyclase species in olfactory cilia that was of a different molecular weight than the brain enzyme. Using protein sequence derived from a highly purified preparation of bovine brain adenylyl cyclase, an oligonucleotide was designed and used to screen both bovine brain and rat olfactory cDNA libraries. Several rounds of cDNA screening led to the identification of three distinct forms of adenylyl cyclase [9]. The type I form corresponds to the bovine brain enzyme and is expressed only in that tissue. Type II adenylyl cyclase is expressed in the brain as well as several peripheral tissues. A third adenylyl cyclase species, type III, was isolated from the rat olfactory library and its expression appears to be confined to that tissue. Moreover, when the abundance of the type III mRNA was examined in normal and neuron-depleted olfactory neuroepithelium, there was a dramatic decrease in the mRNA levels in the neuron-depleted epithelium. This decrease paralleled that seen for G_{olf}. Localization of the type III protein by immunohistochemistry was also shown to match that seen for G_{olf}, namely, that type III adenylyl cyclase was concentrated in the olfactory cilia.

The biochemical properties of type III adenylyl cyclase were examined in a human kidney cell line (293 cells) that has low endogenous adenylyl cyclase activity [9]. Cell homogenates from stably-transformed cell lines were assayed for enzyme activity. In the presence of forskolin, a direct activator of cyclase, cell lines expressing both type I and type III adenylyl cyclase showed about 5-fold increased activity as compared to untransformed cells. A similar result was obtained in the presence of aluminum fluoride, a

direct activator of the endogenous stimulatory G protein, G_s. This suggests that the endogenous G_s present in 293 cells can activate the type III as well as the type I adenylyl cyclase. Interestingly, the type III enzyme had a low basal activity, similar to that seen in control cells, while the type I enzyme had a 30-fold higher basal activity. This low basal activity suggests that the concentration of cAMP in unstimulated olfactory cilia is low and therefore even small increases in cAMP may be sufficient to trigger the depolarization of the sensory neuron.

cAMP-GATED CATION CHANNEL

Using the coding region for the cGMP-gated channel from bovine rod, a rat olfactory cDNA library was screened at low stringency and a single class of clones was isolated [10]. Sequence analysis indicated that this cDNA could encode a protein with 57% homology to the photoreceptor channel. The most highly conserved region between the two channels lies at the carboxy terminus in a region implicated as the cyclic nucleotide-binding site. The two channels diverge greatly at the amino terminus in a region predicted to reside on the cytoplasmic side of the plasma membrane. Northern blot analysis revealed an abundant message in RNA from olfactory epithelium. The level of expression of this message is significantly reduced in RNA from bulbectomized animals. This pattern was also seen for G_{olf} and type III adenylyl cyclase, and indicates that the channel is specifically expressed in the mature neurons.

The electrophysiological properties of the olfactory channel were investigated by transient expression in 293 cells [10]. Inside-out patches of plasma membrane were excised from the transfected cells and tested for sensitivity to bath-applied cyclic nucleotides. Only cells transfected with the olfactory channel, and not mock-transfected cells, showed a cyclic nucleotide-induced current. All three of the cyclic nucleotides (cAMP, cGMP and cCMP), tested at saturating concentrations, resulted in a maximum current of about 1 nA. The olfactory channel was most sensitive to cGMP ($K_{1/2}$ for activation = 2 μM), followed by cAMP ($K_{1/2}$ = 40 μM) and cCMP ($K_{1/2}$ = 80 μM). Voltage–current relationships measured under different ionic conditions indicated that the channel was about equally permeable to K^+ and Na^+, and only slightly permeable to Ca^{2+}. These findings are consistent with those reported for the native channel in olfactory cilia [5] and suggests that this novel cDNA species encodes the channel responsible for olfactory signal transduction. Also of interest is the finding that both the native and the transiently expressed olfactory channel were 20-fold more sensitive to cGMP than to cAMP in spite of the central role that cAMP is suggested to play in odorant detection.

ODORANT RECEPTORS

The most recent elements of the olfactory signal transduction pathway to be identified are the genes encoding putative odorant receptors. Many neurotransmitter and hormone receptors which transduce intracellular signals by activating specific G proteins have been cloned and sequenced. The deduced protein sequences indicated that all of these receptors traverse the membrane seven times while sharing modest amino acid homology in the transmembrane domain regions [11]. The similarity between the predicted pathway of olfactory signal tranduction and that of neurotransmitters and

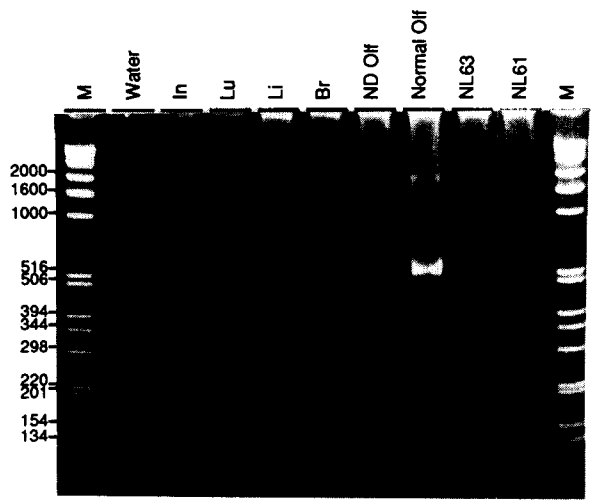

Fig. 2. PCR products obtained with degenerate oligonucleotides homologous to olfactory receptors. Oligonucleotides were designed to the second and sixth transmembrane domains of olfactory receptors [12]. The reactions contained 1 ng of 1st strand cDNA in a 25 μl vol. Reaction conditions were as described in the GeneAmp Kit protocol from United States Biochemical Corp. with the following temperature selections: 48°C for 1 min, 72°C for 1 min, 94°C for 45 s, all repeated for 35 cycles. Samples were concentrated and run on a 5% acrylamide gel. All the reactions contained 1 μM of each degenerate oligonucleotide (NL61 and NL63) with the exception of the two reactions which contained only the oligonucleotide indicated at the top. The templates used in the three rightmost reactions were from normal olfactory (olf) tissue. Other tissues analyzed were: ND-neuron depleted olfactory tissue, Br—brain, Li—liver, Lu—lung and In—intestine. M—DNA markers—sizes indicated on the left are in base pairs.

hormones which act through G proteins suggested that olfactory receptors would also be members of this seven-transmembrane-domain superfamily of receptors. Degenerate oligonucleotides homologous to conserved regions in the second and seventh transmembrane domains were used in a polymerase chain reaction (PCR) to amplify receptor sequences from olfactory cDNA [12]. One of the PCR products was subsequently shown to contain a heterogeneous population of molecules which shared homology to the superfamily of G protein-coupled receptors.

Screening of a rat olfactory cDNA library with PCR probes resulted in the isolation of 18 unique receptor species [12]. Sequence analysis of ten of these clones showed that the receptors share many sequence motifs yet they displayed notable sequence divergence in the third, fourth and fifth transmembrane domains. Expression of these receptors was shown by Northern blot analysis to be restricted to olfactory epithelium.

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                TM II                                                TM III
F3    PMYFFLSNLSFVDICFISTTVPKML----VNIQTQNNVITYAGCITQIYFFLLFVELDNF
F5    PMYFFLSNLSFVDVCFSSTTVPKVL----ANHILGSQAISFSGCLTQLYFLAVFGNMDNF
F6    PMYFFLCNLSFLEIWFTTACVPKTL----ATFAPRGGVISLAGCATQMYFVFSLGCTEYF
F12   PMYFFLANLSFVDICFTSTTIPKML----VNIYTQSKSITYEDCISQMCVFLVFAELGNF
I3    PMYLFLSNLSFSDLCFSSVTMPKLL----QNMRSQDTSIPYGGCLAQTYFFMVFGDMESF
I7    PMYFFLANMSFLEIWYVTVTIPKMLAGFIGSKENHGQLISFEACMTQLYFFLGLGCTECV
I8    PMYLFLSNLSFVDICFISTTVPKML----VNIQTQNNVITYAGCITQIYFFLLFVELDNF
I9    PMYLFLSNLSFADLCFSSVTMPKLL----QNMQSQVPSIPYAGCLAQIYFFLFFGDLGNF
I14   PMYLFLSNLSFSDLCFSSVTMPKLL----QNMQSQVPSISYTGCLTQLYFFMVFGDMESF
I15   PMYLFLSNLSFSDLCFSSVTMPKLL----QNMQSQVPSIPFAGCLTQLYFYLYFADLESF

OR1   PMYLFLASLACVDTWLSSTVTPKML----QDIFAKSKLISFSECVIQFFSFVVSATTECF
OR2   PMYLFLSNLAFVDTWLSSTVTPKML----FNLLDKGKVISIAECMTQFFSFAISVTTECF
OR4   PMYFFLSNLSFIDVCHSTVTVPKML----SDTLSDEKVISFDACVVQIFFLHLFACTEIF
OR5   PMYLFLTFLSFSETCYTLGIIPRML----SGLVMGGQVISFMGCATQMFFSASWACTNCF
OR6   PMYLFLSYLSLVEVCYCSVTAPKLI----FDSLLKRKVISLQGCITQIFFLHFFGGTGIF
OR7   PMYLFLASLAFVDTWLSSTVTPKML----LDFFTKSKLISFSECMIQFFSFGISGTTECF
OR8   PMYLFLSNLSFVDICFISTTVPKML----VNIQTQNNVITYAGCITQIYFFLLFVELDNF
OR9   PMYLFLSNLAFVDTWLSSTVTPKML----FNLLDKGKVISVAECMTQFFSFAISVTTECF
OR10  PMYLFLSNLAFVDTWLSSTVIPKML----FNLLDKGKVISIAECKTQFFSFAISATTECF
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                                                TM IV
F3    LLTIMAYDRYVAICHPMHYTVIMNYKLCGFLVLVSWIVSVLHALFQSLMMLALPFCTHLEI
F5    LLAVMSYDRFVAICHPLHYTTKMTRQLCVLLVVGSWVVANMNCLLHILLMARKSFCADNMI
F6    LLAVMAYDRYLAICLPLRYGGIMTPGLAMRLALGSWLCGFSAITVPATLIARLSFCGSRVI
F12   LLAVMAYDRYVAXCHPLCTYVIVNHRLCILLLLLSWVISIFHAFIQSLIVLQLTFCGDVKI
I3    LLVAMAYDRYVAICFPLHYTSIMSPKLCTCLVLLLWMLTTSHAMMHTLLAARLSFCENNVV
I7    LLAVMAYDRYVAICHPLHYPVIVSSRLCVQMAAGSWAGGFGISMVKVFLISRLSYCGPNTI
I8    LLVAMAYDRYVAICFPLHYTNIMSHKLCTCLLLVFWIMTSSHAMMHTLLAARLSFCENNVL
I9    LLVAMAYDRYVAICFPLHYMSIMSPKLCVSLVVLSWVLTTFHAMLHTLLMARLSFCEDSVI
I14   LLVVMAYDRYVAICFPLRYTTIMSTKFCASLVLLLWMLTMTHALLHTLLIARLSFCEKNVI
I15   LLVAMAYDRYVAICFPLHYMSIMSPKLCVSLVVLSWVLTTFHAMLHTLLMARLSFCADNMI

OR1   LLAAMAYDRYVAICKPLLYPVIMTNRLCVRLLTLSLVGGFIHALIHGGFLFRLIFCNSNII
OR2   LFAAMAYDRYAAICNPFLYPVVMTNSLCIRLLALSFVGGFLHAVIHESFLSRLTFCNSNIL
OR4   LLSVMAYDRYVAICKPFEDMTIMNWKVCTVLGVAMWTAGTVHSISFTSLTIKLPYCGPDEI
OR5   LLSVMGFDRYVAICAPLHYPSRMNPTVCAQLVGTSFLSGYLFGMGMTLVIFRLSFCSSHEI
OR6   LLIVMAYDRYVAICKPLHCVTIMNRRVCGLLVGAAWSGGLLHSAGQTFLIFQLPFCGPNVI
OR7   LLAAMAYDRYVAICKPLLYPVIMTNRLCVRLLTMSFIGGFIHTFIHAVFLFRLTFCDSNII
OR8   LLTIMAYDRYVAICHPMHYTVIMNYKLCGFLVLVSWIVSVLHALFQS
OR9   FLAAMAYDRYAAICNPLLYPVVMTNRLCIRLLALSFVGGFLHAVIHGS
OR10  LLAAMAYDRYAAICNPFLYPVVMTNRLCIRYGLVIFRVAFFMTVIHES
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Fig. 3. Amino acid sequence comparison of olfactory receptors described by Buck and Axel [12] and receptors cloned from a 550 bp PCR product made with degenerate oligonucleotides NL61 and NL63. Putative membrane spanning regions are indicated by a horizontal line along the top of the sequence. Boxed amino acids indicate regions where the receptors generated with NL61 and NL63 (OR1–OR10) diverge from conserved residues in previously reported olfactory receptors (F3–I15). OR8–OR10 contain a Hind III site shortly after the fourth transmembrane domain. Sequences downstream of this site were not recovered during cloning. The first six amino acids in the second transmembrane domain, PMY(L/F)FL, are contained in NL61. NL63 encodes the amino acid sequence TC(G/A/S)SHL from transmembrane domain six.

Genomic blot analysis showed that individual cDNA clones were able to recognize several bands, indicating the presence of many subfamilies within the larger odorant receptor family. The number of odorant receptors was estimated to be between 100 and several hundred based on screens of rat genomic libraries.

We have designed degenerate oligonucleotides to regions in the second and sixth transmembrane domains that were highly conserved among the olfactory-specific receptors and not present among other members of the seven-transmembrane-domain receptor superfamily. Using these oligonucleotides in a PCR reaction with olfactory cDNA, we obtained a singe band at the appropriate molecular weight (550 bp) for the predicted size of an olfactory receptor (Fig. 2). The intensity of the band was decreased about 5-fold in a similar PCR reaction using cDNA from bulbectomized animals as a template. No bands were apparent using cDNA derived from other tissues in the PCR reaction.

This PCR product was cloned into a plasmid vector and nine recombinants were chosen randomly and sequenced. The results revealed nine unique receptor-like sequences with homology to the previously reported sequences but with many differences at the amino acid and nucleotide level (Fig. 3). Some amino acids which were completely conserved among the ten previously reported sequences were divergent in this new set of receptors. Examples include the well conserved tryptophan in transmembrane four which is shared by non-olfactory-specific receptors as well. Six out of nine sequences have this amino acid replaced by phenylalanine or serine. In transmembrane domain two, a well conserved asparagine is replaced in four out of nine sequences. This indicates that the PCR probes used in our experiment are detecting a larger proportion of this putative olfactory receptor family. Screening of a rat olfactory cDNA library resulted in approx. 0.02–0.05% positive clones. This figure is 5–10-fold greater than the number of clones reported previously, and may result from a greater complexity present in the PCR-generated probe that we have used. Screening of a rat genomic library at low stringency with the same 550 bp PCR probe resulted in approx. 0.1% positive phage and suggests a very large family of related receptors.

CONCLUSIONS

The major molecular components of the cAMP-mediated olfactory signal transduction pathway have been identified. Interestingly, the olfactory system has evolved novel forms of a G protein, adenylate cyclase, and cation channel involved in signal transduction. The existence of a large family of G protein-coupled receptors expressed by the sensory neurons may provide for the diversity in odorant recognition. The mechanism for the regulation of expression of this complex gene family and the specificity of ligand binding by individual receptors can now be addressed.

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