Chemical reception in vertebrate olfaction: evidence for multiple transduction pathways

BERNARDO MORALES[1] and JUAN BACIGALUPO[2]

[1] Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
[2] Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

Odorant detection takes place at the receptor neurons of the olfactory epithelium and odorant discrimination relies in an important degree on these chemosensory cells. Here we review the evidence for the participation of multiple transduction pathways in the mechanisms of odor recognition in olfactory neurons.

Key terms: *cAMP, chemotransduction, excitation, inhibition, IP3, membrane conductance, olfactory neuron.*

INTRODUCTION

The sense of smell is essential for most animal species. Among vertebrates, some species are capable of recognizing a remarkable variety of odorants; for example, humans have the ability of perceiving several thousand different odorants. In other mammals, like the dog and the rabbit, the variety of detectable odorants is even wider. How do these animals accomplish this impressive feature?

The initial events in olfaction takes place at the olfactory epithelium, the peripheral olfactory organ of vertebrates. This is a pseudostratified epithelium formed by three major cells types: olfactory neurons, sustentacular cells, and basal cells. The olfactory neurons are primary chemosensory cells, specialized on odorant detection. They are bipolar cells with their somata located near the center of the epithelial layer. They project from their apical end a single dendrite that reaches the external surface of the tissue, where it forms a spherical dilatation termed the dendritic knob. This knob is the site of insertion of a variable number of chemosensory cilia. The early steps in olfaction takes place in these cilia and, perhaps to some extent, in the olfactory knob. The opposite end of the neuronal cell body projects a thin unmyelinated axon that reaches the olfactory bulb, where it makes synapse with a mitral cell. The sustentacular and the basal cells of the olfactory epithelium do not directly participate in olfactory reception and can be easily distinguished from the receptor neurons both morphologically and electrophysiologically.

The interest of numerous laboratories have focused on the study of the mechanisms involved in the generation of the responses to odorants at the level of the olfactory epithelium. Modern techniques from electrophysiology, biochemistry and molecular biology have allowed considerable progress on the understanding of olfactory transduction, the process by which the receptor cells of the olfactory epithelium generate electrical responses to odorants. Several chemotransduction mechanisms have been proposed, including mechanisms that involve

Correspondence to: Dr Bernardo Morales. Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda B O'Higgins 340, Casilla 114-D, Santiago, Chile. Fax: (56-2) 222-5515. Phone: (56-2) 222-686-2870. E-mail: bmorales@genes.bio.puc.cl

second messenger cascades and others in which odorants would directly gate ionic channels. This review discusses the evidence for each of them.

OLFACTORY TRANSDUCTION MECHANISMS INVOLVING SECOND MESSENGER PATHWAYS

Two different cascade processes have been identified in vertebrate olfactory neurons, one involving cyclic AMP (cAMP) and another one involving inositol trisphosphate (IP_3). Of both, by far the best understood is the cAMP pathway, which underlies the excitatory response to odors. In this cascade process, the binding of an odor molecule to a membrane receptor activates a GTP-binding protein, which in turn induces the activation of adenylyl cyclase. This enzyme is responsible for elevating cAMP levels; this cyclic nucleotide directly gates ionic channels at the ciliary membrane (Restrepo *et al*, 1996).

The available evidence for the IP_3 pathway is still incomplete and neither the molecular components participating on it nor its electrophysiological target have been clearly defined.

Olfactory receptors

A key component of chemotransduction pathways involving second messengers are the olfactory receptor proteins. These receptors are integral membrane proteins that specifically bind odorants. They are confined to the ciliary membrane, where they make direct contact with incoming odorants. The presence of receptor proteins was inferred from biochemical (Rhein and Cagan, 1983) and electrophysiological (Caprio and Byrd, 1984) studies in fish, and from the existence of some selective anosmias in humans (Doty *et al*, 1978). However, their existence was very difficult to demonstrate. The most relevant contribution in this field came from the work of Buck and Axel (1991) in the rat olfactory system. Based on the notion that olfactory transduction involves the participation of a G-protein, these authors reasoned that the odorant receptors may be members of the superfamily of G-protein coupled receptors. Using the polymerase chain reaction and oligonucleotide primers with conserved regions of several receptors belonging to this superfamily, they were able to clone and characterize a gene family that encodes for putative odorant receptors. The gene products that they obtained presented the seven transmembrane domains that are characteristic of the structure G-proteins coupled receptors and their expression was confined to the sensory neurons of the olfactory epithelium (Buck and Axel, 1991). The amino acid sequences of the different putative receptors exhibited great divergence in the third, fourth, and fifth transmembrane segments, corresponding to the ligand-binding regions of other G-protein coupled receptors, suggesting that the recognition of odorants may be related to these variable regions of the receptor sequence. Members of this new multigene family have also been found in human (Selbie *et al*, 1992), rat (Raming *et al*, 1993), and fish (Ngai *et al*, 1993a) olfactory neurons and, interestingly, also in spermatozoa, which respond to chemical signals present in the environment (Parmentier *et al*, 1992).

The ability of cloned odorant receptor proteins to bind odorants and activate second messenger cascades was addressed by expressing receptor-encoding complementary DNA from rat in an insect cell line (Raming *et al*, 1993). Only one member of the cloned receptor family was effective on conferring to the transfected cells the ability to respond to odorants. Stimulation with a mixture of the odorants lyral and lilial induced an increase in IP3 levels in a membrane preparation derived from these cells (Raming *et al*, 1993). This evidence supports the notion that the cloned proteins are the odorant receptors. It remains to be demonstrated whether a similar effect of odorants occurs *in vivo*.

Recent evidence obtained from *in situ* hybridization studies have suggested that an individual olfactory neuron expresses only one or very few receptors of the same subfamily (Ngai *et al*, 1993b; Ressler *et al*, 1993; Vassar *et al*, 1993). Electrophysiological evidence have demonstrated that an olfactory neuron may generate excitatory and inhibitory responses to different odorants

(Morales *et al*, 1994; Kang and Caprio, 1995), consistent with the notion that olfactory neurons express more than one receptor protein. The presence of different receptor proteins associated to distinct electrophysiological responses in the same cell would increase the diversity of firing patterns, which may be relevant for odor recognition.

The cyclic AMP pathway in olfactory transduction

Cyclic AMP role as second messenger in olfactory chemotransduction was demonstrated by biochemical studies done on ciliary-enriched membrane preparations. With a few exceptions, stimulation of these membranes with floral, fruity, minty and herbaceous odorants increased adenylyl cyclase activity (Pace *et al*, 1986; Sklar *et al*, 1986). However, another important group of odorants, including putrid odors and organic solvents, failed to activate this enzyme (Sklar *et al*, 1986), suggesting that transduction of these odorants operates by a different mechanism. Moreover, Breer and colleagues found that those odors that failed to induce a raise in cAMP, incremented the IP_3 levels (Boekhoff *et al*, 1990). Ronnett and collaborators (1993) found that both, IP_3 and cAMP were increased with different potencies by all tested odorants in cultured rat olfactory neurons.

The odorant-induced increase on cAMP levels was GTP-dependent and it was inhibited by cholera toxin, suggesting the participation of G_s-type protein (Pace *et al*, 1986; Sklar *et al*, 1986). Molecular cloning revealed that this G-protein was peculiar of olfactory neurons, and was given the name of G_{olf} (Jones and Reed, 1989). However, this protein has also been found in the central nervous system (Herve *et al*, 1995). Using fast kinetics methodology on membranes derived from rat olfactory cilia, Breer and colleagues (1990) found that the elevation of cAMP induced by odorants presented a fast early transient that developed in less than 50 ms, followed by a much smaller plateau phase. The relaxation of the response could be prevented by walsh inhibitor, a blocker of protein kinase A, indicative that phosphorylation of the receptor protein determines the transient nature of the odorant-induced changes in cAMP (Boekhoff and Breer, 1992). The time course of the increase in cAMP is compatible with the latency of the excitatory current triggered by odorants (Firestein *et al*, 1991a,b).

The involvement of the cAMP cascade in olfaction received strong support from electrophysiological studies using the patch-clamp technique. The earliest electrophysiological evidence that cAMP mediated the olfactory transduction was obtained by Nakamura and Gold (1987), who showed that excised patches from toad olfactory cilia contained a conductance that was directly gated by cyclic nucleotides. This cyclic nucleotide-gated (CNG) current presented a reversal potential similar to that of the macroscopic odor-induced current (Trotier and MacLeod, 1983). They also found that cyclic GMP (cGMP) was more effective on activating this conductance than cAMP. The physiological significance of this result is still unclear (see below), since the available evidence shows that adenylyl cyclase rather than guanylyl cyclase is the main target of the odorants (Breer *et al*, 1990; Lancet and Pace, 1987). These evidence supported the idea that cAMP is the main natural gating messenger for the odorant-dependent channel. Studies using on-cell patches showed that a channel identical than the one activated by cAMP in excised patches could be activated both by odorant stimulation and by extracellular application of membrane-permeant analogues of cyclic nucleotides (8-Br-cAMP and 8-Br-cGMP) or of the phosphodiesterase inhibitor, isobutyl-methyl-xanthine (IBMX; Firestein *et al*, 1991a,b). These results established the identity of the CNG channel with the one activated by odorants. The α-subunit of the CNG channel has been cloned and expressed in *Xenopus laevis* oocytes. The cloned channel presents close similarity with the cGMP-gated channel of vertebrate photoreceptors (reviewed by Zufall *et al*, 1994; Kaupp, 1991).

Receptor currents triggered by odorants have been recorded in isolated olfactory neurons from various vertebrate species using the whole-cell patch-clamp technique (Trotier, 1986; Firestein and Werblin, 1989;

Firestein *et al*, 1990; Morales *et al*, unpublished). Cells stimulated with brief odorant pulses (< 1 s) developed a cationic current after a latency of a few hundred milliseconds, consistent with the involvement of an enzyme cascade (Firestein and Werblin, 1989; Firestein *et al*, 1990; Morales *et al*, unpublished). Dialysis of olfactory neurons with GTP-Γ-s (a hydrolysis-resistant analog of GTP), forskolin (cyclase activator) and cyclic nucleotide analogues gave rise to ionic currents that resembled the odorant-activated current (Kurahashi, 1990; Firestein *et al*, 1991a; Frings and Lindemann, 1991). A similar effect was induced by the external application of IBMX, 8-Br-cAMP, and 8-Br-cGMP onto isolated olfactory neurons (Firestein *et al*, 1991a; Morales *et al*, unpublished). It is believed that the cyclic nucleotide-triggered inward current is carried mainly by Na^+ and Ca^{2+} during the odor response (reviewed by Zufall *et al*, 1994), resulting on an increase in intracellular Ca^{2+}. This Ca^{2+} in turn activates a Ca^{2+}-dependent Cl^- channel at the ciliary membrane, originally described by Kleene (1993). The Cl^- current accounts for a substantial fraction of the transduction current, amounting 40% in the newt and 85% in the rat (Kurahashi and Yau, 1993; Lowe and Gold, 1993). This

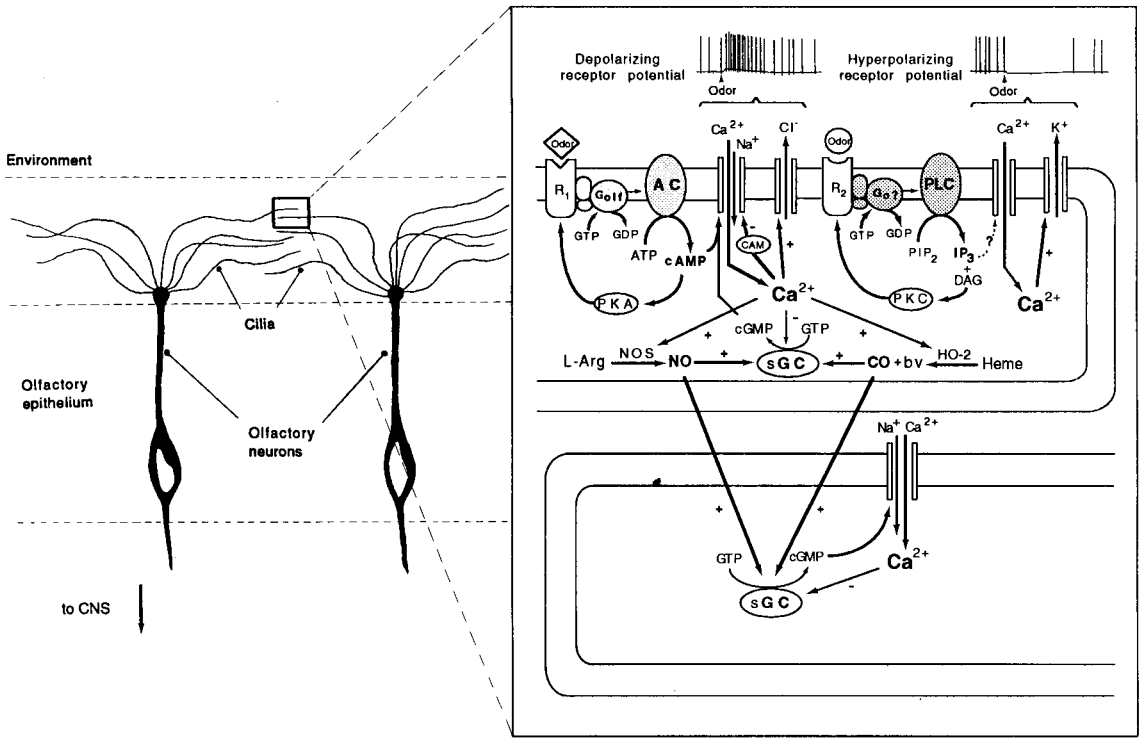

Fig 1. Model for chemotransduction integrating the various second messenger-mediated pathways implicated in odorant recognition. Left part: Diagram of two chemosensory cells of the olfactory epithelium. Right part: Schematic representation of an enlarged view of two cilia, illustrating the main transduction mechanisms. The cAMP pathway involves an odorant receptor protein (R1), a GTP-binding protein (Golf), an adenylyl cyclase type III (AC) that induces an increase in cAMP levels, and two ionic channel species: a cation selective channel and a Ca^{2+}-dependent Cl^- channel, both responsible for a depolarizing receptor potential that leads to action potential firing. Ca^{2+}/calmodulin (CAM) inhibits the CNG channel. Another pathway involves a different odorant receptor protein (R2), a G-protein (Go or Gi), a phospholipase C (PLC) that produces IP_3 and diacylglycerol (DAG), and a Ca^{2+} channel which is directly or indirectly gated by IP_3 (Tareilus *et al*, 1995). A Ca^{2+}-activated K^+ channel is also included as part of this pathway. This latter channel is responsible for a hyperpolarizing receptor potential that decreases the firing frequency (Morales *et al*, 1994, 1995). Both second-messenger pathways are terminated by the action of protein kinases on the receptor proteins. Two other mechanisms implicating gaseous second messengers are included. One of them involves the NO/cGMP system: L-arginine (L-arg), the enzyme NO synthase (NOS) and the diffusible messenger nitric oxide (NO) that stimulates soluble guanylyl cyclase (sGC), incrementing cGMP levels. cGMP gates the CNG channel, increasing intracellular Ca^{2+} levels. Ca^{2+} inhibits the sGC. The other mechanism involves the CO/cGMP system, which implicates the degradation of heme by the enzyme heme oxygenase 2 (HO-2), producing CO and biliverdin (bv). CO also stimulates the production of cGMP by acting on sGC. The + sign indicates activation and the - sign, inhibition.

seems to be the case also in *Xenopus* olfactory neurons (Zhainazarov and Ache, 1995). Thus, the CNG channel has two major roles, to carry a fraction of the transduction current and to increase intracellular Ca^{2+}, activating the Cl^- channels.

In summary, the interaction of odorant molecules with receptor proteins in the olfactory cilia trigger a sequence of biochemical events that increases intracellular cAMP levels (Fig 1). G_{olf} and adenylyl cyclase are involved in this cascade. Cyclic AMP directly gates a membrane conductance permeant to Ca^{2+} and other cations. These Ca^{2+} ions in turn activate a Ca^{2+} sensitive Cl^- conductance. Both currents are responsible for an excitatory receptor potential, which produces an increase on action potential firing. Protein kinase A participates in the termination of this cascade, by phosphorylating the receptor protein.

The IP_3 pathway in olfactory transduction

Unlike the cAMP pathway, the IP_3 pathway is still poorly understood. Huque and Bruch (1986) found that certain odorants like L-alanine and L-arginine, activate phospholipase C (PLC) in a membrane fraction derived from catfish olfactory cilia, suggesting that IP_3 is a second messenger in chemotransduction. The activation of PLC could be induced either by GTP or GTP-Γ-s, indicating the participation of a G-protein in this transduction mechanism, as in the cAMP pathway. Boekhoff and collaborators (1990), using fast kinetics measurements in rat olfactory cilia membranes, showed that odors that fail to affect the levels of cAMP (*i.e.*, putrid odorants) incremented the IP_3 levels. This increase reached a peak 25-50 ms after of onset of the odorant stimulus, decaying to basal level in ~500 ms (Breer *et al*, 1990). The relaxation of the response was dependent on protein kinase C, because it could be blocked by sphingosine, suggesting that this relaxation involved phosphorylation of olfactory receptors (Boekhoff and Breer, 1992). Furthermore, IP_3 stimulation was blocked by pre-treatment with pertussis toxin, consistent with the participation of a $G_{o\alpha}$-protein in the IP_3 pathway (Breer *et al*, 1990). Accordingly, rat and toad olfactory cilia present high levels of G_o and G_i (Jones *et al*, 1990; Schmidt and Monasterio, 1990). Similar results have been obtained in catfish (Restrepo *et al*, 1993).

The electrophysiological data for the IP_3-mediated odor transduction are not conclusive and the target for IP_3 has not been determined. Attempts to mimic the action of odors by artificially increasing intracellular IP_3 levels in salamander (Firestein *et al*, 1991a) and frog olfactory neurons (Lowe and Gold, 1993) have been unsuccessful, casting doubt on the possible role of IP_3 as a second messenger in olfactory transduction. Dialysis of catfish olfactory neurons with IP_3 gave rise to a transient depolarization that was sensitive to ruthenium red, a known blocker of the ryanodine-sensitive Ca^{2+} channels of muscle cells (Restrepo *et al*, 1990; Miyamoto *et al*, 1992). These results, taken together with the observation that L-amino acids induced a Ca^{2+} current in these receptor neurons (see above), suggest that IP_3 mediates an excitatory response by activating a plasma membrane Ca^{2+} conductance in the catfish; the associated Ca^{2+} influx would subsequently open Ca^{2+}-activated K^+ channels, repolarizing the membrane and making the response transient (Restrepo *et al*, 1990). However, *in vivo* recordings from catfish individual olfactory neurons showed that L-alanine induce an inhibition of action potential firing (Kang and Caprio, 1995), in disagreement with the previously mentioned conclusion. Further work is required to clarify this controversy.

A sustained depolarization induced by IP_3 in rat olfactory neurons has recently been reported (Okada *et al*, 1994). In addition, the direct activation of single-channel currents by IP_3 has been observed in bilayers doped with a membrane preparation from rat olfactory cilia (Restrepo *et al*, 1992; Lischka *et al*, 1995). These results are consistent with an involvement of IP_3 in mammalian olfactory transduction. In support of this notion, immunocytochemical evidence have indicated the presence of IP_3 receptors in the membrane of rat olfactory cilia (Cunningham *et al*, 1993).

An alternative model that may explain the effect of IP_3 in olfactory neurons derives from recent results reported by Morales *et al*

(1994, 1995). These authors found that odorants that trigger an increase in IP_3 in rat olfactory cilia inhibited action potential firing in isolated toad olfactory neurons. In contrast, these cells could respond with a depolarizing receptor potential and an associated increase in firing to odorants that activate adenylyl cyclase in cilia from the same toad (Schmidt and Monasterio, 1990) and from rat (Boekhoff *et al*, 1990). However, there is no evidence that odorants that increase IP_3 in rat olfactory cilia have the same effect on the toad. A similar observation was more recently reported in catfish olfactory neurons, in which L-alanine, an amino acid that stimulates IP_3 production, inhibited firing (Kang and Caprio, 1995), whereas an amino acid that induced an increase cAMP triggered an increase in firing.

Odorant inhibition in the toad was shown to be due to a hyperpolarizing receptor potential generated by the activation of a charybdotoxin-sensitive, Ca^{2+}-dependent K^+ conductance (Morales *et al*, 1994; Morales *et al*, 1995). This channel was shown to be present in olfactory cilia by experiments in which ciliary membranes were incorporated into phospholipid planar bilayers (Jorquera *et al*, 1995). An inward Ca^{2+} current appears to be responsible for the increment of intracellular Ca^{2+} that causes the activation of the K^+ conductance (Morales *et al*, unpublished). Both, the odor-activated K^+ outward current and Ca^{2+} inward current developed hundreds of milliseconds after the onset of the odorant stimulus; this delay is consistent with the participation of a second messenger cascade in the activation of such currents (Fig 1). Working on isolated rat olfactory neurons, Tareilus *et al* (1995) reported that odorants that trigger a raise in ciliary IP_3 induced an elevation in apical Ca^{2+}. However, whether the physiological target of this IP_3-induced Ca^{2+} increase is the same in the rat as in the toad remains to be established. If such were the case, the IP_3 pathway in these species would be understood to a considerable extent (see Fig 1).

Although this review concerns with vertebrate olfactory transduction, it is important to mention that the experimental evidence obtained in lobster support the participation of IP_3 in invertebrate olfactory transduction. Certain odor molecules induce a rapid and transient increase in IP_3 in the outer dendritic membranes *in vitro* and the activation of ionic channels in isolated lobster olfactory neurons. These odor-dependent channel could be activated by the application of IP_3 to the intracellular face of excised membrane patches (reviewed by Ache and Zhainazarov, 1995).

Taken together, the available evidence suggest that an IP_3 pathway plays a role in olfactory transduction (Fig 1).

Gaseous second messengers in olfactory transduction

Gaseous compounds as nitric oxide (NO) and carbon monoxide (CO) also appear to have roles in olfactory transduction (Breer and Shepherd, 1993; Leinders-Zufall *et al*, 1995). These membrane-permeant molecules regulate the activity of guanylyl cyclase in various cellular systems (for a review, see Dawson and Snyder, 1994).

As previously mentioned, the role of the cGMP in the olfactory transduction is poorly understood. The high affinity of the olfactory CNG channel for cGMP may reflect its common evolutionary origin with the vertebrate photoreceptor cGMP-dependent channel. However, the finding that an odor-dependent cGMP increase is abolished by L-N^G-nitroarginine, a selective inhibitor of NO formation, suggested that the NO/cGMP system may be involved in olfaction (Breer *et al*, 1992). Subsequent studies showed that application to olfactory neurons of the NO-donor sodium nitroprusside activated a cation conductance with properties similar to that induced by odorants (Lischka and Schild, 1993), supporting the notion that NO is an intercellular messenger in the olfactory epithelium.

The increment in intracellular Ca^{2+} in response to high doses of odorants trigger the production of NO from L-arginine, by stimulation of the NO synthase in a Ca^{2+}/calmodulin-dependent manner. NO, an endogenous activator of guanylyl cyclase, may rapidly diffuse to neighbor cells and increase their own cGMP levels, inducing the activation of their cAMP-sensitive channels

(Fig 1). Thus the NO/cGMP system may be important on recruiting adjacent receptor neurons during intense odor stimulation. This transduction pathway would be terminated by a decrease in the concentration of NO, a molecule with a very short half-life, and by the inhibitory effect of Ca^{2+} on the guanylyl cyclase.

Recent evidence suggests that carbon monoxide (CO), rather than NO, may be the activator of guanylyl cyclase in olfactory neurons. This notion is based on the observation that high levels of heme oxygenase (isoform HO-2), the enzyme that degrades the heme group to biliverdin and CO, have been found in olfactory epithelium (Verma *et al*, 1993). On the contrary, the enzyme NO synthase appears to be absent in mature olfactory neurons (Roskams *et al*, 1994). It has been proposed that NO may play a role in the development and regeneration of the olfactory epithelium. Recently, Zufall and colleagues found that micromolar concentrations of CO depolarized isolated olfactory neurons by tonically activating the CNG channel (Leinders-Zufall *et al*, 1995). CO responses were GTP-dependent and were abolished by LY85383, a blocker of guanylyl cyclase (Leinders-Zufall *et al*, 1995). These evidence supports a role for CO as a messenger in vertebrate olfactory transduction (Fig 1). Its mode of action would be analogous to the one proposed for NO, described above.

TRANSDUCTION MECHANISM INVOLVING DIRECT IONIC CHANNELS GATING BY ODORANTS

Direct gating of ion channels by odorants has been proposed as an additional chemotransduction mechanism in olfaction. Reconstitution studies with membrane vesicles prepared from rat olfactory epithelia into planar lipid bilayers, showed that nanomolar concentrations of the odorants diethyl sulfide and (-)-carvone activated a K^+-selective channel (Vodyanoy and Murphy, 1983). The effect of diethyl sulfide required ATP and GTP, suggesting the involvement of a G-protein (Vodyanoy and Vodyanoy, 1987). Labarca and colleagues (1988), using vesicles derived from olfactory cilia of *Rana catesbeiana*, found that bell pepper odor and citralva activated a cation channel with multiple conductance states. This effect was specific on the olfactory epithelium, since that it was not observed on vesicles prepared from the respiratory epithelium. More recent studies reported that histamine directly gates ion channels in the soma of lobster olfactory neurons (McClintock and Ache, 1989).

Direct activation of ion channels may generate a receptor potential in a manner similar to the nicotinic acetylcholine receptors in other cell types. This direct gating mechanism would involve low gain (number of ionic channels opened per activated odorant receptor protein), but could be advantageous on allowing an extremely rapid response. It remains to be established whether these odorant-gated conductances actually operate *in vivo* and, if so, it would be necessary to evaluate its relative importance for the physiology of olfactory neurons.

CONCLUSIONS

The evidence reviewed here demonstrate that vertebrate olfactory transduction is more complex than initially thought. It appears that recognition and discrimination of odorants is achieved when these molecules interact with specific membrane receptor proteins –responsible in part for the specificity of the olfactory system– and trigger one or more different transduction pathways. Among these pathways, the best characterized is the cAMP pathway, were the principal molecular components —receptor protein, G_{olf}, adenylyl cyclase, cyclic nucleotide gated cation channel and Ca^{2+}-activated Cl^- –channel– have been identified and some of them cloned and sequenced. Another pathway involves IP_3 as a second messenger. Unlike the cAMP-pathway, the identification of the molecular components of the IP_3 pathway is incomplete. Gaseous messengers also appear to have a role in olfactory chemotransduction. They would regulate sGC-activity, controlling the levels of cGMP, a messenger that can gate the CNG channel.

Mechanisms involving direct gating of ion channels by odorants may also be present in olfactory neurons. However, the possible importance of such mechanisms in olfactory chemotransduction remains to be established.

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