Co-Expression of Putative Pheromone Receptors in the Sensory Neurons of the Vomeronasal Organ

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Two large and divergent families of G-protein-coupled receptors (V1Rs and V2Rs) are expressed in subsets of neurons in the vomeronasal organ. These receptors are likely to mediate pheromone responses, but it appears that many V2R genes may encode expressed pseudogenes rather than functional proteins. Therefore we have raised antibodies to representative V2Rs and show labeling of vomeronasal neurons demonstrating that V2R genes encode expressed receptors. V2R immunoreactivity was detected at the sensory surface of the vomeronasal organ in dendritic terminals, indicating these receptors are capable of directly interacting with pheromones and mediating physiological responses. Immunohistochemistry confirmed that three V2R receptors are expressed in small subsets of sensory neurons. However, surprisingly we found that a subfamily of V2R genes is broadly expressed in the Gai-layer of the vomeronasal organ and are coexpressed in the same cells as other V2Rs. This is in direct contrast to the main olfactory epithelium where sensory neurons express only a single receptor. Thus, our results suggest that different modes of the information processing may occur in the main and accessory olfactory systems.

Key words: vomeronasal organ; pheromone receptors; G-protein-coupled receptors; immunohistochemistry; coexpression; olfaction; pheromone; receptors and signal transduction; chemosensory receptors; sensory coding

Mammalian pheromones are thought to be diverse chemical signals that play a role in controlling interactions between individuals of a single species (Keverne, 1983; Novotny et al., 1986; Halpern, 1987; Tirindelli et al., 1998). For example, under specific conditions, pheromones evoke neuroendocrine responses among conspecifics resulting in mating (Keverne, 1983). Thus subtle changes in response to pheromones may provide an important mode of speciation.

The vomeronasal organ (VNO) is a chemosensory organ located at the base of the nasal septum of most terrestrial vertebrates that plays a major role in pheromone responses in many mammalian species (Halpern, 1987). Molecular analysis of VNO neurons has revealed unexpected functional differences between this organ and the main olfactory epithelium (Berghard et al., 1996; Wu et al., 1996). In the VNO, the G-proteins Go1,2 and Goα, are expressed in distinct subsets of mature sensory neurons (Halpern et al., 1995; Berghard and Buck, 1996; Jia and Halpern, 1996). Two large and unrelated families of G-protein-coupled receptors, the V1Rs and V2Rs, are expressed in small subsets of the neurons containing Go1,2 and Goα, respectively, and may encode pheromone receptors (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsumuki and Buck, 1997; Ryba and Tirindelli, 1997). It has been demonstrated that a V1R (VN6) is found in the sensory microvilli, supporting this idea (Takigami et al., 1999). Moreover, the pattern of cellular activation of VNO neurons in response to pheromones parallels the expression pattern of receptors (Holy et al., 2000; Leinders-Zufall et al., 2000). However, there is no direct evidence that any of these molecules are pheromone receptors.

The V2Rs are homologous to the extracellular Ca-sensing receptors (Brown et al., 1993), metabotropic glutamate receptors (Nakanishi, 1992), taste receptors (Hoon et al., 1999), and a family of fish receptors that also appear to play chemosensory role (Cao et al., 1999; Naito et al., 1999). All these receptors possess a large N-terminal extracellular domain that is likely to form the ligand-binding site (Nakanishi, 1992; Brown et al., 1993). The ligand specificity of one of these receptors (receptor 5.24) from goldfish olfactory epithelium was recently reported (Speca et al., 1999). This receptor is activated primarily by the basic amino acids, arginine and lysine, which are odors for the fish. One intriguing facet of the expression of receptor 5.24 is that it is widely expressed in the olfactory epithelium. In contrast, many other receptors are expressed in small subsets of cells (Cao et al., 1998; Naito et al., 1998; Speca et al., 1999).

We raised antibodies to several V2Rs to examine expression of these proteins and to investigate their cellular distribution. Immunohistochemistry demonstrates that V2R genes encode proteins that are expressed in the VNO and are likely to function as pheromone receptors. Surprisingly, we observed that V2R2 is expressed at a lower level in almost all the VNO neurons of the Gao-positive layer, suggesting that cellular responses to pheromones may involve the interaction between receptors of the same family.

MATERIALS AND METHODS

Isolation and expression of the VNO receptors, Escherichia coli expression systems were used to produce peptides encoding part of the extracellular domain of V2Rs. A fragment of the mouse receptor, V2R2 (bases 1053–1807), was subcloned in pET28 (Novagen, Madison, WI). The equivalent region of the extracellular domains of other V2Rs: mouse
V2R1 (991–1669), rat Go-VN1 (986–1738), rat Go-VN2 (975–1730), rat Go-VN3 (1577–2323) and rat Go-VN4 (1110–1868) (Herrada and Ducal, 1997; Ryba and Tirindelli, 1997), were amplified from cDNA, sequenced and subcloned in the plasmid pTrcHis2 (Invitrogen, San Diego, CA). The untranslated region of three distinct members of the V2R2 subfamily were amplified and subcloned in pCRII. A fragment encoding the rat homolog of the human receptor related to goldfish receptor 5.24 was generated by degenerate PCR of genomic DNA using primers preceding the first and sixth transmembrane helices. Peptide expression was induced with isopropylthiogalactoside according to standard methods (Invitrogen; Novagen). Bacterial pellets were resuspended in 10 mM Tris, 150 mM NaCl, and 1 mM PMSF and sonicated for 1 min. After centrifugation, pellets were dissolved in 6 M guanidinum-HCl in resuspension buffer. Purification of the peptide was performed by affinity chromatography onto a Talon metal affinity resin (Clontech, Palo Alto, CA) according to the manufacturer’s instruction. Approximately 2–4 mg was obtained from 200 ml culture.

For Southern hybridization, a fragment of mouse V2R2 corresponding to a single extracellular exon (540–1388) and the 3′ nontranslated region of rat V2R2, V2R2a, and V2R2b were amplified by PCR. Southern blots were washed at high stringency (1 hr at 65°C in 0.1× SSC for the extracellular probe and 20 min at 65°C in 0.5× SSC for the 3′ nontranslated region probes). The rat cDNA library was screened at moderate densities. Antibody generation and Western blotting. The V2R extracellular domain fragments were extensively dialyzed against PBS, and the precipitate that formed was used to immunize rabbits (500–1000 μg each injection). Antibody purification was performed by ammonium sulfate precipitation followed by DEAE exclusion chromatography (Harlow and Lane, 1988). Because the fusion proteins all contained hexahistidine tags, antibodies were preabsorbed with a saturated solution of polyhistidine to reduce cross-reactivity. Antibodies were used at a concentration of 20 ng/ml for Go-VN2, 45 ng/ml for Go-VN3, 4 ng/ml for Go-VN4, and 1–20 ng/ml for V2R2. Antibodies were assayed by Western blot analysis of crude plasma membrane preparation from rat VNO and control tissues (Tirindelli and Ryba, 1996).

In situ hybridization and immunohistochemistry. Tissue was obtained from adult Wistar rats and C57BL/6 mice. Frozen sections were cut at 14 μm and attached to silanized slides. Probe preparation and in situ procedures were essentially as described previously (Ryba and Tirindelli, 1997). Riboprobes were labeled with digoxigenin, and signal was developed using an alkaline phosphatase-conjugated antibody and chromogenic substrate. For double-label fluorescent detection, probes were labeled with fluorescein or with digoxigenin. An alkaline–phosphatase-conjugated anti-digoxigenin antibody was used in combination with fast red and tyramide fluorogenic substrates (Boehringer Mannheim, Indianapolis, IN; New England Nuclear, Boston, MA). Confocal images were obtained with a Leica (Nussloch, Germany) TSC confocal microscope using an argon–krypton laser; 1 μm optical sections were recorded to ensure that any overlapping signal originated from single cells.

For immunohistochemistry, sections were prepared as for the in situ hybridization, blocked in 1% albumin and 0.3% Triton X-100 (blocking solution) for 20 min and incubated with the anti-V2R2 antibody in blocking solution. For double-label immunohistochemistry, anti-V2R2 antibody was labeled with N-hydroxysuccinimide biotin (Sigma, St. Louis, MO) at a ratio of 5:1. Sections were first incubated with the anti-VN antibody and developed with an anti-rabbit IgG conjugated with Alexa-586. After blocking with normal rabbit serum, sections were incubated with 20 ng/ml biotinylated anti-V2R2 antibody (Harlow and Lane, 1988) in presence of 10% normal rabbit serum, and immune complexes were visualized with fluorescein avidin (Vector Laboratories, Burlingame, CA). For preabsorption controls, 5 μg of anti-V2R2 antibody was incubated with 10 μg of the polypeptide against which it was raised or a mix of 10 μg of each of the other V2R polypeptides. Fluorescent images were obtained using a Zeiss fluorescent microscope and a Leica TSC confocal microscope equipped with an argon–krypton laser.

RESULTS
We grouped V2Rs according to their sequence conservation and immunized rabbits with expressed extracellular domains of representative V2R genes that encode members of distinct subfamilies. Antibodies against the N-terminal extracellular domain of three V2R-family receptors: Go-VN2, Go-VN3, and Go-VN4 labeled small subsets of VNO neurons (Fig. 1). For all the V2R antibodies, strongest immunoreactivity was in the cell body of VNO neurons, and as indicated in Figure 1d, expression of receptors was detectable in the sensory dendrites extending to the surface of the epithelium. In contrast, no specific immunostaining was observed in the axon bundles and accessory olfactory bulb of either adult or neonatal animals (data not shown).

The pattern of immunoreactivity observed with an antibody to V2R2 was markedly distinct. Unlike the punctate pattern of expression observed for the other V2Rs that we examined, the antibody to V2R2 labeled all neurons in the basal half of the sensory cell layer of the VNO (Fig. 2a,b). The cellular distribution of V2R2 was very similar to that of other V2Rs with prominent labeling of soma (Fig. 2a,b) and sensory microvilli (Fig. 2c) but no labeling of the axon bundles or accessory bulb (data not shown; Fig. 2d). To rule out the possibility that this antibody recognized a truncated receptor or other proteins expressed in a
Figure 2. V2R2 is expressed in the cell bodies and sensory dendrites of a large subset of VNO neurons. Anti-mouse V2R2 stained all neurons in the basal half of the VNO epithelium in mouse (a) and rat (b); c, higher magnification of the luminal region of b showing staining of dendrites and knobs. Scale bars: a, 100 μm; b, 30 μm; c, 4 μm. d, Western blot of rat membrane protein extracts stained for anti-V2R2 immunoreactivity shows that this antibody recognizes an ~100 kDa protein that is expressed in the VNO, but not MOE, olfactory bulb (OB), spleen (SP), or testis (TE); the position of the 94 kDa marker is indicated.

Figure 3. Specificity of antibodies to V2Rs. a, Western analysis of the four V2R-family antigens using the four antibodies; each antibody only recognizes the polypeptide against which it was raised. b, c, To demonstrate specificity of immunohistochemistry, the anti-V2R2 antibody was incubated with the polypeptide against which it was raised (b) or a mix of three polypeptides encoding the same region of Go-VN2, Go-VN3, and Go-VN4 (c). Preincubation with the V2R2 antigen abolished immunostaining (b), although preincubation with the other polypeptides had no effect on the pattern of immunohistochemistry (c). Scale bar, 100 μm.

To unambiguously demonstrate co-expression of V2R receptors, we performed double-label immunohistochemistry experiments and analyzed 1-μm-thick optical sections using confocal microscopy. As expected, almost all the neurons expressing the V2R family receptors: Go-VN2, Go-VN3, and Go-VN4, also contained V2R2 (Fig. 6). This was observed along the entire length of the VNO and also was found for the apically located neurons that expressed Go-VN2 in male rats. We confirmed these results using in situ hybridization double labeling (Fig. 6). Therefore our results strongly suggest that many Gaαi-positive neurons express more than one V2R receptor.
DISCUSSION

V2R expression in VNO sensory neurons

Recently it was shown that members of large family of putative pheromone receptors (V2Rs) are expressed in distinct subsets of VNO sensory neurons (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). However, the sequence of several V2R cDNAs indicated that many of these putative receptors might be expressed pseudogenes (Herrada and Dulac, 1997; Matsunami and Buck, 1997). Moreover, in the absence of functional data, other roles for these proteins (for example in directing axon projection) must also be considered (Belluscio et al., 1999; Rodriguez et al., 1999). Therefore we raised antibodies to investigate whether V2Rs are expressed proteins and to examine their distribution in VNO neurons.

The expression pattern of the V2R proteins revealed by the antibody staining was remarkably similar to that obtained by in situ hybridization experiments (data not shown; Herrada and Dulac, 1997). For instance, anti-Go-VN3 antibodies labeled neurons localized to the basal layer of the Goα-positive zone, whereas anti-VN2 antibodies labeled cells extending to the apical region of the VNO. The number of neurons stained by the anti-Go-VN4 antibodies was approximately three times greater than those identified by in situ hybridization with a corresponding cRNA probe, suggesting that this antibody may recognize an epitope found in more than one V2R. However, control experiments using high concentrations of the expressed N-terminal domains of four representative V2Rs showed that the antibodies are specific within this group only recognizing the protein against which they were raised (Fig. 3).

Expression of receptors was detected in the sensory dendrites extending to the surface of the epithelium, consistent with a role for V2Rs as pheromone receptors (Figs. 1, 2). Nonetheless, the high degree of cellular staining is surprising for sensory receptors that might be expected to be concentrated at the luminal surface of the VNO. One possibility is that this distribution might reflect the fast turnover of the receptors exposed to the lumen of the VNO. A second potential role of the V2Rs might be in directing axon guidance (for example, see Belluscio et al., 1999; Rodriguez et al., 1999). However, the lack of immunoreactivity in the olfactory bulb (Fig. 2d), while not ruling out a role of these receptors in axon guidance mechanisms, suggests any role of V2Rs in axon guidance may be indirect.

V2R2s are co-expressed with other V2Rs

Both immunohistochemistry and in situ hybridization show that the expression pattern of V2R2 and closely related subfamily members is remarkably different from that of other V2Rs. In earlier studies (Ryba and Tirindelli, 1997), we mistakenly overlooked this difference because the weak signal obtained using
Interestingly, olfactory receptors can detect more than one odorant, and odorants can be recognized by several receptors with similar ligand specificity.

This weak staining suggests that cellular expression of any single V2R subfamily member is significantly lower than that of other V2Rs. However, in contrast to the small subsets of neurons that express most V2Rs, all neurons in the basal half of the sensory cell layer of the VNO appear to express several transcripts from the V2R2 subfamily (Figs. 2, 4, 5). Moreover, double-labeling experiments clearly demonstrate co-expression of several different V2Rs in V2R2 containing sensory neurons (Fig. 6).

These differences in expression raise the possibility that V2R2 plays a distinct role from other V2Rs. Sequence comparison of V2Rs also demonstrates that V2R2 is a divergent member of this family (Fig. 5) and places V2R2 closer to fish olfactory receptors than to other V2Rs. This may suggest closer functional similarity between V2R2 and these fish receptors, but equally may reflect the fact that only a small subset of V2R genes has been sequenced. The expression of V2R2 is restricted to the VNO and specifically to the Goα-expressing cells within this neurosensory epithelium (Herrada and Dulac, 1997; Matsumani and Buck, 1997; Ryba and Tirindelli, 1997). Moreover, the cellular distribution of immunoreactivity is very similar to that seen with antibodies to other V2Rs (Figs. 1, 2). Finally, as is the case for the other V2Rs, only a V2R2 pseudogene is found in the human genome (Speca et al., 1999; Fig. 5).

The V2R-related family of receptors from fish olfactory epithelium also contains receptors that are expressed in a small subset of neurons and others that are more broadly expressed (Cao et al., 1998; Naito et al., 1998; Speca et al., 1999). Specifically, it has been shown that two goldfish receptors, 5.24 and 5.3, are expressed in a large subset of olfactory neurons. Thus here too, it is likely that receptors of this family are co-expressed in the same cells. Again it is notable that receptors 5.24 and 5.3 have the most divergent sequences (Speca et al., 1999; Fig. 5). Receptor 5.24 has been shown to respond to arginine and lysine, which are important odorant cues for fish. However, receptor 5.24 is only distantly related to V2R2, and therefore it is unlikely that V2R2 responds with similar ligand specificity.

What might be the functional significance of expressing multiple receptors in a single neuron? In the MOE, neurons express a single receptor, and neurons expressing a common receptor project to the same set of glomeruli (for review, see Mombaerts et al., 1996). Interestingly, olfactory receptors can detect more than one odorant, and odorants can be recognized by several receptors (Malnic et al., 1999). Thus, mammals use combinatorial codes of glomeruli activation to discriminate odors (Rubin and Katz, 1999). The apical neurons of the rodent VNO are also thought to express only a single sensory receptor, however neurons expressing single V1Rs target multiple glomeruli (Belluscio et al., 1999; Rodriguez et al., 1999). Moreover, glomeruli appear to receive input from neurons expressing distinct V1Rs (Belluscio et al., 1999). This slightly different organization may facilitate stereotyped responses to specific mixes of odorants. Caenorhabditis elegans has a much simpler nervous system and uses a very different strategy that permits detection of many odorants but more limited discrimination between them. To do this, C. elegans chemosensory neurons express many receptors per cell (Troemel et al., 1995). Interestingly, the uniform bitter taste of many toxins also appears to result from the co-expression of several distantly related receptors in mammalian taste receptor cells (Adler et al., 2000; Chandrashekar et al., 2000). The co-expression of V2R2 with another V2R receptor suggests that yet another mode of chemosensory information processing may occur. For example it is possible that ligands for V2R2 significantly alter the effects mediated by ligands to other V2Rs, either by sensitizing or desensitizing the cells. It is even conceivable that ligands for V2R2 might override signals mediated by other V2Rs. However, a recent study of the effects of urine on VNO neurons provided no evidence for such an extreme model (Holy et al., 2000). An alternative hypothesis is that V2R2 might form heterodimers with other V2Rs. For example it has been shown that GABA-b receptors are composed of two distinct but related G-protein-coupled receptors (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). In Drosophila the pattern of receptor expression most closely parallels the V2R receptor expression profile. Sensory neurons each express a single member of a family of ~50 odorant receptors (Vosshall et al., 2000), and in addition express a highly divergent member of this gene family.

In conclusion, our data suggest that V2R2 and the other V2Rs have similar cellular localization and are present in the sensory region of the neurons, as would be expected for receptors involved in pheromone detection. However, the expression pattern of V2Rs is significantly more complex than for other vertebrate olfactory receptors. It will be fascinating to determine how these patterns of expression are regulated and to determine their role in chemosensory signaling and pheromone responses.

REFERENCES

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