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Possible pheromone-carrier function of two lipocalin proteins in the vomeronasal organ

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We report the molecular cloning and characterization of two secretory proteins specifically expressed in vomeronasal and posterior glands of the nasal septum, the ducts of which open into the lumen of the vomeronasal organ. These two proteins are members of the lipocalin superfamily, consisting of hydrophobic ligand carriers. We immunohistochemically localized one of the proteins in the mucus covering the vomeronasal sensory epithelium, where the primary reception of pheromone takes place. The immunoreactivity on the vomeronasal sensory epithelium was evident in the neonatal and post-pubertal periods, when the close contact between animals plays critical roles in suckling and sexual behaviors, respectively. These results suggest that small lipophilic molecules stimulate the accessory olfactory system to regulate the reproductive behavior of mice.

Key words: differential hybridization/lipocalin protein/pheromone/vomeronasal organ

Introduction

The accessory olfactory system with its peripheral sensory organ, the vomeronasal organ (VNO), plays an important role in the perception of pheromones, the molecules that influence the regulation of sexual behavior, hormone levels, aggregation and fertility (Halpern, 1987). We have been interested in the molecular mechanisms by which animals recognize pheromone ligands through the VNO. One of the fundamental differences between the main and accessory olfactory systems has been thought to be the chemical nature of substances that stimulate them (Halpern, 1987). For terrestrial vertebrates, most odors are volatile; they migrate over distance from the stimulus sources to the main olfactory system. The detection of these molecules requires some events taking place at the air–aqueous interface, thereby the volatile molecules traverse the aqueous mucus layer to interact with the membrane-associated receptors (Getchell *et al.*, 1984; Carr *et al.*, 1990). In the olfactory epithelium, odorant binding proteins (OBPs) are thought to concentrate and deliver odorants to the receptors (Bignetti *et al.*, 1985, 1988; Pevsner *et al.*, 1985, 1988a,b). OBP belongs to the lipocalin superfamily, members of which bind and transport small hydrophobic molecules (Godovac-Zimmermann, 1988).

By contrast, it has been thought that the substances which stimulate the vomeronasal system are of relatively high molecular weight and non-volatile (Halpern, 1987; Wysocki *et al.*, 1980, 1985). Animals investigate conspecific secretions or excretions by placing their snouts in direct contact with the materials. Nose rubbing and facial grimacing enlarge the duct to the VNO, thereby facilitating the access of non-volatile substances in a liquid medium toward the VNO. In fact, Wysocki *et al.* (1980, 1985) demonstrated that a non-volatile substance is capable of entering the VNO; they allowed guinea pigs to investigate urine containing rhodamine, a non-volatile fluorescent dye, and observed the VNO filled with the fluorescent dye. On the other hand, some volatile molecules in mouse urine have also been shown to cause pheromonal effects in mice (Jemiolo *et al.*, 1986; Novotny *et al.*, 1986).

In the present study, we found two lipocalin proteins specifically expressed in the vomeronasal complex. One of the proteins was immunolocalized on the vomeronasal sensory epithelium. Our findings support the idea that small lipophilic (volatile) substances also stimulate the accessory olfactory system. We further discuss the functional significance of the lipocalin proteins in terms of animal behavior.

Results

Two lipocalin proteins specific to the vomeronasal system

The question of how the accessory olfactory system could be differentiated from the main olfactory system led us to perform differential hybridization analyses between the two systems. We have sought clones manifesting cell-specific expression in the vomeronasal complex (VNC), which contains the VNO, the vomeronasal gland (VNG) and blood vessels. Approximately 120 000 clones of a mouse VNC cDNA library in λgt11 were screened with single-stranded ^{32}P-labeled cDNA probes prepared by random-primed reverse transcription of mRNAs from the VNC and olfactory epithelium (OE). After two rounds of screening, we isolated 36 clones that hybridized with the VNC probe, but not with the OE probe. Cross-hybridization at high stringency and restriction enzyme map analyses resolved the 36 clones into four independent clones: 371, 841, 111 and 611. We then carried out Northern blot analyses using the four clones as probes. Probes 371 and 841 detected VNC-specific mRNA species of 0.9 kb (Figure 1). The faint signal bands that appeared in OE of Figure 1A and B were derived from contaminated tissue of the caudal part of the posterior glands of the nasal septum (see below). Clone 111 gave a 6.0 kb transcript in the VNC, colon and small intestine; clone 611 gave a

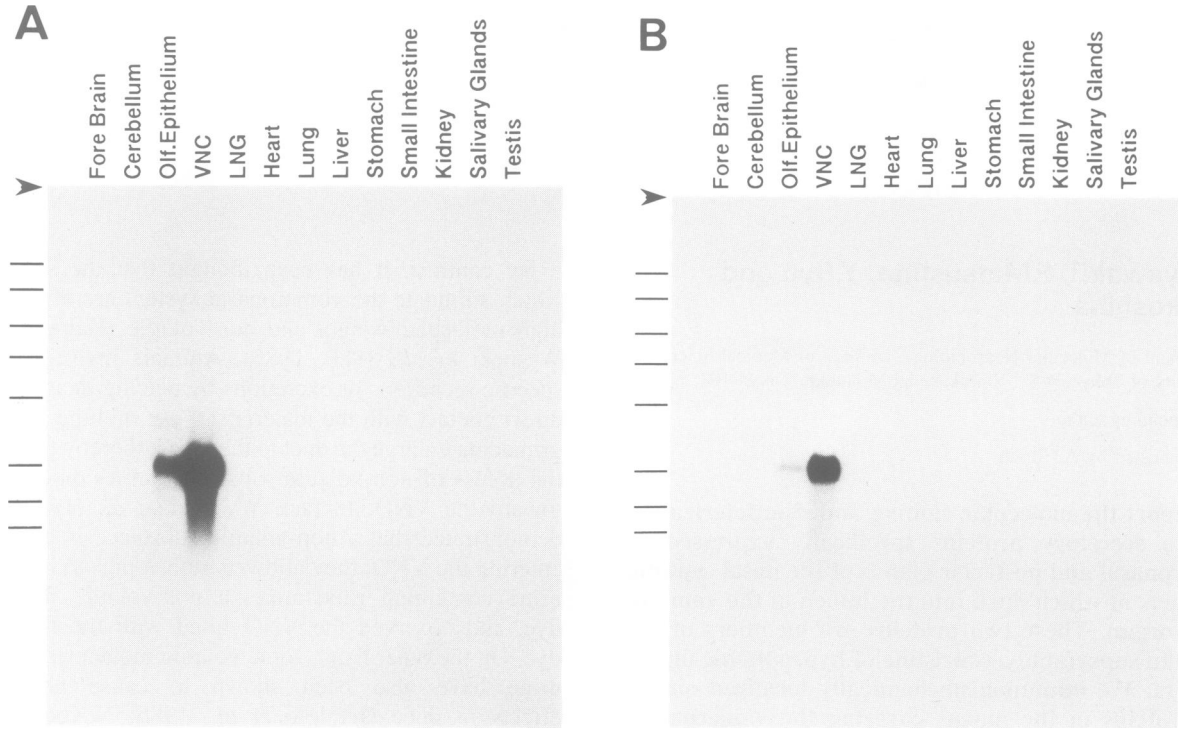

Fig. 1. Distribution of mRNAs of VNSP I (**A**) and II (**B**). Molecular sizes shown on the left are 9488, 6225, 3911, 2800, 1898, 872, 562 and 363 bp. Arrowheads indicate origins of the gels. The signals appearing in olfactory epithelium were due to contamination of the caudal part of the posterior glands of the nasal septum (see Figure 3).

4.5 kb transcript in the VNC and salivary glands (manuscript in preparation).

Sequence analyses of clones 371 and 841 revealed open reading frames encoding 182 and 185 amino acid proteins, respectively (Figure 2). Both the proteins are thought to be secretory ones, since they contain typical amino-terminal signal sequences of ~20 amino acid residues. From this and their restricted expression to the VNC, the proteins encoded by clones 371 and 841 were designated VNSP (vomeronasal secretory protein) I and II, respectively. A single potential *N*-linked glycosylation site occurs at asparagine residue 30 of VNSP I. Comparison of their amino acid sequences with other known protein sequences in the GenBank/EMBL data bases showed that VNSP I and II belong to the lipocalin superfamily: lipophilic molecule carriers (Godovac-Zimmermann, 1988). Members of this family bind and transport small hydrophobic molecules such as retinol, steroids, lipids and odorants. VNSP I and II have significant similarities to rat von Ebner's gland protein (Schmale *et al.*, 1990), rat OBP_{II} (Dear *et al.*, 1991) and human tear prealbumin protein (Redl *et al.*, 1992), showing ~40% identity at the amino acid level in every case; with rat OBP (Pevsner *et al.*, 1988b), the identity is 25–30% (Figure 2). Based on the sequences of rat clones, we carried out reverse transcriptase-polymerase chain reactions (RT-PCR) and obtained two partial cDNAs of mouse OBP and OBP_{II}, either of which was shown to have an almost identical amino acid sequence to the rat counterpart. Northern blot analysis revealed that the mRNAs of mouse OBP and OBP_{II} were most abundant in the lateral nasal gland (LNG) (data not shown), where no VNSP transcripts were detected. We therefore verified the novelty of our VNSP clones; they are not the mouse counterparts of OBP and OBP_{II}. They all present a highly conserved motif: G-X-W at a position around 30. In addition, two cysteine residues that seem to form disulfide linkages are conserved among them: Cys76 and Cys168 in VNSP I; Cys80 and Cys172 in VNSP II. The Chou–Fasman method (Chou and Fasman, 1978) predicts that each VNSP has six β-sheets, which seem to form a β-barrel, a hydrophobic pocket for lipophilic molecules and one α-helix near the C-terminus.

The precise location of VNSP mRNAs was examined by *in situ* hybridization on coronal sections of mouse nasal tissue. As seen in Figure 3A–D, hybridization of the antisense probe of VNSP I resulted in a high density of silver grains over the VNG and posterior glands of the nasal septum (PGNS) throughout the nose. These signals are specific, since the sense cRNA probe yielded no silver grains (Figure 3E). No positive signals were detected in cells of the LNG or anterior glands of the nasal septum (AGNS). The PGNS was discriminated from the AGNS by staining the serial sections with periodic acid–Schiff; the PGNS produces neutral glycoproteins, whereas the AGNS does not (Cuschieri and Bannister, 1974; Klaassen *et al.*, 1981). VNSP II mRNA exhibited exactly the same distribution pattern as VNSP I mRNA; one representative section probed with an antisense cRNA of VNSP II is given in Figure 3F.

Immunological characterizations of VNSPs

We raised two polyclonal antibodies, AbIC and AbIIC, against the C-terminal regions of VNSP I and II, respectively. We then performed immunoblot analysis of crude extract from the mouse VNC. AbIC detected a major

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VNSP I   MRALLLIISFC-LVAVLQAQDSSFLAFNNGNFSGKW---FLKALVSEDDIPI-----  48
VNSP II  MKSLLLTVTLSSLVATLQTYDDLPFISEEDKLSGVW---FIKATVSQRREVEGETL-  53
VEGr     MKALLLT-FGLSLLAALQAQ-AFPTTEENQDVSGTW---YLKAAAWDKEIPDKKFGS  52
OBPr     MVKFLLIVLALGVSCA--HHENL--DISPSEVNGDWRTLYIVADNVEKVAEGG---S  50
OBPrII   MKSRLLTVLLLGLMAVLKAQEAPP--DDQEDFSGKW---YTKATVCDRNHTDGK-RP  51
TPh      MKPLLLAVSLGLIAALQAHHLL-ASDEEIQDVSGTW---YLKAMTVDREFPEMNLE-  52
         mk lll v    l a l              sg w   y ka

VNSP I   NKVSPMLILVLNNGD--IELSITHMIY--DQCLEVTTILEKTDVPGQYLAFEGKTHL 101
VNSP II  -VAFPIKFTCPEEGT--LELRHTLASK--GECINVGIRLQRTEEPGQYSAFWGHTLF 105
VEGr     VSVTPMKIKTLEGGN--LQVKFTVLIA--GRCKEMSTVLEKTDEPAKYTAYSGKQVL 105
OBPr     LRAY---FQHMECGDECQELKIIFNVKLDSECQTHTVVGQKHED-GRYTTDYSGRNY 103
OBPrII   MKVFPMTVTALEGGD--LEVRITFRGK--GHCHLRRITMHKTDEPGKYTTFKGKKTF 104
TPh      -SVTPMTLTTLEGGN--LEAKVTMLIS--GRCQEVKAVLEKTDEPGKYTADGGKHVA 104
           v pm     e g   le   t      g c      l ktdepg yta  gk

VNSP I   QVQLSSVKGHYMLYCDGEIEGMRFLMTQLI-GRDPQENLEALEEFKVFTQIKGLVAE 157
VNSP II  YIYDLPVKDHYIIYCESHPFQKISQFGYLI-GKYPEENQDTLEVFKEFIQHKGFLQE 161
VEGr     YIIPSSVEDHYIFYYEGKIHRHHFQIAKLV-GRDPEINQEALEDFQSVVRAGGLNPD 161
OBPr     F-HVLKKTDDIIFFHNVNVDESGRRQCDLVAGKREDLNKAQKQELRKLAEEYNIPNE 159
OBPrII   YTKEIPVKDHYIFYIKGQRHGKSYLKGKLV-GRDSKDNPEAMEEFKKFVKSKGFREE 160
TPh      YIIRSHVKDHYIFYCEGELHGKPVRGVKLV-GRDPKNNLEALEDFEKAAGARGLSTE 160
         y     vkdhyify  g           lv grdp  n eale f       g   e

VNSP I   NLVILEQMEKCEPESFYELPSRPSE                                 182
VNSP II  KIGVPEQRDRCIPIHDSAHQDHKC                                  185
VEGr     NIFIPKQSETCPLGSN                                          177
OBPr     NTQHLVPTDTCNQ                                             172
OBPrII   NITVPELLDECVPGSD                                          176
TPh      SILIPRQSETCSPGSD                                          176
         ni  p q   c p s
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Fig. 2. Amino acid sequence (single-letter code) alignment of VNSP I and II with other lipocalin proteins. The putative *N*-linked glycosylation site is indicated by an arrowhead. Alignments were done manually following previous reports (Schmale *et al.*, 1990; Redl *et al.*, 1992). Identical amino acids in at least four proteins including either VNSP are indicated below in lower case. VEGr, rat von Ebner's gland protein; OBPr, rat odorant binding protein; OBPrII, rat OBP_{II}: RY2G12 protein; TPh, human tear prealbumin protein. The VNSP I and II cDNA sequences have been assigned the GSDB/DDBJ/EMBL/NCBI accession numbers D38580 and D38581 respectively.

broad band of protein at 23 kDa and a minor one at 19 kDa (Figure 4A, lane 1). AbIIC detected two bands at 27 and 20 kDa (Figure 4A, lane 4). The selectivity of the antibodies was examined with proteins derived from the cDNAs. Insect cells (Hi Five™) infected with recombinant baculovirus produced cDNA-derived VNSPs that were secreted into the serum-free medium. The antibodies reacted with two secretory products for each VNSP (VNSP I, 21 and 19 kDa, Figure 4A, lane 2; VNSP II, 23 and 20 kDa, Figure 4A, lane 6). There was no cross-immunoreactivity between the two VNSPs (Figure 4A, lanes 3 and 5). The lower bands, which were commonly detected in both cDNA-derived and native samples, exhibited the relative molecular masses (M_r) predicted from the amino acid sequences of the mature forms of the proteins. After cleavage of the signal peptides (Perlman and Halvorson, 1983), the calculated M_r of VNSP I and II are ~19 and 20 kDa, respectively. When extract from the VNC was treated with *N*-glycosidase F, the AbIC-reactive 23 kDa band disappeared (Figure 4B, lane 2), indicating that a majority of native VNSP I have oligosaccharide chains at Asn30. By contrast, the AbIIC-reactive 27 kDa protein in the VNC extract was resistant to all of the glycosidases tested: *N*-glycosidase, *O*-glycosidase and neuraminidase. Furthermore, another antibody raised against the bacterially expressed VNSP II (52 C-terminal amino acids) detected only the 20 kDa protein band in the VNC extract. It was therefore concluded that VNSP II is not a glycosylated protein; the 27 kDa band in the VNC extract is due to a cross-reactivity of AbIIC.

Immunolocalization of VNSP I to the mucus layer of vomeronasal sensory epithelium

In the main olfactory epithelium, OBPs secreted from the LNG are thought to function as cofactors in olfactory transduction by concentrating and delivering odorants to the receptors. Because the ducts of the VNG and PGNS empty into the lumen of the VNO (Bojsen-Møller, 1964), the VNSPs seem to reside in the vomeronasal mucus, possibly for pheromone perception. However, owing to the difficulty of fixing the secreted proteins, no lipocalin proteins in vertebrates have been immunohistochemically identified in the regions where they actually function, but only in the cells where they are synthesized. For instance, rat OBP was immunohistochemically localized only in the LNG (Pevsner *et al.*, 1988a). It was thought to be washed away during fixation procedures and did not appear in the mucus of the OE. This is the case with the VNSPs; immunohistochemistry with AbIC on sections fixed by conventional procedures [perfusion transcardially with phosphate-buffered saline (PBS), followed by fixative such as Zamboni and 4% paraformaldehyde] gave signals only in the VNG and PGNS. Immunohistochemistry with AbIIC showed very faint stainings, because of weak affinity of the antibody. To immobilize the secreted proteins, we fixed the mucus of a deeply anesthetized mouse with formalin vapor before immersing the excised tissue in fixative. We were then able to observe the AbIC staining in the mucus covering the vomeronasal epithelia of the anterior portion of the VNO (Figure 5A). Immunostaining on the sensory epithelium was continuous, and

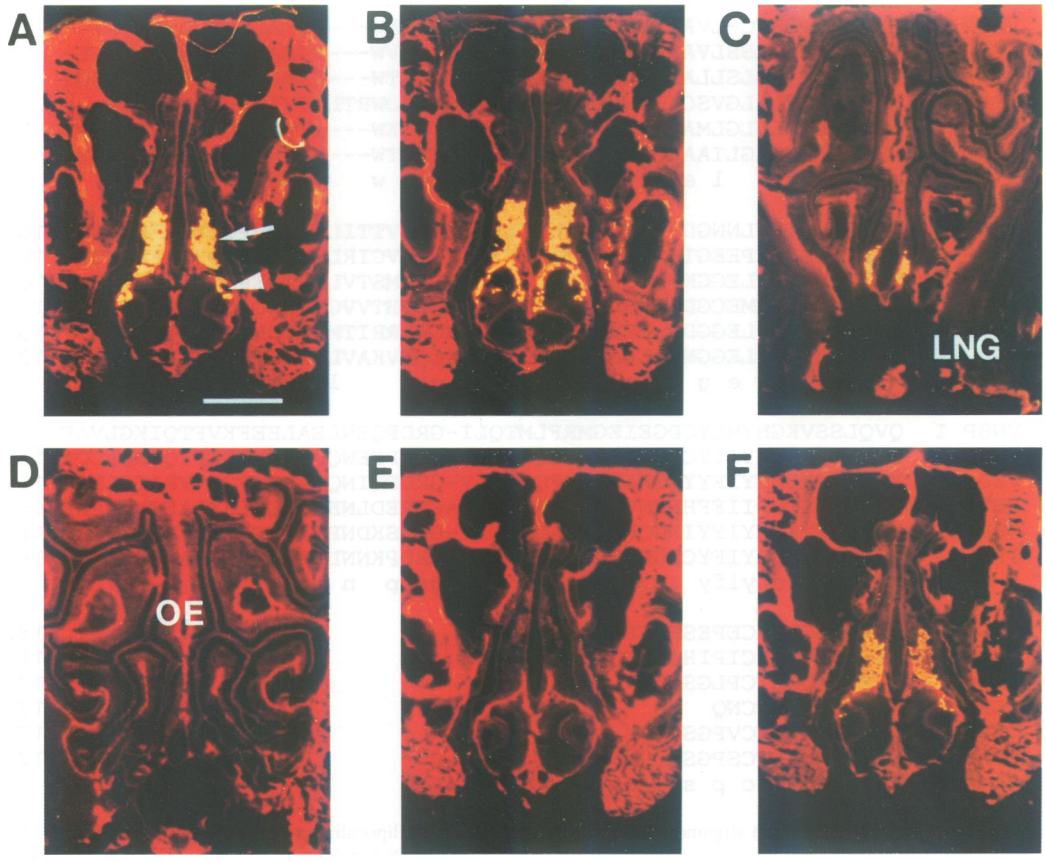

Fig. 3. Localization of mRNAs of VNSP I and II in coronal sections of mouse nasal cavity. *In situ* hybridization with an antisense (**A**–**D**: rostral–caudal) or a sense cRNA probe of VNSP I (**E**), and with an antisense cRNA probe of VNSP II (**F**). Sections (E) and (F) correspond to section (A), which was cut at the rostral level of the cavity. An arrow and an arrowhead in (A) indicate VNG and PGNS, respectively. OE, olfactory epithelium. Scale marker, 1 mm.

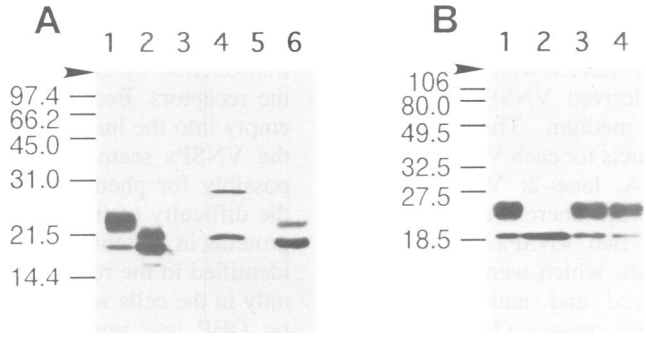

Fig. 4. (**A**) Immunoblot analysis of native and cDNA-derived VNSPs. The extract from the VNC (lane 1, 5 μg; lane 4, 30 μg) and the culture media of Hi Five™ cells producing cDNA-derived VNSPs I (lanes 2 and 5, 0.15 μg of medium protein) and II (lanes 3 and 6, 1.5 μg of medium protein) were electrophoresed. Lanes 1–3, AbIC (4 μg/ml). Lanes 4–6, AbIIC (16 μg/ml). (**B**) Glycosidase digestions of native VNSP I. Extract from the mouse VNC was treated with *N*-glycosidase F (lane 2), *O*-glycosidase (lane 3), or a combination of *O*-glycosidase and neuraminidase (lane 4). Lane 1, untreated sample. Arrowheads in (A) and (B) indicate the origin of the gels.

was abolished by pre-incubation of AbIC with the peptide. By contrast, staining on the non-sensory epithelium was intermittent, and was slightly reduced in the presence of the peptide (Figure 5B). By using normal rabbit IgG, we observed similar non-specific staining on the non-sensory epithelium. We therefore conclude that VNSP I is present predominantly on the sensory epithelium. Intensive observation using serial sections revealed that VNSP I is less distributed in the posterior portion of the VNO (results not shown). Anteriorly, the VNO opens into the nasal cavity. The preference of VNSP I to distribute in the sensory epithelium of the anterior or middle portion of the VNO strongly supports the idea that VNSP I is involved in the primary perception of pheromones. This characteristic distribution in the VNO is interesting in light of the selective transport mechanisms of VNSP I, in which the *N*-linked oligosaccharide chains might be involved. VNSP I was not detected in the mucus of the OE or the respiratory epithelium.

We next examined the immunolocalization of VNSP I

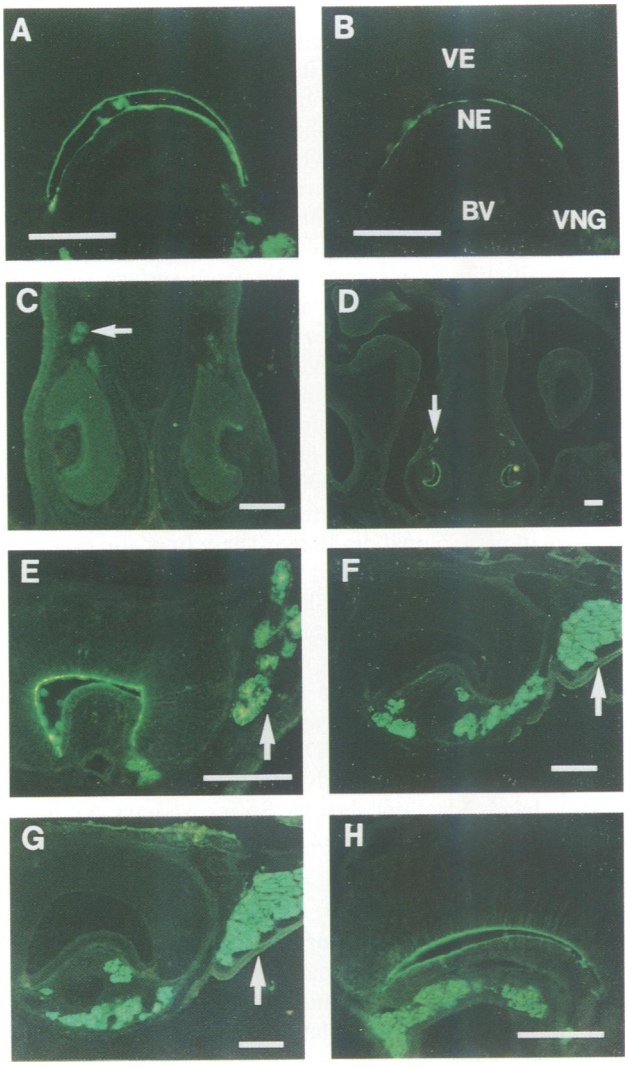

Fig. 5. Indirect immunofluorescence localization of VNSP I in the VNO of adult [15 weeks of age; (**A**) and (**B**)], E18 (**C**), P1 (**D**), P3 (**E**), P28 (**F**), P49 (**G**) and P84 (**H**) mice. Tissue sections (16 μm thick) were incubated with AbIC (A, C, D, E, F, G and H) or AbIC pre-incubated with the synthetic C-terminal peptide (B). The abbreviations in (B) are: VE, vomeronasal sensory epithelium; NE, non-sensory epithelium; VNG, vomeronasal gland; BV, blood vessel. Scale marker, 200 μm. Arrows in (C), (E), (F) and (G) indicate the PGNS.

during the development of mice. At embryonic day 18 (E18), the immunoreactivity was detected in the PGNS and VNG faintly (Figure 5C). At postnatal days 1 (P1) and 3 (P3), the vomeronasal sensory epithelium was markedly immunolabeled (Figure 5D and E, respectively). This immunoreactivity was evident within ~10 days after birth. During the neonatal period, chemical communication through the VNO is an important part of infant–mother interaction. It might be that VNSP I carries a nipple-search pheromone. Between 2 and 8 weeks of age, however, VNSP I was not detected on the sensory epithelium, in spite of a great deal of synthesis of the protein in the PGNS and VNG (Figure 5F: P28; Figure 5G: P49); and in post-pubertal mice, VNSP I appeared on the sensory epithelium (Figure 5H: P84). The developmental profile of the expression and distribution of VNSP I (see Table I) is attractive in view of changes in the style of intraspecific communications throughout life.

Table I. Developmental change in the immunostaining of VNSP I on the vomeronasal sensory epithelium

Age	Sex	Intensity of immunolabelling	
E18	?	−	(2)
P0	M	++	(1)
P1	F	++	(1)
P3	?	++	(1)
P5	?	++	(1)
P7	M	++	(1)
P2W	M	−	(1)
	F	−	(1)
P3W	M	−	(1)
P4W	M	−	(2)
P5W	M	−	(1)
P6W	M	−	(1)
	F	−	(1)
P7W	M	−	(1)
P8W	M	−	(1)
	F	−	(1)
P9W	F	++	(1)
P10W	M	+	(1)
	F	+	(1)
P12W	M	++	(1)
P15W	M	++	(1)
Pregnant	F	++	(1)
Lactating parent	F	++	(2)

Serial sections (rostral–caudal) of each mouse vomeronasal complex were stained with AbIC, among which the most intense labelling was estimated on the following scale: −, not detected; +, weak signal; ++, strong signal. The numbers of mice analyzed are shown in parentheses. M, male; F, female; ?, not discriminated.

Native form of VNSP I

When extract from the VNC was treated with an amine-reactive homobifunctional cross-linking reagent (*bis*(sulfosuccinimidyl)suberate, BS^3), a faint 45 kDa protein band reactive with AbIC appeared (Figure 6, lane 3). The band is thought to represent the homodimer of VNSP I, since self-association was observed when we used the cDNA-derived VNSP I (Figure 6, lanes 5 and 6). However, a majority of VNSP I were detected as monomers after treatment with as much as 10 mM of BS^3. Similar results were obtained by using two other homobifunctional cross-linking reagents: disulfosuccinimidyl tartarate and ethylene glyco*bis*(sulfosuccinimidylsuccinate).

Discussion

We performed differential screening to isolate cDNAs present within the vomeronasal system, but not within the main olfactory system. We obtained two genes (VNSP I and II) which encode new members of the lipocalin family. *In situ* hybridization demonstrated that expression of the two genes is restricted to the vomeronasal gland and the posterior glands of the nasal septum. Immunohistochemistry confirmed the sites of expression; by fixation with formalin vapor, VNSP I was localized to the mucus of the vomeronasal sensory epithelium.

Functional organization of VNSPs; why two lipocalins in the VNO?

Bovine OBP was reported to make a dimeric complex (Bignetti *et al.*, 1985; Pevsner *et al.*, 1985). Gel filtration

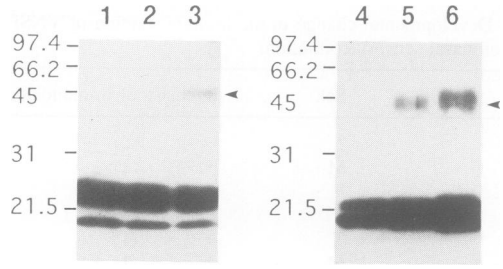

Fig. 6. Immunoblot analysis of the cross-linked native (lanes 1–3) and cDNA-derived (lanes 4–6) VNSP I. Fifty micrograms of extract from the VNC of adult male mice were treated with 0 mM (lane 1), 1 mM (lane 2) and 10 mM (lane 3) of cross-linker (BS^3; Pierce). In each lane, 5 μg of protein were loaded. In the same way, 8 μg protein from the culture medium of Hi Five™ cells producing VNSP I were treated with 0 mM (lane 4), 1 mM (lane 5), and 10 mM (lane 6) BS^3. In each lane, 0.5 μg of protein was loaded. The 45 kDa protein bands that seem to represent a homodimer of VNSP I are indicated by arrowheads.

and sucrose centrifugation analyses showed that the apparent size of the native OBP is 38 kDa (the M_r of OBP estimated by SDS–PAGE is 19 kDa). The three-dimensional structure of bovine OBP has been reported (Monaco and Zanotti, 1992): two loops were shown to be involved in dimer formation. We have been interested in the fact that the distribution patterns of the mRNAs of VNSP I and II are identical. Also, the expression level of both transcripts is extremely high. Among the 36 clones initially obtained by differential hybridization, 17 clones were VNSP I, 11 were VNSP II and there were no other clones encoding lipocalin molecules. Thus, we rule out the possibility of the existence of other types of lipocalin family molecules which would exhibit the same expression pattern as VNSPs. Although VNSP II has not been immunolocalized, it is conceivable that the protein is also present in the mucus of the vomeronasal sensory epithelium. What does it mean that two lipocalin molecules are expressed in the same places and in the same amounts? The co-localization suggests complementary functions of the two proteins. It was, therefore, speculated that VNSP I and II associate to make a heterodimeric complex. However, AbIIC did not react with the proteins in the VNC that had been immunoprecipitated with AbIC (data not shown). VNSP I and II are not likely to associate with each other. *In situ* hybridization analysis using P3 mice revealed that the mRNA of VNSP I is expressed in the VNG and PGNS in the neonatal period, whereas that of VNSP II is absent (data not shown). In contrast to VNSP I, VNSP II may not be involved in suckling behavior. These findings suggest that the two VNSPs work independently of each other.

The native form of VNSP I in the VNC extract was examined by cross-linking experiments. A majority of VNSP I were recognized as monomers after treatment with cross-linking reagents (Figure 6). In addition, an immunoblot analysis with the sample separated on native polyacrylamide gel (results not shown) gave rise to similar protein bands to those detected in SDS–PAGE under reducing conditions. We thus assume that VNSP I exists mainly as a monomer.

Lipocalin proteins in stimulus sources

A well-characterized lipocalin, retinol-binding protein (RBP), delivers retinol from its storage site in the liver to various vitamin A-dependent tissues (for a review on RBP, see Goodman, 1984; Blomhoff *et al.*, 1990). RBP is synthesized in hepatocytes, where it obtains one molecule of retinol in the endoplasmic reticulum. After its secretion into the plasma, the protein forms a non-covalent complex with prealbumin (transthyretin). RBP, free or in complex with prealbumin, is recognized by a cell surface receptor that transfers the vitamin A into the cells. Our concern was whether stimulus sources for the accessory olfactory system, such as urine and vaginal discharge, have any proteins which associate with VNSPs. Interestingly, the stimulus sources also contain lipocalin proteins: major urinary protein (MUP) in mouse urine (Clark *et al.*, 1984; Cavaggioni *et al.*, 1990) and possibly a lipocalin corresponding to hamster aphrodisin (Singer and Macrides, 1990) in mouse vaginal discharge. These lipocalin proteins may come into the lumen of the VNO and may deliver pheromones to VNSPs by making dimeric complexes (see Figure 7, pathway C). We combined male or female mouse urine and vaginal discharge with the VNC extract. The samples were separated on a 15% native polyacrylamide gel, or treated with some amine-reactive cross-linking reagents and subjected to SDS–PAGE. Immunoblotting analyses with AbIC or AbIIC detected no shifts of the reactive bands (data not shown). So far we have not been able to observe any complex formations of VNSPs that would occur in the VNO.

Pre(peri)receptor events of pheromone perception

Our present study suggests that small lipophilic molecules (they tend to be volatile) stimulate the accessory olfactory system. How would the volatile pheromones come into the lumen of the VNO? Some of them may travel from stimulus sources by themselves (Figure 7, pathway A). In fact, Jemiolo *et al.* (1986) synthesized two volatile constituents which are found in male mouse urine, and showed that the synthesized compounds alone induced the estrous cycle in female mice. Although, in many cases, high-molecular-weight and non-volatile fractions of stimulus sources have been found to stimulate the accessory olfactory system, it is possible that the active components (pheromones) are small volatile molecules which are associated with proteins. The volatile pheromones may be transported to the VNO lumen by lipocalin proteins in stimulus sources (Figure 7, pathways B, C and D). After entering the VNO, the pheromones may traverse the mucus layer by binding VNSPs (Figure 7, pathways A, B and C) or exogenous lipocalins (Figure 7, pathway D; Bacchini *et al.*, 1992).

The exact roles of lipocalin proteins in chemical sense organs are still elusive (Pevsner *et al.*, 1985, 1988a,b; Lee *et al.*, 1987; Schmale *et al.*, 1990; Dear *et al.*, 1991; Breer, 1994). The proteins are thought to bring chemostimulants to the receptors; alternatively they might act as scavengers by binding and thereby clearing the stimulative molecules after reception. In any case, the discovery of the two lipocalin proteins (VNSP I and II) has led to the notion that small lipophilic molecules are involved in the chemo-signals through the VNO. It is likely that the accessory

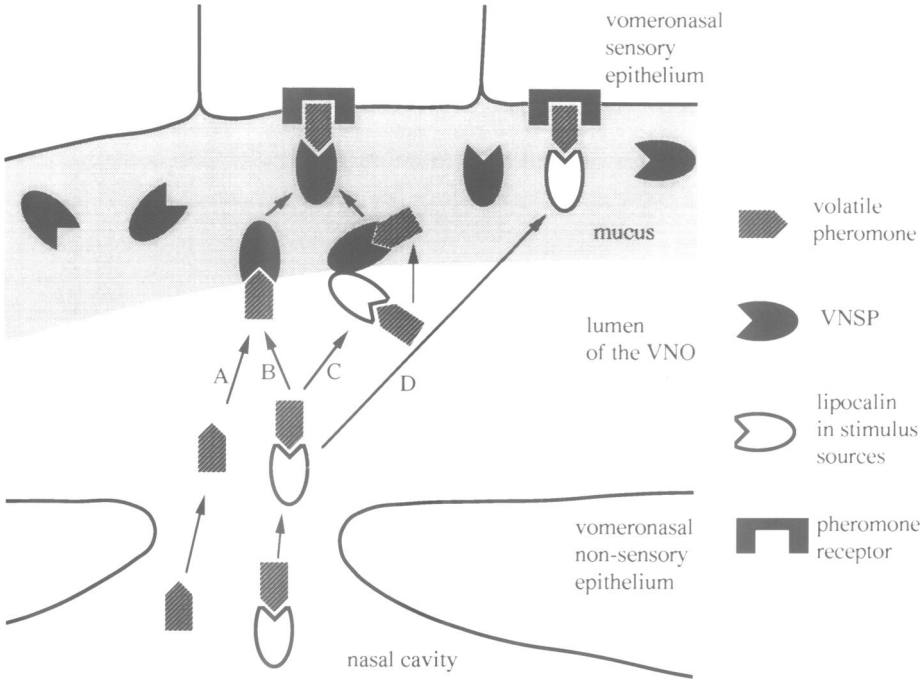

Fig. 7. Prereceptor events of the perception of volatile pheromones in the vomeronasal organ.

olfactory system also has such pre(peri)receptor events as discussed for the main olfactory system (Breer, 1994).

Bacchini *et al.* (1992) analyzed the CH_2Cl_2 extract of purified MUPs by gas chromatography, and showed that MUP selectively binds two male mouse pheromones: 2-(*sec*-butyl)thiazoline and 2,3-dehydro-*exo*-brevicomin. It is necessary to identify the ligands of VNSPs, and to know each VNSP spectrum for lipophilic ligands. We have obtained large amounts of cDNA-derived VNSPs (apo-VNSPs), with which we hope to discover novel pheromones from stimulus sources such as mouse urine and vaginal discharge. Then, by using the holo-VNSPs, we hope to see the specific reception of pheromones taking place on the vomeronasal receptor cells.

Materials and methods

Northern blot

Total RNA (10 μg) was subjected to electrophoresis on a 1.2% agarose gel and blotted to a nylon membrane (Gene Screen Plus, NEN). The blots were hybridized to ^{32}P-labeled full-length cDNA probes 371 (VNSP I) and 841 (VNSP II). Hybridization was performed at 45°C in the presence of 50% formamide, followed by two washes at 60°C, 0.1× SSC, for 30 min. Autoradiography was for 5 h at −70°C.

In situ hybridization

Male or female mice (ddY, 10 weeks old) were perfused transcardially with PBS. Whole nasal tissue was embedded in OCT compound, frozen, and 14 μm thick cryostat sections were fixed for 20 min in 4% paraformaldehyde (PFA) in PBS. Hybridization was performed as described previously (Furuichi *et al.*, 1993) with ^{35}S-labeled cRNA probe corresponding to the *Not*I fragment (full-length cDNA) of VNSP I or II. After washing and RNaseA treatment, the sections were dipped in NTB2 emulsion (Kodak), developed after a 2 day exposure and counterstained with cresylviolet. Dark-field photomicrographs were taken with an Olympus BH2 photomicroscope.

Preparation of VNC extract

Mouse VNCs were frozen and ground to a powder in liquid nitrogen in a mortar. The powdered tissue was added to several volumes of homogenizing buffer (50 mM sodium phosphate buffer, pH 7.8). After incubation on ice for 1 min, precipitated bony components were eliminated, and then homogenized in a glass−Teflon Potter homogenizer. The suspension was centrifuged at 12 000 *g* for 20 min. The supernatant was used as the extract from the VNC.

Production of AbIC and AbIIC

Peptides corresponding to the C-terminal portions were synthesized (CEPESFYELPSRPSE, VNSP I; PEQRDRCIPIHDSAHQDHK, VNSP II), and coupled to keyhole limpet hemocyanin through the cysteine residues. Polyclonal antibodies were raised in rabbits and affinity purified.

Expression of VNSP I and II from the cDNAs

*Not*I fragments of the full-length cDNAs of VNSP I and II were inserted into the polylinker site of transfer vector pVL1392. The recombinant plasmid (pVL1392/VNSPI, pVL1392/VNSPII) was transfected into Sf9 cells with linearized BaculoGold™ virus DNA. The recombinant baculovirus obtained (AcMNPV/VNSPI, AcMNPV/VNSPII) was used to infect 8×10^6 Hi Five™ cells in 10 ml serum-free culture medium (EX-CELL 400™). The medium was collected and used 3 days later.

Indirect immunofluorescence microscopy

Under deep anesthesia with Nembutal, a ddY mouse was placed in a container filled with formalin vapor for 10 min. The tip of the nose was removed and the animal was again put in the container. Then the nasal tissue was fixed in 4% PFA solution at room temperature for 2 h, cryoprotected in PBS containing 20% sucrose and embedded in OCT compound. Sixteen micrometer thick cryosections were incubated in 10% normal goat serum in PBS for 1 h and then kept overnight in the primary antibody (AbIC, 5 μg/ml). As a control, sections were stained with AbIC pre-absorbed with the peptide. AbIC (5 μg/ml) and 100 μg/ml of the peptide were incubated for 1 h. After centrifugation, the supernatant was used. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (Vector, diluted 1:200 in PBS) was used as the second antibody.

Cross-linking experiments

Cross-linking experiments were performed as described previously (Miyawaki *et al.*, 1991). Extract from the VNC or medium of Hi Five™ cells was suspended in 50 mM sodium phosphate buffer (pH 8.0) at a protein concentration of 1 mg/ml. Aliquots (50 μl) of the solution were treated with various concentrations of BS^3 (Pierce) for 30 min on ice.

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