2002

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Published in final edited form as:
J Neurochem. 2002 December ; 83(6): 1452–1460.

Type-specific inositol 1,4,5-trisphosphate receptor localization in the vomeronasal organ and its interaction with a transient receptor potential channel, TRPC2

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Abstract

The vomeronasal organ (VNO) is the receptor portion of the accessory olfactory system and transduces chemical cues that identify social hierarchy, reproductive status, conspecifics and prey. Signal transduction in VNO neurons is apparently accomplished via an inositol 1,4,5-trisphosphate (IP_3)-activated calcium conductance that includes a different set of G proteins than those identified in vertebrate olfactory sensory neurons. We used immunohistochemical (IHC) and SDS–PAGE/western analysis to localize three IP_3 receptors (IP_3R) in the rat VNO epithelium. Type-I IP_3R expression was weak or absent. Antisera for type-II and -III IP_3R recognized appropriate molecular weight proteins by SDS–PAGE, and labeled protein could be abolished by pre-adsorption of the respective antibody with antigenic peptide. In tissue sections, type-II IP_3R immunoreactivity was present in the supporting cell zone but not in the sensory cell zone. Type-III IP_3R immunoreactivity was present throughout the sensory zone and overlapped that of transient receptor potential channel 2 (TRPC2) in the microvillar layer of sensory epithelium. Co-immunoprecipitation of type-III IP_3R and TRPC2 from VNO lysates confirmed the overlapping immunoreactivity patterns. The protein–protein interaction complex between type-III IP_3R and TRPC2 could initiate calcium signaling leading to electrical signal production in VNO neurons.

Keywords

calcium signaling; inositol 1,4,5-trisphosphate (IP_3); olfaction; protein–protein interaction; vomeronasal; vomeronasal organ (VNO)

The vertebrate vomeronasal organ (VNO) contains the sensory neurons of the accessory olfactory system, through which the animal is informed about aspects of its environment such as conspecific reproductive status or social organization. Vomeronasal neurons (VNs) are functionally analogous to vertebrate olfactory sensory neurons, but the VN signaling mechanism is distinct in that it is apparently driven by phospholipase C (PLC)-induced production of inositol 1,4,5-trisphosphate (IP_3) and the subsequent increase in intracellular

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calcium concentration (Luo *et al*. 1994; Kroner *et al*. 1996; Wekesa and Anholt 1997; Sasaki *et al*. 1999; Holy *et al*. 2000; Leinders-Zufall *et al*. 2000).

The VN signal cascade is initiated by binding of specialized chemical signals to transmembrane proteins of the V1R/V3R and V2R receptor families (Dulac and Axel 1995; Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997; Pantages and Dulac 2000). The guanine nucleotide binding (GTP-binding) proteins $G_{\alpha i2}$ and $G_{\alpha o}$ are coexpressed with V1R/V3Rs and V2Rs, respectively, in rodent VNs (Halpern *et al*. 1995; Berghard and Buck 1996; Jia and Halpern 1996; Halpern *et al*. 1998; Wekesa and Anholt 1999). This segregation is not present in other species such as goat, horse, dog or turtle, where $G_{\alpha o}$ is not expressed in the VN epithelium (Takigami *et al*. 2000a,b; Murphy *et al*. 2001). The subunits of G_o and G_i GTP-binding proteins are coupled to PLC activation that leads to the production of IP_3 (Hamm 1998).

The importance of the IP_3 interaction with IP_3 receptors (IP_3Rs) in the maintenance of intracellular calcium levels (see Berridge *et al*. 1998) and in VN signaling is suggested by the production of inward currents in patch-clamped VNs that were dialyzed with IP_3 (Taniguchi *et al*. 1995; Inamura *et al*. 1997; Iida and Kashiwayanagi 2000; Kashiwayanagi *et al*. 2000; Taniguchi *et al*. 2000), and by the IP_3 production by VNs in response to a purified prey item secretion or urinary proteins (Wang *et al*. 1997; Wekesa and Anholt 1997). Further, pheromones and urine induce calcium release in VNs as demonstrated by live calcium imaging (Leinders-Zufall *et al*. 2000), multielectrode array recording (Holy *et al*. 2000) and calcium-dependent whole-cell current recordings (Inamura and Kashiwayanagi 2000).

The transient receptor potential 2 (TRPC2), a putative capacitative calcium entry channel, is expressed in the VNO and highly localized to the microvilli of rat VN sensory neurons (Liman *et al*. 1999). Members of the TRPC family are said to be ‘special assignment’ channels, which are recruited to diverse signaling pathways (Minke and Cook 2002). Although TRPC2 is highly expressed in the VNO, where it functions to elicit proper mating behaviors through identification of sexual partners (Leypold *et al*. 2002; Stowers *et al*. 2002), TRPC2 is also expressed in erythroid cells where it is modulated by erythropoietin (Chu *et al*. 2002) and is found to mediate the acrosomal reaction for sperm penetration during fertilization (Jungnickel *et al*. 2001). TRPC carboxyl termini contain a common IP_3R and calmodulin binding site (Tang *et al*. 2001) and the interaction between IP_3Rs and TRP channels has been demonstrated by coimmunoprecipitation of IP_3R-2 with TRPC1 (Rosado and Sage 2000), IP_3R-3 with TRPC1 (Lockwich *et al*. 2000), and IP_3R-3 with TRPC3 and TRPC6 (Boulay *et al*. 1999). The TRP family of ion channels is quite diverse; some members of this family are calcium-selective whereas others are non-selective (Birnbaumer *et al*. 1996; Harteneck *et al*. 2000; Hofmann *et al*. 2000). TRP family members are known components of plasma membrane calcium entry channels, suggesting a mechanism for external calcium influx (Vannier *et al*. 1999). These data provide strong evidence that IP_3-induced calcium currents are of central importance in VN signaling, although IP_3R expression in the VNO has not been directly demonstrated. Preliminary findings have reported IP_3R immunochemical localization in the VNO of snake and mice (Lin *et al.* 2001; Wang *et al*. 2001).

Here, we use immunohistochemical and protein biochemical procedures to examine VN expression of IP_3R-1, IP_3R-2 and IP_3R-3. Our immunohistochemical data demonstrate an overlap of TRPC2 and IP_3R-3 expression that is supported by coimmunoprecipitation of these two proteins. Such a protein–protein interactive complex suggests a link in the downstream signaling process, by which transduction of the chemical (pheromone) to electrical event is accomplished by calcium release.

Materials and methods

Solutions

Homogenization buffer (HB) contained: 320 mM sucrose, 10 mM Tris base, 50 mM KCl, and 1 mM EDTA; pH7.8. Lysis buffer (LB) contained: 25 mM Tris, 150 mM NaCl, 100 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4, 1% Triton-X 100 (Triton); pH7.5. Protease inhibitor (PI) solution was added to either HB or LB for final concentrations as follows: 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL phenylmethylsulfonyl fluoride, and 10 mM Na_3VO_4. Phosphate-buffered saline (PBS) contained: 137 mM NaCl, 3 mM KCl, 5 mM Na_2PO_4, 2 mM KH_2PO_4; pH7.4. PBS Triton-X-100 (PBST) contained 1% Triton. Wash buffer (WB) contained: 25 mM Tris, 250 mM NaCl, 5 mM EDTA, 1% Triton; pH7.5.

Antibodies

T1NH, T2NHand T3NHare antipeptide polyclonal antibodies specific for the type-1, -2 and -3 IP_3R isoforms, respectively, and were raised against the following amino terminal sequences (amino acid position in parentheses): T1NH = CLATGHYLAAEVDPDQEVDPDQDASR (308–326), T2NH = CPDYRDAQNEGKTVRDGKTVRDGELP (320–338) and T3NH = CENPSYKGDVSDPGDVSDPKAAGPGA (319–337). These antibodies and respective antigenic peptides were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and coimmunoprecipitation experiments and are a generous gift of Dr Gregory Mignery (Loyola University Chicago, Stritch College of Medicine, Maywood, IL, USA) (Ramos-Franco *et al*. 1998). IP_3R type-specific polyclonal antibodies (Affinity BioReagents, Golden, CO, USA) used for immunohistochemistry were directed against the following antigenic amino acid sequences: IP_3R-1 = NKKKDDEVDRDAPSRKKAKE (1829–1848), IP_3R-2 = ELNPDYRDAQNEGKTVR (317–334), IP_3R-3 = RQRLGFVDVQNCMSR (2656–2670). Because different antisera are optimized to perform better in either cryopreserved sections or denatured conditions, or to produce multivalent interactions for immunoprecipitation, different sets of isoform specific IP_3R antisera were selected for immunohistochemistry vs. protein biochemical experiments. Two different polyclonal α-rTRPC2 antisera were selected with independent epitopes and each were found to be equally effective for both immunohistochemistry and immunoprecipitation. The first α-rTRPC2 antibody (TRP-EL) was directed against the carboxyl terminus (814–885) of the rat *TRPC2* gene fused to glutathione *S*-transferase and was a gift of Dr Emily Liman (University of Southern California, Los Angeles, CA, USA; Liman *et al*. 1999). The second α-rTRPC2 antibody (TRP-RA) was directed against the N-terminal cytosolic domain CSSDASGAGPGGPLRNVE and was a generous gift of Dr Richard Axel (Columbia University, New York, NY, USA; Leypold *et al*. 2002).

Immunohistochemistry

Sections (10–12 μm) were cut from 4% paraformaldehyde, fixed and cryoprotected VNO that were harvested from two adult male and female Sprague–Dawley rats (postnatal day = 90) as described previously (Sinnarajah *et al*. 2001). Non-specific binding was blocked by incubation for 30 min in PBST containing 2% albumin Fraction V (PBST-Block; Sigma Chemical Co., St Louis, MO, USA). Sections were incubated in type-specific IP_3R antisera (from 1 : 50 to 1 : 200; Affinity BioReagents) for 90 min at room temperature (25°C), washed with three changes of PBS, and then were incubated for 2 h at rt with fluorescein-conjugated goat anti-rabbit secondary antibody (Roche Molecular Biochemicals, Indianapolis, IN, USA) in PBST-Block. Sections were mounted in 60 : 40 glycerol–PBS with 0.1% *p*-phenylene diamine added to prevent photobleaching. Photomicroscopy was performed at 40× with a Neofluare objective using a Axiovert S-100 microscope equipped

with epifluorescence, an AxioCam digital camera, and Axiovision imaging software (Carl Zeiss Inc., Thornwood, NY, USA).

Protein chemistry

VNOs from male and female Sprague–Dawley rats (postnatal 20–25 day) were quickly harvested and homogenized using 50 strokes by Kontes tissue grinder (size 20) (Kontes Glass, Vineland, NJ, USA) in ice-cold LB with PI solution. Lysis was continued on a Roto-Torque slow-speed rotary (Cole-Palmer Instrument, Vernon Hills, IL, USA) for 30 min at 4°C. The lysates were clarified by centrifugation at 14 000 *g* for 10 min at 4°C and pre-cleared for 1 h with 3 mg/mL protein A-sepharose (Amersham-Pharmacia, Newark, NJ, USA), which was followed by another centrifugation step to remove the protein A-sepharose. Type-III specific IP_3R protein was immunoprecipitated from the clarified lysates by overnight incubation with 5 μg/mL T3NHat 4°C. The samples were then incubated for 2 h with protein A-sepharose and centrifuged as above. The immunoprecipitates were washed four times with WB. Lysates and washed immunoprecipitates were diluted in SDS gel loading buffer (Sambrook and Russell 2001) containing 1 m_M Na_3VO_4 and stored for subsequent analysis.

Purified VNO membrane proteins (see Murphy *et al*. 2001) or immunoprecipitated proteins were separated on 10% acrylamide gels by SDS–PAGE and electro-transferred to nitrocellulose membranes. The nitrocellulose membrane was blocked with 5% non-fat milk and incubated overnight at 4°C in primary antibody against rTRPC2 (1 : 750) or type-specific IP_3R proteins (1 : 2000; Dr Gregory Mignery). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody (1 : 3000) (Amersham-Pharmacia) for 90 min at rt. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure on Fuji Rx film (Fisher Scientific, Suwanee, GA, USA) was used to visualize labeled proteins. The film autoradiographs were analyzed by quantitative densitometry using a Hewlett-Packard Photosmart Scanner (model 106–816; Hewlett Packard, San Diego, CA, USA) in conjunction with Quantiscan software (Biosoft, Cambridge, UK) as described in Murphy *et al*. (2001).

Results

Type-specific IP_3Rs are expressed in the VNO

We investigated the expression patterns of the three IP_3Rs in cryosections of adult male and female rat VNO. Normal rabbit sera produced no significant immunoreactivity in the VNO epithelium but did yield some immunoreactivity in the glands of the lamina propria (Figs 1a and b). αIP_3R-1 weakly labeled the apical surface in both sexes (Figs 1c and d). Interpretation is problematic as the signal is weak and, at high concentrations, normal rabbit serum rendered a similar signal pattern in some preparations (data not shown). Coupled with SDS–PAGE data (Fig. 2), type-I immunoreactivity is likely to be non-specific. αIP_3R-2 immunolabeling occurred in the zone of the supporting cell somata but not in the sensory cells or in the processes apical to the supporting cell somata (Figs 1e and f). The αIP_3R-2 signal was consistently stronger in sections of male vs. female VNO. αIP_3R-3 immunolabeling was present throughout the sensory epithelia of both sexes except in the zones of the supporting cell somata (Figs 1g and h).

We used SDS–PAGE/western analysis to determine whether or not the immunoreactivity observed in tissue sections was specific. Membrane preparations of male and female adult rat VNO were separated, electro-transferred to nitrocellulose, and probed with type-specific IP_3R antisera. Cerebellum was used as a positive IP_3R-1 control. All three IP_3R isoforms have an approximate molecular weight (M_r) greater than 200 kDa (Ramos-Franco *et al*.

2000). No bands were visible in the VNO preparation when probed for IP_3R-1 (Fig. 2a, left panel). Bands of the appropriate M_r (arrow) were found in VNO membrane preparations following incubations with αIP_3R-2 (middle panel) and αIP_3R-3 (right panel) (Fig. 2a). These bands were not present on blots probed with antisera that were pre-adsorbed with the respective antigenic peptide (Fig. 2b), incubated with non-immune rabbit sera, or blots in which primary antisera were omitted (data not shown). Quantitative immunodensity analysis indicated that male VNO preparations contain significantly greater band densities for type-II IP_3R in comparison to those measured in female VNO preparations (Student's *t*-test, arc-sin transformation of percentile data, $\alpha = 0.05$, $n = 12$; four preparations run in triplicate per sex; Fig. 2c). There was no difference in the band density for type-III IP_3R protein between males and females (Student's *t*-test, arc-sin transformation of percentile data, $\alpha = 0.05$, $n = 12$; four preparations run in triplicate per sex). Equal protein loading (30 μg) was controlled by Bradford protein assay and confirmed by Fast Green staining (Sambrook and Russell 2001). Membrane proteins from each sex were also electrophoretically separated in the same PAGE apparatus, transferred to the same piece of nitrocellulose, and then exposed to the same X-ray film to standardize any variance in immunoreactivity or chemiluminescence exposure. Pixel units were determined for each labeled band, background subtracted within that same lane, and then ratios of pixel density were calculated as male : female. The average ratio was calculated for 10 or more experiments to determine the significant difference in the mean expression of a receptor subtype between males and females at the 95% confidence level.

TRPC2 and IP_3R-3 form a protein–protein interaction complex

Adjacent VNO sections that were labeled with αTRPC2 or αIP_3R-3 and counterstained with the nuclear stain DAPI (Figs 3a, b, d and e) showed overlap of immunoreactivity in the microvillar region of epithelium. Double labeling of the two proteins was not desirable because the only available antisera were both generated in the rabbit. Incubation of sections (data not shown) and western blots (Fig. 4a) with preimmune rabbit sera confirmed the specificity of αTRPC2 staining. Because of the immunoreactivity overlap of TRPC2 and IP_3R-3, and in light of a demonstrated common binding site for IP_3Rs and calmodulin on TRP family channels in heterologous expression systems (Tang *et al*. 2001), we questioned whether IP_3R-3 and TRPC2 could exist in a protein–protein interaction complex. We immunoprecipiated IP_3R-3 protein from male and female VNO cell lysates, separated them by SDS–PAGE, and electro-transferred the separated protein to nitrocellulose. Membranes were probed with both T3NH(demonstrating a single band with M_r similar to that in Fig. 2) and αTRPC2 (M_r = 97 kDa) to indicate coimmunoprecipitation of the two signaling proteins (Fig. 4b). The interaction was similar in VNO from males or females ($n = 4$). A second reciprocal coimmunoprecipitation (IP with αTRPC2 and probe with T3NH) demonstrated an association from both protein partners; a strong argument for *in vivo* binding (Fig. 4c). Furthermore, we were able to coimmunoprecipiate TRPC2 and IP_3R-3 using the two independent antisera sources for TRPC2 (TRPC2-EL and TRPC2-RA). Thus, two antibodies were shown to recognize independent epitopes and precipitate the interacting partner (Fig. 4).

Discussion

We used antisera directed against the calcium signaling protein TRPC2 and the three type-specific IP_3Rs to determine the expression pattern of those proteins in adult male and female rat VNO. Immunohistochemistry, in concert with immunoblot analysis, showed that type-I IP_3R either is not expressed or is expressed at levels below the resolution threshold in male and female VNO. Type-II IP_3R is expressed in the supporting cell somata of both sexes but is up-regulated in males compared with females. Type-III IP_3R is expressed throughout the

epithelium except in the supporting cell somata zone; and in the microvilli, type-III IP_3R expression overlaps with that of TRPC2. Co-immunoprecipitation of type-III IP_3R and TRPC2 proteins may suggest a link between chemically-activated G protein coupled VN receptors, IP_3-activated calcium-sensitive VN conductances and transient receptor potential channels localized to the VN microvillar surface.

Combined immunohistochemical and biochemical techniques showed that type-II IP_3R is unusually sexually dimorphic in its expression (see Figs 1e,f and 2a,c). Although other putative VNO signal transduction components have demonstrated a degree of developmental or sex-dependent expression (Herrada and Dulac 1997; Murphy *et al*. 2001), the functional implication of such dimorphism of type II is unclear given that the signal is restricted to the zone of the supporting cell somata, but not visible in either the sensory cell somata or the processes apical to the supporting cell somata.

In contrast to the rather restricted type-II IP_3R expression pattern, type-III IP_3R immunolabeling was found throughout the VN sensory epithelium including the receptor zone and the apical processes. Although the overlap between the type-II and -III IP_3R is not completely exclusionary, such a separation may imply a division of function across cell types. Such differential expression of type-II and -III IP_3R has been previously reported, especially in tissues with secretory functions (Fujino *et al*. 1995). All three isoforms of IP_3Rs are 60–70% homologous with one another (Taylor *et al*. 1999), yet subtle differences in IP_3 binding affinity (Newton *et al*. 1994), calcium-dependent regulation of open probability (Bezprozvanny *et al*. 1991), and modulation by phosphorylation (Bezprozvanny and Ehrlich 1995; Jayaraman *et al*. 1996) could shape different spatial and temporal patterns of cytosolic calcium signals within neurons. It is interesting that across both the VN epithelium and in the circumvallate papillae governing bitter taste transduction, type-III IP_3R appears to be widely expressed, and for the taste receptor it is the dominant isoform, with either little or no evidence for immunoreactivity to type-I or -II IP_3R (Clapp *et al*. 2001). In conjunction with other regulators such as calcium, ATP and phosphorylation, IP_3 gates IP_3Rs in an isoform-specific manner (Thrower *et al*. 2001). In fact, as type-III IP_3R can be activated in the presence of very high levels of IP_3 (for the VNO, such as in the confines of a cellular structure like the microvilli), it has been theorized that this type of IP_3R is best suited and responsible for initiating calcium release in single cells (Thrower *et al*. 2001), as opposed to establishing calcium oscillations or regenerative responses (type-I and -II IP_3R).

Both the localization of type-III IP_3R and TRPC2 proteins in light microscopic sections and the protein–protein interaction between these two molecules, as confirmed by coimmunoprecipitation, provide a strong contention for calcium signaling as a means for encoding species-specific chemical information. Available electron microscopy studies are inconclusive as to whether or not IP_3Rs are in fact present in the microvilli of VN neurons (Menco *et al*. 2001). It is possible that the site of interaction between IP_3R-3 and TRPC2, as associated *in vivo*, could interfere with the binding of IP_3R antisera directed at shielded epitopes. A common binding site for IP_3Rs and calmodulin has been found on all TRPC family members; TRPC2 has two binding sites at the carboxyl terminus (901–936 and 944–1072) to which IP_3R isoforms may bind (Tang *et al*. 2001). Conversely, TRPC3 binds N-terminal amino acids (range: 654–698) depending upon the IP_3R isoform. T3NHand αIP_3R-3 (ABR) antisera used in this study were generated against an upstream amino terminal sequence (319–337) and a nearly terminal carboxyl sequence (2656–2670), respectively, and did not interfere with the TRPC2/IP_3R binding site in either our immunoprecipitation or our immunohistochemical experiments.

Type-III IP_3R is expressed throughout the neuroepithelium, whereas the TRPC2 protein expression is highly restricted to the microvillar layer. These data could suggest an additional function for type-III IP_3R beyond the initial chemical–electrical transduction event at the microvilli. Nonetheless, the two proteins demonstrate overlapping expression and their coimmunoprecipitation argues strongly for protein– protein interaction *in vivo*. There are several important controls that were tested and designed to demonstrate *in vivo* binding. First, both T3NH (this report) and TRPC2 antisera (Murphy *et al*. 2001) were highly specific and recognized only one protein by western analysis. Secondly, the use of two TRPC2 antisera that recognize independent epitopes and precipitate the same interacting partner supports interactive binding. Thirdly, the interaction could be demonstrated by both protein partners (reverse coimmunoprecipitation). Finally, confirmation of TRPC2 (Fig. 4a) and type-III IP_3R (data not shown) immunolabeling in unprecipitated lysates and immunoprecipitation of type-III IP_3R followed by immunoblot of type-III IP_3R (Fig. 4c) demonstrate that association of TRPC2/IP_3R-3 represents a reasonable proportion of the total protein in solution (greater than 10%; Harlow and Lane 1999). Although a formal possibility, it is unlikely that TRPC2 and type-III IP_3R coassembled *in vitro* following cell lysis. Affinity of interactions between two proteins ranges from 10 μ_M to 10 n_M, the molar abundance for signaling molecules and ion channels is extremely low, and the large volume of the immunoprecipitate sample (1 mL) relative to original VNO cell volume (5.2×10^{-13} mL; cell volume estimated as a sphere $4\pi r^3/3$, using $r = 5$ μm, Trotier *et al*. 1998; i.e. representing 13 orders of magnitude difference) would all contend for *in vivo* assembly (Harlow and Lane 1999).

Several recent reports provide evidence of signaling activation or gating of TRPC channels through interaction with IP_3Rs (Kiselyov *et al*. 1998). Such activation of capacitative calcium entry channels requires close physical interactions between endoplasmic reticulum and plasma membrane constituents (Putney 1999). In the confines of microvillar processes that may physically constrain possible endoplasmic reticulum stores, type-III IP_3R may be expressed in close proximity to TRPC2 as an adjacent protein within the plasma membrane to regulate influx of extracellular calcium as a modification of the conformational coupling model (Irvine 1990). Although IP_3Rs have been classically associated with the endoplasmic reticulum, strong support of a plasma membrane IP_3R has been demonstrated in the main olfactory epithelium (Restrepo *et al*. 1990; Fadool and Ache 1992; Kalinoski *et al*. 1992; Restrepo *et al*. 1992; Cunningham *et al*. 1993; Munger *et al*. 2000).

Although we do not know the physical orientation of the IP_3R-3/TRPC2 complex within the membrane(s) and whether additional unknown scaffolding proteins such as PDZ-domain-containing or other adaptor molecules are involved in the assembly of this calcium entry signaling complex (Tsunoda *et al*. 1997; Yao *et al*. 1999), our findings provide further evidence for putative components of the signal transduction cascade present in vomeronasal sensory neurons. It is plausible that the interaction between IP_3R/TRPC2 could be indirect under our conditions of lysis, and we are currently pursuing this utilizing a heterologous expression system to overexpress cloned components. Thus far, in native VNO, we are not able to detect INAD-like proteins (INAD, inactivation no after potential D) (Brann, unpublished data), a common adaptor protein associated with TRPC channels in *Drosophila* (Li and Montell 2000). Gene-targeted deletion of the TRPC2 protein has demonstrated that this channel is required in the VNO to detect sexual partners and elicit male–male aggression (Leypold *et al*. 2002; Stowers *et al*. 2002); however, a ligand to functionally gate the TRPC2 channel has yet to be reported. In T-cells, protein–protein interactions with the IP_3R have been found to shift Ca^2-dependent regulation of the gating of the channel (Jayaraman *et al*. 1996). This report thus furthers the recognition of putative signal transduction machinery in the vomeronasal organ to suggest that regulation of calcium

signaling and ultimately pheromone detection may depend upon isoform-specific IP_3R activation in association with interaction with the TRPC2 channel.

Abbreviations used

HB	homogenization buffer
IHC	immunohistochemical
IP_3	inositol 1,4,5-trisphosphate
IP_3R	IP_3 receptors
LB	lysis buffer
M_r	molecular weight
PBS	phosphate-buffered saline
PBST	PBS Triton-X-100
PI	protease inhibitor
PLC	phospholipase C
rt	room temperature
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TRPC2	transient receptor potential channel 2
VN	vomeronasal neurons
VNO	vomeronasal organ
WB	wash buffer

Acknowledgments

The authors would like to express their gratitude to Ms Kristal Tucker and Dr James M. Fadool for technical and microscopic assistance, respectively. We would like to thank Dr Todd C. Holmes for helpful technical suggestions. We would like to thank Drs Emily Liman and Richard Axel for TRPC2 antisera and thank Dr Greg Mignery for donation of type-specific IP_3R antisera and antigenic peptides. This research was supported by a First Year Assistant Professor Award from the Florida State University (DAF) and a FAA grant 01-6-022 to EEM. JHB was supported by an NIH/NIDCD Predoctoral Chemosensory Training Grant T32-DC00044 to the Florida State University.

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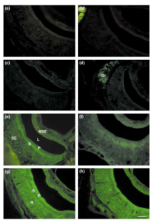

Fig. 1. Type-specific inositol 1,4,5-trisphosphate receptor (IP_3R) protein immunolabeling of male and female rat vomeronasal organ (VNO)

Representative 10-μm cryosection of male (a, c, e and g) and female (b, d, f and h) VNO incubated with αIP_3R antisera and visualized using species-appropriate fluorescein-conjugated secondary antibodies. (a and b) Non-immune rabbit sera control: no immunolabeling in the epithelium with some immunoreactivity in the glands surrounding the epithelium. (c and d) αIP_3R-1: weak immunolabeling visible along the apical surface in both sexes. (e and f) αIP_3R-2: immunolabeling in both sexes in the zone of the supporting cell somata but not in the apically distributed sensory cells or processes. Note greater signal intensity in male vs. female VNO. (g and h) αIP_3R-3: immunolabeling occurs in both sexes throughout the epithelium, except the zone of the supporting cell somata. (a–h) Glands (Gl), lumen (L), non-sensory epithelium (NSE), sensory epithelium (SE), supporting cell somata (S), apical sensory cell processes (P), VN receptor cell zone (R); scale bar = 20 μm.

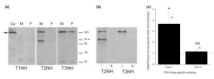

Fig. 2. Type-specific IP_3R proteins visualized by SDS–PAGE-separated vomeronasal (VN) membranes

(a) Western analysis of cerebellum (Ce), male (M) VNO and female (F) VNO probed with type-I (T1NH), type-II (T2NH), and type-III (T3NH) IP_3R antisera. (b) Western analysis of male VN membranes probed with IP_3R type-specific alone (−) or following pre-adsorption (+) with appropriate antigenic peptide. As originally reported (Ramos-Franco *et al*. 2000), the second band in T2NH is likely to represent either proteolysis of full-length receptor or premature translational termination. (c) Histogram showing quantitative densitometric average pixel counts (mean ± SEM) for immunolabeled bands in 12 SDS–PAGE gels (four VNO preparations run in triplicate for each sex) as shown in (a) for the type-II and -III isoforms, respectively. Data are reported as a male/female ratio measured within blot to eliminate variability across epichemiluminescence (ECL) exposure of the autoradiographic films. Background line: male : female ratio = 1.0. *Significantly different by Student's *t*-test, $\alpha = 0.05$, arc-sin transformation of percentile data. NS, not significantly different (i.e ratio close to 1.0).

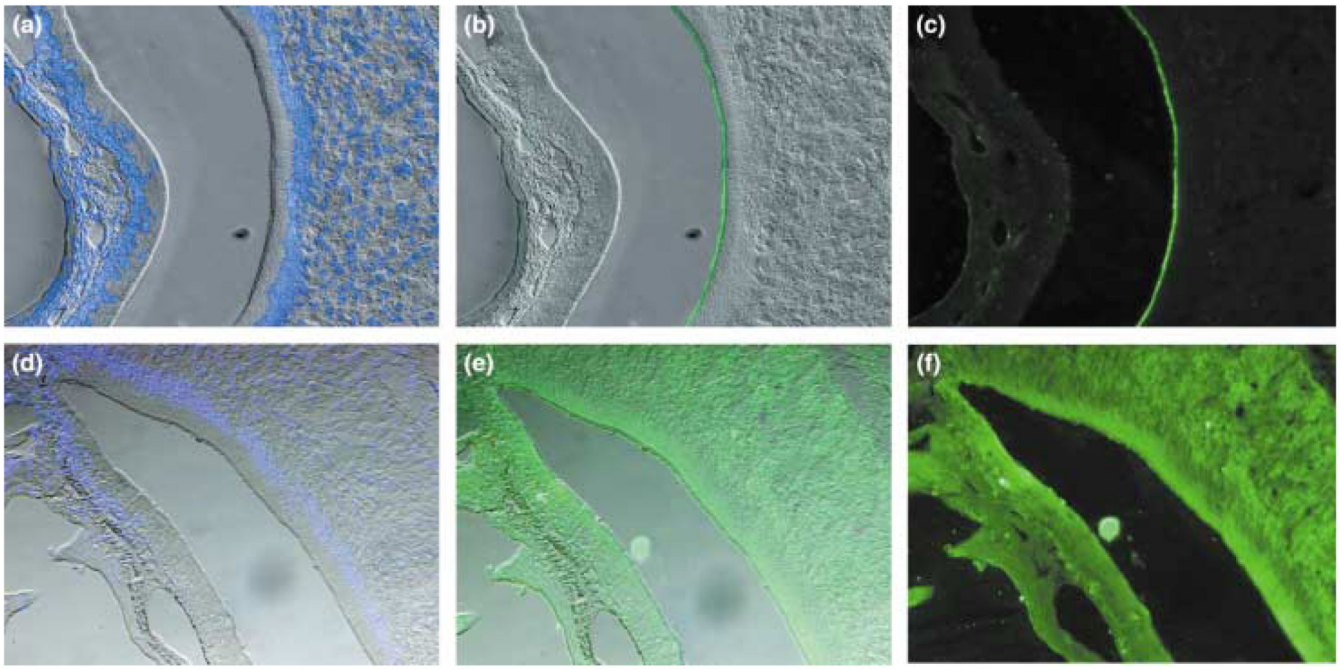

Fig. 3. Type-III IP_3R and transient receptor potential (TRPC2) channel proteins are colocalized in the VNO

(a–c) A cryosection of a male VNO was prepared as in Fig. 1 and labeled with the nuclear stain, DAPI (a) and αrTRPC2 (TRPC2-EL) (b and c). (a) and (b) represent bright-field images digitally merged with epifluorescent signal as shown alone in (c). (d–f) Same as (a)–(c) but for αIP_3R-3.

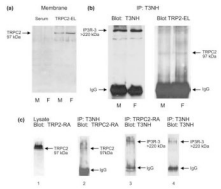

Fig. 4. Type-III IP_3R transient receptor potential (TRPC2) channel proteins are associated in a protein–protein interaction complex

(a) VNO membranes from male (M) and female (F) rats were separated by SDS–PAGE and then the nitrocellulose was probed with rabbit serum (serum) or TRPC2-EL antiserum. TRPC2 protein labeling was detected at 97 kDa (arrow). (b) Type-III IP_3R protein was immunoprecipitated from male (M) and female (F) VN lysates using T3NH (IP : T3NH). Western blots were probed (Blot) with either T3NH or TRPC2-EL. Band intensity reflects the quantity of TRPC2 complexed with type-III IP_3R protein using these antisera. IgG, the heavy chain of Immunoglobulin G. (c) Type-III IP_3R or TRPC2 proteins were immunoprecipitated from whole-cell lysates using T3NH and TRPC2-RA antisera as in (b). (c1) Whole-cell lysates were probed with TRPC2-RA to indicate total quantity of TRPC2 recognized by an antibody with a different epitope than that of (a) and (b). (c2 and c3) Western blots were probed with reciprocal partner to demonstrate coimmunoprecipitation. Band intensity reflects the quantity of T3NH complexed with TRPC2 using these antisera. (c4) T3NH-precipitated proteins were probed with T3NH to indicate total qunatity of the IP3-R protein available for a complex (arrow, > 200 kDa). All SDS–PAGE results in panels (a)–(c) are representative of between four and six different VNO preparations.