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The TRPC2 ion channel and pheromone sensing in the accessory olfactory system

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Abstract The mammalian vomeronasal organ (VNO) has emerged as an excellent model to investigate the signaling mechanisms, mode of activation, biological function, and molecular evolution of transient receptor potential (TRP) channels in real neurons and real physiological systems. TRPC2, a member of the canonical TRPC subfamily, is highly localized to the dendritic tip of vomeronasal sensory neurons. Targeted deletion of the *TRPC2* gene has established that TRPC2 plays a fundamental role in the detection of pheromonal signals by the VNO. TRPC2-deficient mice exhibit striking behavioral defects in the regulation of sexual and social behaviors. A novel Ca^{2+} -permeable, diacylglycerol-activated cation channel found at the dendritic tip of vomeronasal neurons is severely defective in TRPC2 mutants, providing the first clear example of native diacylglycerol-gated cation channels in the mammalian nervous system. The *TRPC2* gene has become an important marker for the evolution of VNO-dependent pheromone signaling in primates.

Keywords Vomeronasal organ · TRP channel · TRPC2 · *trp2* · Diacylglycerol · Diacylglycerol kinase · Phospholipase C · Ca^{2+} signaling

Introduction

The transient receptor potential (*TRP*) genes constitute a large family of ion channels with a wide variety of functions. One major subfamily within this group contains the *TRPC* genes (classical, canonical, or short TRPs), which, in mammals, consist of seven structurally related members: *TRPC1* through *TRPC7* (for recent reviews, see Freichel

et al. 2004; Vazquez et al. 2004). There is currently enhanced interest in the functional role of these genes because evidence is accumulating that they play key roles in phospholipase C-regulated Ca^{2+} signaling pathways.

Here, I provide a brief summary of recent progress that enabled us to define the specific function of one particular member of the *TRPC* genes, *TRPC2* (also known as *TRP2*), which is specifically expressed in sensory neurons of the vomeronasal organ (VNO) in the mammalian accessory olfactory system (Fig. 1). It is now clear that TRPC2 plays a fundamental role in the signal transduction machinery that is necessary for the sensing of pheromonal and other chemical social recognition signals in the rodent VNO (see below). As such, this work has become an excellent example for the usefulness of TRPC-deficient mouse models in determining the biological role of TRPC channels, not only in native cells but also at the systems level in behaving animals.

Initial identification of TRPC2

The search of an expressed sequence tag data base for human orthologs of the *Drosophila* *trp* channel led to the initial discovery of the human *TRPC2* gene (Wes et al. 1995), followed by cloning of mouse (Zhu et al. 1996; Vannier et al. 1999; Hofmann et al. 2000; Yildirim et al. 2003), bovine (Wissenbach et al. 1998), and rat (Liman et al. 1999) orthologs. Whereas the primary structure of *hTRPC2* contains stop codons, full length transcripts were found in mouse and rat, leading to the suggestion that *TRPC2* is a pseudogene in man but not in rodents. Phylogenetic analysis of the bovine ortholog suggests a closer relationship to *mTRPC2* than to *hTRPC2* (Wissenbach et al. 1998). Initial sequence analysis predicted an ion channel containing six membrane-spanning domains, a putative pore region between the fifth and sixth regions, and an intracellular N-terminus with ankyrin repeats (Liman et al. 1999; Vannier et al. 1999; Hofmann et al. 2000), but a systematic analysis of the structure of TRPC2 has not yet been carried out (for a discussion of structure–function

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Fig. 1 Organization of the sense of smell in the mouse. **a** Mid-sagittal view of the rodent nasal cavity (NC) and forebrain. Sensory neurons in the main olfactory epithelium (MOE) project their axons to glomeruli in the main olfactory bulb (MOB). These neurons use cAMP and cyclic nucleotide-gated channels for sensory transduction. GC-D neurons, which likely use a cGMP-mediated signal transduction cascade, are also located in the MOE, but project to a limited number of necklace glomeruli in the MOB (adapted from Zufall and Munger 2001). Sensory neurons in the vomeronasal organ (VNO), located at the base of the nasal septum (S), project to the anterior (gray) or posterior (black) accessory olfactory bulb (AOB). **b, c** Coronal section through the nasal cavity, demonstrating the typical crescent-shaped organization of the VNO sensory epithelium. The VNO epithelium is segregated into two distinct zones, both of which express a unique set of transduction-related molecules: an apical or superficial zone (gray sensory neurons) that expresses the G protein $G\alpha i2$ as well as members of the V1R family of vomeronasal receptors (~150 genes), and a basal or deep zone that characteristically contains sensory neurons (black) that express $G\alpha o$ and members of the V2R receptor family (>150 genes). Both neuron types also contain TRPC2.

relationships of the TRPC subfamily, see Vazquez et al. 2004).

A major advance occurred with the cloning of *TRPC2* from rat VNO by Liman et al. (1999). This study provided three particularly striking results. First, northern analysis showed expression of *rTRPC2* in the VNO but not in main olfactory system or brain, suggesting a highly specific role for the gene product. Second, in situ hybridization revealed an expression pattern that was restricted to all vomeronasal sensory neurons (VSNs). Third, immunolabeling of TRPC2 demonstrated intense staining only in a highly

limited region at the dendritic tip of the sensory neurons containing the sensory microvilli, the proposed site of sensory transduction. Based on these findings, *TRPC2* was proposed to encode an ion channel participating in VNO sensory transduction (Liman et al. 1999; see also Hofmann et al. 2000). This was further supported by immunoelectronmicroscopy localization of TRPC2 (Menco et al. 2001).

Pheromone responses in the vomeronasal organ

To demonstrate a functional involvement of TRPC2 in VNO sensory transduction, it was necessary to develop techniques enabling the recording of sensory responses in the VNO and to define the nature of the sensory stimuli detected by the VSNs. A major breakthrough came with the development of reduced VNO preparations and physiological recording strategies by Leinders-Zufall et al. (2000) and Holy et al. (2000). Recent reviews have summarized this quest (Liman 2001; Zufall et al. 2002; Dulac and Torello, 2003; Liman and Zufall 2004).

Both groups used approaches for simultaneous recording from large populations of mammalian VNO neurons to overcome the problem of identifying a sufficient number of responsive cells. Leinders-Zufall et al. (2000) first developed a method to record local field potentials from the microvillous surface of the intact VNO sensory epithelium. These field potentials, the electrovomeronasogram (EVG), register summed local activities of a large number of VSNs, allowing efficient screening of potentially bioactive chemicals. This approach revealed excitatory, electrical responses in the VNO following stimulation with six prospective pheromones known to be secreted in mouse urine, demonstrating that the VNO indeed can transduce specific pheromonal ligands into electrical membrane signals. Leinders-Zufall et al. (2000) also developed a VNO slice preparation, which allowed for the optical recording of sensory responses in large numbers of individual VSNs by means of confocal Ca^{2+} imaging, as well as patch clamp recording from single, visually identified neurons. Holy et al. (2000) used a 64 microelectrode array to record extracellular action potential activity from large subsets of sensory neurons in an explant of mouse VNO epithelium, employing dilute urine, a rich source of natural pheromones, as a sensory stimulus. All these methods proved highly useful in characterizing the basic response and coding properties of VNO neurons (for recent reviews see Liman and Zufall 2004; Luo and Katz 2004).

TRPC2-deficient mice reveal an essential role for TRPC2 in VNO pheromone detection

With this methodology in place, the next step was to generate TRPC2-deficient mice and compare functional VNO responses in wildtype and mutant animals (Fig. 2a, b). Again, this was done independently by two groups (Leypold et al. 2002; Stowers et al. 2002). Several review articles have summarized these results, providing a detailed

Fig. 2 **a–f** Generation of *TRPC2*-deficient mice and electrophysiological characterization of VNO responses. **a, b** In situ hybridization on VNO sections with antisense *TRPC2* probe. **a** Signal is detected in all wildtype (*WT*) vomeronasal sensory neurons (VSNs), but **b** is absent in *TRPC2*^{−/−} mice (adapted from Leybold et al. 2002). **c** Strongly diminished field potential responses in *TRPC2*-deficient VSNs to stimulation with the prospective pheromone 2-heptanone (Leybold et al. 2002). **d–g** *TRPC2*^{−/−} VSNs display a striking defect in the activation of a diacylglycerol (DAG)-activated conductance. **d** Representative families of whole-cell currents to a series of depolarizing and hyperpolarizing voltage steps (as indicated in the figure) recorded from an isolated WT VSN. Experiments are performed in the presence of 1 μ M tetrodotoxin to block voltage-gated Na⁺ channels; voltage-activated K⁺ channels are blocked by using a Cs⁺-based pipette solution. Dotted line zero current level. **e** A prominent DAG-gated conductance is observed in these cells following application of the endogenous DAG analog 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG, 100 μ M). **f** In VSNs from *TRPC2*^{−/−} mice, SAG application fails to activate a large conductance. **g** However, a drastically diminished residual response to SAG still exists in these cells (adapted from Lucas et al. 2003).

comparison of the two studies (McCarthy and Auger 2002; Keverne 2002; Brennan and Keverne 2004).

At the cellular level, the *TRPC2* knockout mouse clearly reveals an important role of *TRPC2* in the generation of sensory responses induced by pheromonal ligands in the VNO (Stowers et al. 2002; Leybold et al. 2002). In the absence of *TRPC2*, pheromone-induced VNO field potential responses were either absent or strongly diminished, depending on the stimulus concentration (Fig. 2c; Leybold et al. 2002). Similarly, extracellular action potential recordings showed that individual VSNs from *TRPC2*^{−/−} mice are electrically active but unable to respond to cues

present in dilute urine (Stowers et al. 2002). Both studies concluded that *TRPC2* is crucial for the generation of electrical responses in VSNs to sensory stimulation, i.e., functions as an essential component of the VSN signal transduction machinery.

In contrast to its function in the VNO, it is still unclear whether *TRPC2* plays important roles in other cell types, especially in spermatocytes. Although low levels of *TRPC2* transcripts were detected in testis (Wissenbach et al. 1998; Hofmann et al. 2000), immunohistochemistry failed to confirm this (Stowers et al. 2002). Other results suggested a specific role for *TRPC2* in the sperm acrosome reaction (Jungnickel et al. 2001). Because *TRPC2*^{−/−} mice are fertile and do not differ significantly from wildtype mice in the number of offspring (Stowers et al. 2002; Leybold et al. 2002), the role of *TRPC2* in sperm physiology requires further investigation.

TRPC2-deficient mice exhibit striking behavioral deficits

TRPC2-deficient mice have afforded an excellent opportunity to define the role of the VNO in the generation of innate sexual and social behaviors in mice. This work has shown that deletion of only a single gene from the genome can lead to striking defects in complex social behaviors (Leybold et al. 2002; Stowers et al. 2002). Two key results have emerged from these investigations thus far. First, *TRPC2* is essential for pheromone-evoked male–male aggression. In a resident-intruder assay, which tests for intermale aggression, resident *TRPC2*^{−/−} males fail to initiate attack behavior, although they are physically and neurologically capable of displaying aggressive interactions (Leybold et al. 2002; Stowers et al. 2002). Interestingly, presumably as a result of the lack of an aggressive response, *TRPC2*^{−/−} males fail also to establish dominance hierarchies and instead display urine marking behavior typical of subordinate males (Leybold et al. 2002). Aggressive behavior is also severely attenuated in lactating female *TRPC2*^{−/−} mice that are confronted with a male intruder, indicating that signals transduced by the VNO initiate aggressive behavior in both males and females (Leybold et al. 2002).

Second, a striking defect is also seen in the sexual behavior of *TRPC2*^{−/−} males. Although *TRPC2*^{−/−} males mate normally with females, they display increased sexual behavior towards other males, i.e., mounting other males at a much higher rate (Leybold et al. 2002; Stowers et al. 2002). This behavior had not been observed previously in animals in which the VNO was surgically ablated. This unexpected result has been interpreted as evidence that *TRPC2*-mediated signaling may be essential for gender discrimination (Stowers et al. 2002). One possible model consistent with these data is that mounting is an innate behavior that is inhibited by male pheromones acting through the VNO. *TRPC2*^{−/−} males, therefore, persist in mounting other males. Because of the absence of major defects in male–female sexual behavior in *TRPC2*^{−/−} mice,

pheromones or other sensory cues essential for mating may not be detected by the VNO, but rather by other sensory systems such as the main olfactory epithelium.

Hence, it is now clear that TRPC2 is essential for the detection of male-specific cues in the VNO that, in turn, regulate the expression of complex behavioral repertoires including aggressive and sexual interactions.

TRPC2 is required for a diacylglycerol-activated, Ca^{2+} -permeable cation channel in vomeronasal neuron dendrites

An important question that was until recently unresolved is whether TRPC2 forms a functional ion channel in VNO neurons, and if so, by which mechanism this channel would be gated. Early findings using heterologous expression systems yielded somewhat conflicting results. Whereas one set of studies showed that TRPC2 is not detectable in the plasma membrane but rather accumulates in intracellular organelles when expressed in a variety of cell lines (Hofmann et al. 2000; Hofmann et al. 2002), another study concluded that *TRPC2* encodes a store depletion-activated capacitative Ca^{2+} entry channel (Vannier et al. 1999; see also Vazquez et al. 2004).

To resolve this matter and to define the nature of the signal transduction mechanism in the VNO, Lucas et al. (2003) set out to identify and characterize the native ion channels that are encoded by *TRPC2*. To do this, they used an experimental design that was based on four assumptions:

1. The native channels are likely to be found at the dendritic tip of VSNs
2. They are likely to be regulated, directly or indirectly, by products of phospholipase C (PLC) activity
3. They are likely to be defective in *TRPC2*^{-/-} VSNs
4. They are expected to exhibit functional properties consistent with those of the pheromone-induced conductance

Using a combination of whole-cell and inside-out patch clamp recordings in freshly dissociated VSNs and VNO tissue slices from wildtype and mutant mice, Lucas et al. (2003) indeed found ion channels that meet these four criteria (Fig. 2d–g). Unexpectedly, however, these channels were neither activated by store depletion nor by inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$]. Instead, they were gated by the lipid messenger diacylglycerol (DAG), resembling in their biophysical properties the DAG-gated cation channels formed by hTRPC3 or hTRPC6 (Hofmann et al. 1999). The DAG-gated channel in the VNO clearly depended on TRPC2, because, in TRPC2-deficient VSNs, its activation by the DAG analog 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) was highly diminished, by as much as 90% (Lucas et al. 2003). Thus it is very likely that TRPC2 is a major component, if not the principal subunit, of this DAG-gated channel.

An interesting finding was that the deletion of TRPC2 did not fully abolish activation of the DAG-gated channel in VSNs but left a small but significant residual conductance intact (Fig. 2f, g; Lucas et al. 2003). The presence of

a residual conductance in *TRPC2*^{-/-} VSNs suggests that other channel subunits activated by DAG may exist in these cells, although with strongly reduced efficacy. It remains to be seen whether these predicted channel subunits associate with TRPC2 to form a heteromultimeric channel complex or whether they form independent channels. A search for additional members of the TRP channel family in VSNs has consistently failed (Stowers et al. 2002). It is interesting to note, in this respect, that TRPC2 does not seem to interact with any known TRPC protein (Hofmann et al. 2002), although other work provided evidence for an interaction of TRPC2 with TRPC6 in primary erythroblasts isolated from mouse spleen (Chu et al. 2004).

TRPC2 as a genetic marker for the evolution of VNO-dependent pheromone sensing

The *TRPC2* gene has become an important marker for the evolution of vomeronasal signaling in primates. Because *TRPC2* is expressed uniquely in the VNO and is essential for VNO function, the loss of a *TRPC2* gene can serve as a marker for the loss of VNO function. Based on this reasoning, Liman and Innan (2003), Zhang and Webb (2003) and Webb et al. (2004) examined sequences of the *TRPC2* gene from a large number of primate species. The human *TRPC2* gene has six mutations that generate premature stop codons, resulting in a severely truncated protein. The earliest mutation is a nonsense mutation that is shared by all old world (OW) monkeys and apes and that is predicted to generate a protein that is missing much of its C-terminus (Liman and Innan 2003; Zhang and Webb 2003; see also Liman and Zufall 2004). Because this mutation occurs in a well-conserved region of the protein, it is likely to impair functioning. Thus based on the observation that this mutation is found in all OW monkeys and apes but not in new world (NW) monkeys, one can date the loss of a functional *TRPC2* gene in the human lineage to 25–40 million years ago. This dating is further supported by an examination of selective pressure on the *TRPC2* gene, which was also relaxed at this time (Liman and Innan 2003).

A model for VNO pheromone transduction

Identifying the signal transduction mechanism in pheromone-sensitive VSNs is an important step in understanding how the accessory olfactory system encodes social and reproductive information that is essential for reproductive fitness. Based on the work summarized here, we have proposed a model in which the TRPC2 channel functions as the primary conductance pathway in a pheromone-stimulated second messenger cascade of mouse VSNs (Lucas et al. 2003). Our results strongly support the notion that pheromones and other chemosignals activate specific G-protein coupled receptors (see Del Punta et al. 2002; and references therein), which in turn leads to the generation of DAG or its endogenous analogs by the activity of phos-

pholipase C. DAG then initiates, by gating of the TRPC2 cation channel, the sensory current that underlies the depolarizing receptor potential in these neurons. Termination of DAG signaling occurs, at least in part, through the activity of a DAG kinase (Lucas et al. 2003). This model differs significantly from the mechanisms underlying sensory transduction in the vertebrate visual and main olfactory systems, which both employ cation channels directly gated by cyclic nucleotides (Zufall and Munger 2001). Hence, distinct molecular cascades have evolved in the mammalian main and accessory olfactory systems for the detection of chemosensory signals.

It must be noted, however, that this model is far from being complete and needs to be refined over the next few years. Numerous important questions remain to be answered: what is the role of $\text{Ins}(1,4,5)\text{P}_3$? How does Ca^{2+} entry through the TRPC2 channel contribute to sensory signaling? Which receptors are activated by which ligands and how do they couple to the second messenger cascade? Does phosphatidylinositol-4,5-bisphosphate (PIP_2) play any role in channel activation? Which subunits form the DAG-gated channel? What is the molecular identity of other components of this signaling cascade such as G-proteins, PLC, and DAG kinase? Can the phenotype in TRPC2-deficient mice be rescued by TRPC2 gene delivery? Does TRPC2 form a functional DAG-activated channel in a heterologous cell type? What is the molecular gating mechanism induced by DAG in this new family of ion channels?

Conclusions

The past few years have delivered substantial progress in understanding the functional role of TRPC2 in the mammalian vomeronasal (accessory olfactory) system. On the basis of this work, we can now assign a clear function to TRPC2 at both the cellular and systems levels, showing that TRPC2 occupies a critical role in the detection of pheromonal signals in the VNO and thus is essential for social recognition of conspecifics. The experimental strategy employed in the mouse VNO now serves as a powerful model for examining the native functions of other TRP genes. This work serves also as a prime example of the importance of investigating the function of TRP channels within their native cellular environment and with respect to their biological role at the whole-animal level.

Our work has shown that TRPC2 is essential for a DAG-gated cation channel in VSNs. In fact, this DAG-gated channel represents the first native DAG-gated channel in the mammalian nervous system. Hence, our findings may have important implications beyond the sense of smell in that they provide a general mechanism by which phospholipase C signaling can produce neuronal excitation and Ca^{2+} entry, independently of protein kinase C, $\text{Ins}(1,4,5)\text{P}_3$, and Ca^{2+} stores. TRPC2 does not seem to be expressed in other neurons outside the VNO, but the closely related TRPC3, TRPC6, and TRPC7 subunits are (Ricchio et al. 2002). Because all three proteins can function as DAG-

gated channels (Hofmann et al. 1999; Okada et al. 1999; Lintschinger et al. 2000; in this issue see also Dietrich et al. 2005; Groschner and Rosker 2005), it is reasonable to propose that channel activation by DAG may serve as a general signal transduction mechanism in the brain, as well as in other non-neuronal cell types such as portal vein myocytes (Albert and Large 2003). Only very recently has it been recognized that DAG not only activates its classical target, protein kinase C, but is in fact involved in a multitude of interconnected signaling processes (Brose et al. 2004). Ca^{2+} -permeable, DAG-gated cation channels of the TRPC family can now be added to the 'non-classical, non-PKC' targets of DAG.

Update

A recent report has shown that small peptides that serve as ligands for major histocompatibility complex (MHC) class I molecules function also as sensory stimuli for a subset of vomeronasal sensory neurons (Leinders-Zufall et al. 2004). MHC peptides constitute a previously unknown family of chemosensory stimuli by which MHC genotypic diversity can influence social behavior.

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