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The elusive crypt olfactory receptor neuron: evidence for its stimulation by amino acids and cAMP pathway agonists

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SUMMARY

Crypt olfactory receptor neurons (ORNs) are a third type of chemosensory neuron along with ciliated and microvillous ORNs in the olfactory epithelium of fishes, but their functional role is still unknown. To investigate their odorant response properties and possible transduction pathways, we recorded crypt ORN activity with calcium imaging and the patch clamp technique in its cell-attached mode in combination with odorant and agonist stimulation. Bile salts and putative fish pheromones did not elicit responses with either method, but the cells frequently responded to amino acids, with excitation and intracellular calcium signals. 8Br-cAMP and IBMX plus forskolin stimulated over 40% of crypt ORNs and triggered calcium signals in a similar percentage. Furthermore, crypt ORNs were immunoreactive to an antiserum against adenylate cyclase III. Together, these data suggest the presence of a cAMP transduction pathway, which might transduce odorants such as amino acids.

Key words: crypt cell, fish, olfaction, chemosensory, olfactory receptor neuron, odorant, transduction.

INTRODUCTION

Besides ciliated and microvillous olfactory receptor neurons (ORNs), crypt ORNs are a third type of chemosensory neuron in the olfactory epithelium of actinopterygians (Hansen and Finger, 2000; Hansen and Zeiske, 1998; Hansen and Zielinski, 2005). Recently, the presence of crypt-like ORNs was also demonstrated in a cartilaginous fish (Ferrando et al., 2006), suggesting that they are a conserved feature among fishes. The crypt ORN has an oval cell body that is almost completely surrounded by one or two supporting cells. Mature crypt ORNs localize to the surface of the sensory area of the olfactory epithelium, and their apical invagination (the 'crypt') opens to the exterior space. Both cilia and microvilli are found within the crypt, and are thought to hold the olfactory receptor proteins and other elements of the transduction machinery.

During recent years, considerable progress has been made in the understanding of the molecular components involved in the transduction cascade of vertebrate ORNs. As in mammals, the tall ciliated ORNs of teleost fishes express OR-type odorant receptors and transduce signals through a cyclic AMP (cAMP) cascade that includes the G-protein $G\alpha_{olf}$ (Cao et al., 1998; Ngai et al., 1993). Homologous to mammalian VNO neurons, the shorter microvillous ORNs in the fish olfactory epithelium express V2R-like receptors molecules, the G-protein $G_{\alpha o}$ and possibly others (Belanger et al., 2003; Hansen et al., 2004; Speca et al., 1999), and the transient receptor potential channel C2 (Sato et al., 2005), but the identity of the intracellular messenger that activates these channels is still unclear. Crypt ORNs apparently lack any homologous counterpart in terrestrial vertebrates, and with the exception of the olfactory G-proteins $G\alpha_o$ and $G\alpha_q$, which were identified by immunohistochemistry (Belanger et al., 2003; Hansen et al., 2004; Hansen et al., 2003), neither their olfactory receptors nor the downstream elements of the transduction cascade have been established.

Based on the projection pattern of crypt ORNs in crucian carps and their seasonal density variation, they were proposed to mediate chemical signaling related to reproductive behavior (Hamdani el and Doving, 2006; Hamdani el et al., 2008). Yet, the responsiveness of crypt ORNs to fish pheromones and the involvement of these cells in behavioral control have not been clarified. Therefore, apart from their odorant specificity and transduction process, the entire functional context of crypt ORNs is still hypothetical.

Since neither the molecular nor the anatomical strategies of the cited studies were successful in resolving the function of crypt ORNs, we decided to pursue this matter with a physiological approach. In a previous article, we described the basic electrophysiological properties of crypt ORNs isolated from the Pacific jack mackerel and provided initial evidence for their chemosensitivity towards amino acids (Schmachtenberg, 2006). Here, we further analyzed the odorant response properties of crypt ORNs and show that they are excited by agonists of the cyclic cAMP pathway.

MATERIALS AND METHODS

Patch clamp recordings

Crypt ORNs were isolated from olfactory rosettes of the Pacific jack mackerel *Trachurus symmetricus* (Ayres 1855) of both sexes captured during the summer months from December to March, as described previously (Schmachtenberg, 2006). Briefly, the fish were sacrificed by fast decapitation on ice, the olfactory organs were removed and transferred to ice-cold saline solution, containing (mmol l^{-1}): 150 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 5 Hepes, 10 glucose, pH 7.4. The sensory area of the olfactory lamellas was cut into small pieces, triturated with a fire-polished Pasteur pipette and the dissociated cells were transferred to a cooled (10–15°C) poly-L-lysine-coated recording chamber that was mounted on an Olympus IMT-2 microscope. Cells were viewed with a 100× oil immersion

objective and identified by their characteristic morphology. Minimum criteria for their identification were a comparatively large size, their ovoid shape and a clear separation between the crypt ORN and the supporting cell. Frequently, these features were complemented by the presence of a supporting cell foot process and an apical brush border. Sometimes the crypt was also visible.

Recording pipettes were drawn from borosilicate electrode glass (WPI, Sarasota, Fl, USA) or hematocrit glass capillaries (Hirschmann, Eberstadt, Germany) in a horizontal puller (P-97; Sutter, Novato, CA, USA) to a tip resistance of 5–10 MΩ. The pipette solution contained (mmol l^{-1}): 120 KCl, 4.6 $MgCl_2$, 10 glucose, 10 Hepes, 10 EGTA, 4 Na-ATP and 0.4 Na-GTP, pH 7.4. Cell-attached recordings were obtained with a Dagan 3900 amplifier (Dagan Corp., Minneapolis, MN, USA; generously provided by John Caprio), low-pass filtered at 5 kHz with a four-pole Bessel filter, and digitalized with pClamp software (Axon Instruments, Union City, CA, USA) at a sampling rate of 20 kHz. Odorants and reagents were supplied to the cells with a custom-built picospritzer connected to a triple-barreled stimulus pipette (WPI), as described previously in detail (Schmachtenberg and Bacigalupo, 2004). Amino acids were applied as a mixture of the L-isomers of alanine, arginine, asparagine, glutamate, glycine, methionine, phenylalanine and tyrosine at a concentration of 1 mmol l^{-1} each in saline solution. A mixture of lithocholic acid, sodium taurocholate and taurolithocholic acid ('bile salts'), reportedly strong odorants to fish (Rolen and Caprio, 2007), was applied alternatively. Two candidate fish pheromones, prostaglandin F2α (PGF2α) and 4-pregnen-17a,20b-diol-3-one (17,20βP) were tested together at two concentrations of 5 and 50 μmol l^{-1} each. 8-Bromo cyclic AMP (8Br-cAMP), a membrane-permeant agonist of cyclic nucleotide-gated ion channels and 3-isobutyl-1-methylxanthine (IBMX) together with forskolin, which elevate intracellular cAMP levels, were used at a concentration of 1 mmol l^{-1} in the stimulus pipette. Most cells were tested with two or three stimuli. We did not measure or calculate the agonist concentrations reaching the cell, since too many variables were involved. The criterion for a response to a stimulus was a >30% increase in the spike rate compared to pre-pulse levels over an interval of 5 s during or immediately after the stimulus.

Calcium imaging

For calcium imaging experiments, crypt ORNs were isolated as described above and were loaded with Fluo-4 AM (5 μmol l^{-1} in 0.1% pluronic acid) at 4°C for 1 h. Images were recorded with a Sensicam QE cooled digital camera (Cooke Corp., Romulus, MI, USA) attached to a Nikon Eclipse 2000 (Nikon Corp., Japan) inverted microscope at intervals of 1 s, and analyzed with the NIH ImageJ program. Additional experiments were performed on an upright Nikon FN-1 microscope (objective 40× water immersion, NA 0.8) with a Nikon DS-2MBWc camera controlled by the program NIS Elements (Nikan Corp., Japan). Cells were perfused continuously with cooled saline solution (at ~10°C) and stimulated with a picospritzer, as in the patch clamp experiments. To obtain the relative fluorescence ratio, a bleaching curve was calculated for every experiment and subtracted from the image sequence. Relative pixel intensities were obtained by dividing all images through the initial (prepulse) image of the series. The criterion for a significant response was a >10% increase in fluorescence intensity compared to pre-pulse levels.

Immunochemistry

For western blots, the olfactory epithelia of one jack mackerel and one adult rat were homogenized in ice-cold lysis buffer containing 1% SDS, 50 mmol l^{-1} Tris (pH 8.3), 5 mmol l^{-1} EDTA and a protease and phosphatase inhibitor cocktail. The homogenates were centrifuged at 13 000 ***g*** for 15 min at 4°C. The supernatant was collected and the total protein contents was determined using the Bradford method. The samples were diluted in Laemmli buffer (62.5 mmol l^{-1} Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 300 mmol l^{-1} dithiothreitol), and denatured by heating to 95°C for 5 min. The wells of a 10% acrylamide–bisacrylamide gel were loaded with 90 μg of protein and a molecular mass standard (Kaleidoscope, Bio-Rad, Hercules, CA, USA) was used adjacently. Proteins were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Buckinghamshire, UK), blocked in 8% non-fat milk with 0.1% Tween 20 in Tris buffer for 1 h at room temperature, and incubated overnight at 4°C with the polyclonal anti-adenylate cyclase III antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:500. After three washes, the horseradish peroxidase-coupled secondary antibody (anti-rabbit-HRP; Jackson ImmunoResearch, West Grove, PA, USA; diluted 1:5000), was applied for 1 h at room temperature. Bound immunoglobulins were visualized with the SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA) on Kodak BioMax Light film.

For immunocytochemistry, olfactory epithelium was dissociated as for the physiological experiments, and the cells were fixed in 2% paraformaldehyde for 25 min on poly-L-lysine-coated slides at room temperature. After permeabilization with 0.2% Triton-X-100 for 5 min, the preparation was blocked in 5% BSA for 1 h. The primary anti-adenylate cyclase III antiserum was applied at a dilution of 1:100 overnight at 4°C. As a control, the antiserum was preadsorbed overnight at 4°C with excess antigen (1 μg ml^{-1}; Santa Cruz). After three washes, the preparation was incubated in the secondary antiserum, donkey anti-rabbit Cy-3-conjugated IgG (Jackson ImmunoResearch, diluted 1:1000) for 1 h at room temperature. Finally, the cells were counterstained with DAPI (Invitrogen, Carlsbad, CA, USA) for 5 min at 5 μg ml^{-1} and mounted in Fluomount (Dako Industries, Carpenteria, CA, USA). Photographs were taken with a CoolSnap-Pro digital camera on an Olympus BX-51 microscope operated by Image-Pro Express software (Media Cybernetics, Silver Spring, MD, USA).

The experimental procedures were approved by the Bioethics Committee of the University of Valparaiso and in accordance with the bioethics regulation of the Chilean Research Council (CONICYT). Reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise indicated.

RESULTS

In this study, we used the techniques of cell-attached patch clamp recording and calcium imaging with a membrane-permeant calcium indicator, to avoid dialyzing the intracellular milieu of the cells and obtain responses to odorants and agonists under stable physiological conditions. Slightly less than half of the analyzed crypt ORNs responded to a mixture of amino acids with a significant transient increase of the action potential discharge frequency under on-cell patch clamp and with an increase of intracellular calcium under fluorescence imaging (Fig. 1A, Fig. 2A and Table 1). The observed excitatory effects were independent of the basal discharge frequency of the ORNs, which ranged from zero to about 5 Hz (Fig. 1B). Odorant-induced spike inhibition was not consistently observed. In contrast to amino acids, a mixture of three bile salts did not excite crypt ORNs or generate any calcium signals (Fig. 2E).

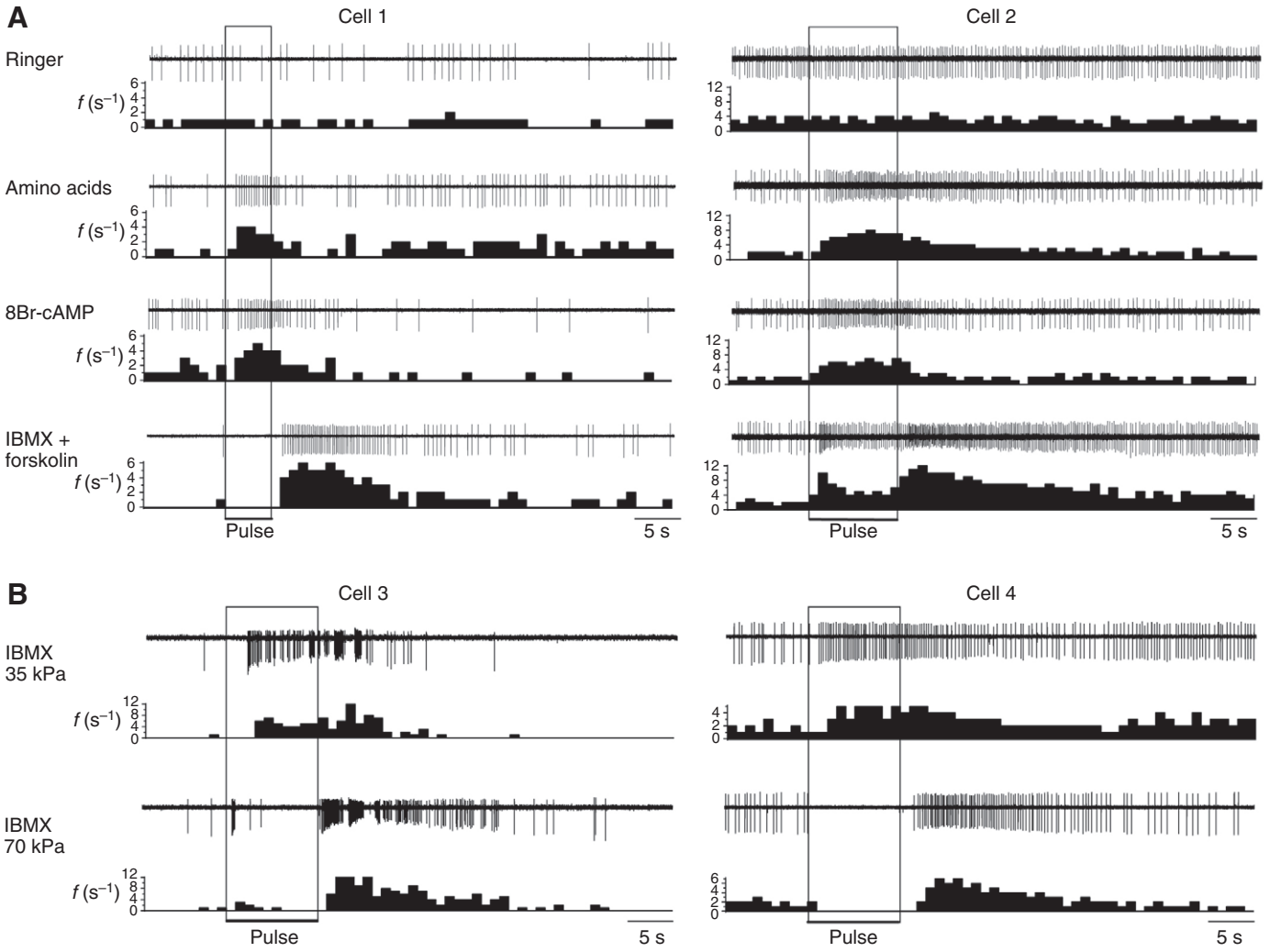

Fig. 1. Cell-attached recordings of isolated crypt olfactory receptor neurons (ORNs) and their respective spike-frequency plots (*f*, s^{-1}). (A) Amino acids, 8Br-cAMP and IBMX plus forskolin caused a transient increase of the spike frequency in crypt ORNs. Individual cells responded to all three stimuli. In experiments with amino acids or 8Br-cAMP, the excitatory effect reverted to baseline levels within seconds following termination of the stimulus, whereas the combination of IBMX and forskolin induced stronger and longer lasting effects. (B) Temporal response characteristics of silent and tonically active crypt ORNs stimulated with IBMX alone. Higher stimulus concentrations, expressed as the pressure applied to the stimulus pipette by the picospritzer, frequently caused spike suppression during the stimulus followed by prolonged excitation during the post-pulse period. Burst-like activity was observed in a significant proportion of cells (cell 3).

To investigate the possibility that a cAMP transduction pathway is expressed and operative in crypt ORNs, we applied 8Br-cAMP through a puffer pipette aimed at the crypt of the cells. Pulses of 8Br-cAMP triggered transient excitation under patch clamp and a rise of intracellular calcium under fluorescence imaging with an intensity and time course similar to the responses to amino acids (Fig. 1A, Fig. 2C). A significantly stronger and longer-lasting effect was observed after the application of IBMX plus forskolin, which triggered larger calcium signals than amino acids or 8Br-cAMP, even more than the positive control KCl (Fig. 2B,D). The moderate

Table 1. Overview of the experimental results

	Patch clamp			Calcium imaging		
Stimulus	No. cells	No. responsive cells	Percentage	No. cells	No. responsive cells	Percentage
Amino acids	41	17	41	27	13	48
Bile salts	14	0	0	7	0	0
PGF2α + 17,20βP	11	1	9	21	0	0
IBMX + forskolin	27	13	48	21	9	43
8Br-cAMP	8	4	50	21	9	43

PGF2α, prostaglandin F2α; 17,20βP, 4-pregnen-17a,20b-diol-3-one; IBMX, 3-isobutyl-1-methylxanthine; 8Br-cAMP, 8-bromo cyclic AMP.

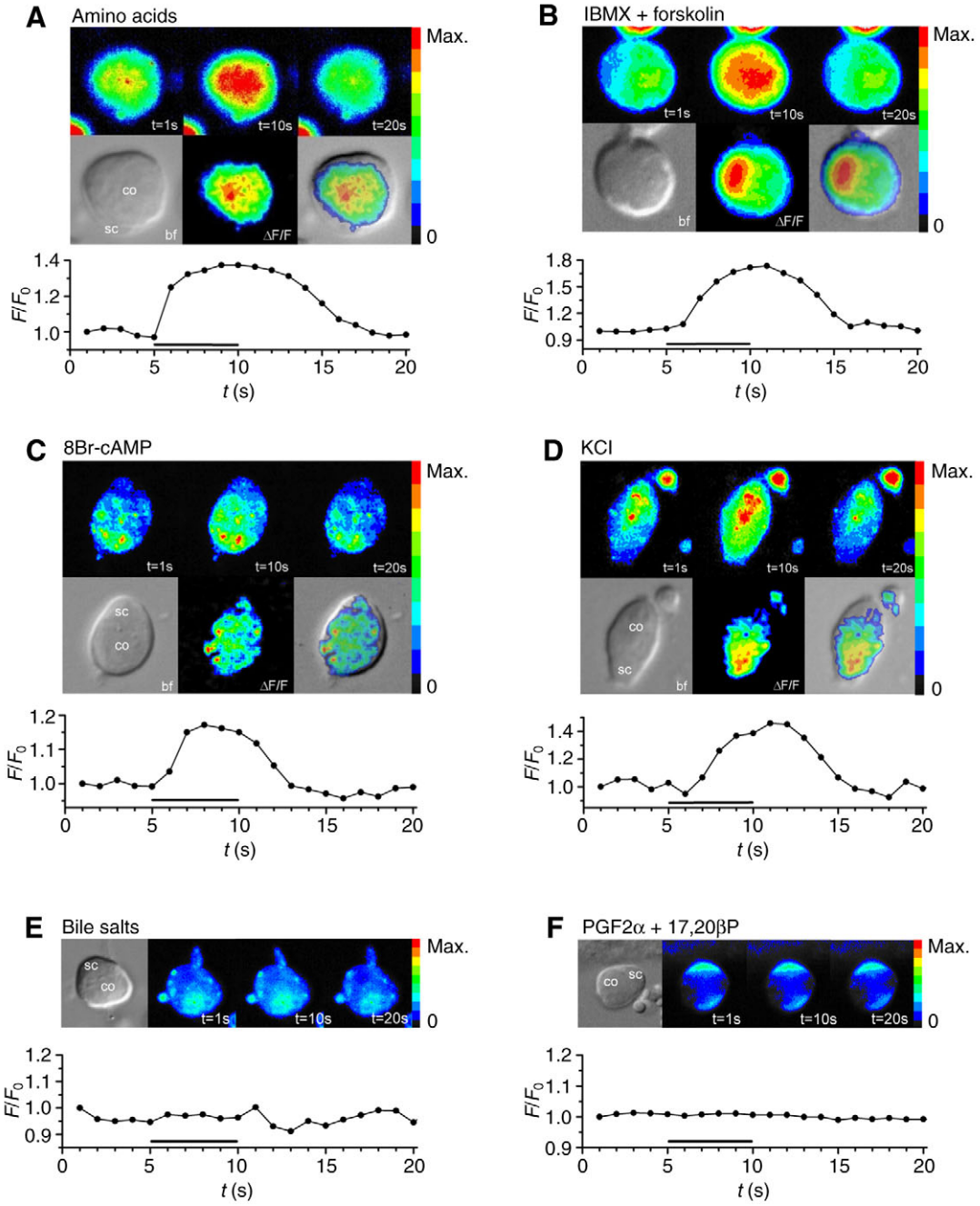

Fig. 2. Calcium imaging of isolated crypt olfactory receptor neurons (ORNs). In each panel the upper row of images shows total fluorescence; the lower row shows a bright-field image, maximum relative fluorescence increase ($\Delta F/F$) and an overlay of both. The corresponding plots display the time course of the normalized fluorescence average of the whole crypt ORN and its supporting cell. Amino acids (A), IBMX plus forskolin (B) and 8Br-cAMP (C) induced a transient rise of intracellular calcium in the crypt ORN. Some supporting cells, which are coupled to the crypt ORN by gap junctions (Schmachtenberg, 2006), were included in the calcium signal (B,C) whereas others were apparently excluded (D). The effects were similar in amplitude and duration to those of stimulation with KCl (50 mmol l^{-1} in the stimulus pipette). Bile salts (E) and the combination of PGF2α and 17,20βP (F) did not cause significant and reproducible effects. bf, bright-field image; co, crypt ORN; sc, supporting cell.

effect of KCl might be due to the fact that the crypt ORN membrane is largely covered by the supporting cell(s). Under patch clamp, IBMX alone elicited prolonged excitation in a dose-dependent manner, which was frequently preceded by an initial suppression of spike activity during the stimulus application (Fig. 1B).

The prostaglandin PGF2α and the steroid hormone 17,20βP were tested to find evidence for the hypothesis that crypt ORNs act as pheromone detectors (Hamdani el et al., 2008). Although isolated effects were observed in one cell under patch clamp, these were just above the defined response threshold and irreproducible in other cells (Table 1). No responses were obtained under calcium imaging (Fig. 2F). Therefore, a direct activation of crypt ORNs by PGF2α and 17,20βP remains to be demonstrated.

In an attempt to complement our physiological evidence for cAMP odor transduction in crypt ORNs with immunocytochemical data, we labeled isolated crypt ORNs with an antibody against

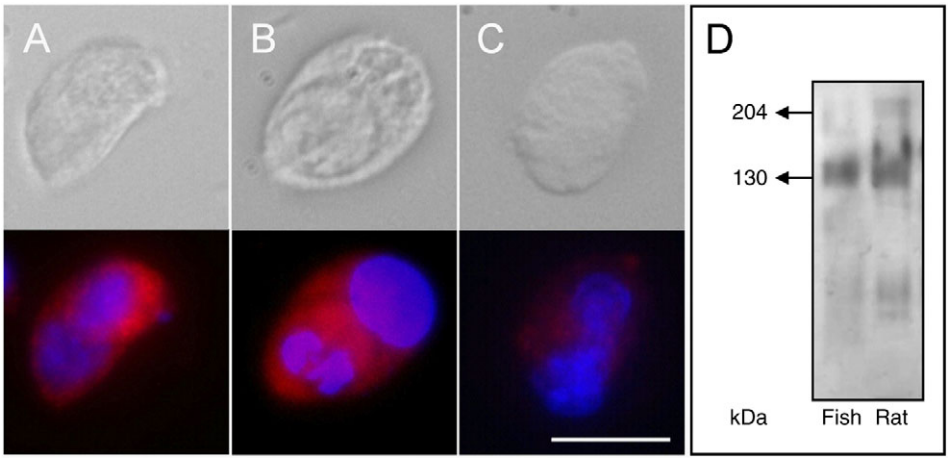

Fig. 3. Immunocytochemistry and western blot of adenylate cyclase III (AC III). (A,B) Bright-field images and immunocytochemical labeling of AC III (red) in isolated crypt olfactory receptor neurons (ORNs) counterstained with DAPI (blue). (C) Near-absence of labeling after preadsorption of the antiserum with excess antigen. Scale bar: 10 mm. (D) Immunoblot of olfactory epithelium from fish and rat. The AC III antiserum marked a band of about 130 kDa in both species. 95 μg of fish protein were loaded as opposed to 15 μg of rat protein.

adenylate cyclase type III (Fig. 3). This antiserum detected a protein with the appropriate molecular mass on western blots of fish olfactory epithelium, which was completely abolished by preadsorption with excess AC-III antigen. Crypt ORNs treated with the antiserum displayed cytoplasmic labeling that tended to be concentrated in the apical part of the cells and around the crypt. This suggests that AC-III is expressed in crypt ORNs and could be a part of the odor transduction pathway as in the ciliated ORNs of fish and terrestrial vertebrates. Together, these results confirm a chemosensory function of crypt ORNs and support the presence of a cAMP transduction pathway.

DISCUSSION

Crypt ORNs represent a third type of chemosensory neuron besides the ciliated and microvillous ORNs in the olfactory epithelium of fishes (Hansen and Finger, 2000; Hansen and Zielinski, 2005). Evidence is mounting that each ORN type is tuned towards distinct odorant classes, which are transduced through different pathways and activate specific regions of the olfactory bulb (Friedrich and Korsching, 1998; Hansen et al., 2004; Hansen et al., 2003; Nikonov and Caprio, 2001; Sato et al., 2005; Sorensen and Caprio, 1998). However, the precise match between ORN and odorant types is still unclear, and there are evidently more chemical odorant classes than morphologically distinguishable ORN types in fish, as pointed out previously (Hansen et al., 2004).

Here, we demonstrate that an amino acid mixture excited a significant percentage of isolated crypt ORNs and induced a transient rise of intracellular calcium in these cells. This demonstrates some degree of chemosensory selectivity as other odorant classes tested in the present and a previous study (Schmachtenberg, 2006), bile salts, polyamines and pheromone candidates, did not trigger any reproducible responses with both approaches.

However, theoretical considerations and indirect evidence suggest that crypt ORNs may function as detectors of conspecific odorants or pheromones in the aquatic environment. Crypt ORN density in the olfactory epithelium is low compared to other ORN types, may vary throughout the year and reach a peak during the spawning season (Hamdani el et al., 2008). Furthermore, crypt ORNs project to small specific areas of the olfactory bulb (Hansen et al., 2003), which have been associated with reproductive behavior (Hamdani el and Doving, 2006; Lastein et al., 2006). Finally, the restricted access to the crypt will limit the exchange rate of odorant molecules at the receptors, which rather suggests a slow, highly sensitive labeled-line type odorant detection than a fast and combinatorial operation.

Unfortunately, our attempts to demonstrate the responsiveness of crypt ORNs to PGF2α and 17,20βP, both reportedly strong odorants with pheromonal function in some fishes (Lastein et al., 2006; Sorensen et al., 1988; Stacey et al., 1989), were unsuccessful. Although a large number of chemicals released by fish may act as reproductive pheromones (Stacey et al., 2003), it is possible that crypt ORNs of *T. symmetricus* are tuned to detect only few specific compounds not tested here, or that their group comprises a variety of odorant receptors responsive to multiple substances acting as conspecific chemosignals, but were under sampled in our experiments. The only odorant class that consistently triggered responses were amino acids, which confirms previous observations (Schmachtenberg, 2006).

In this regard, it is interesting to note that the axons of crypt ORNs terminate in a region of the olfactory bulb that globally responds to amino acids in the channel catfish (Hansen et al., 2003; Nikonov and Caprio, 2001). However, the methodology used in these studies, local extracellular recordings and retrograde labeling, has a limited resolution and may not resolve restricted olfactory bulb zones of different odorant sensitivity and physiology.

Interestingly, amino acids have already been shown to be detected by both ciliated and microvillous ORNs in fish. Why should several receptor cell types be employed for that task? Perhaps each ORN type is the starting point for a different signaling pathway with separate analytical function. On the other hand, the relatively high concentrations necessary to elicit responses in isolated ORNs compared to intact olfactory epithelium in live animals may also activate neurons that are tuned to different substance classes, possibly with similar sterical groups. Finally, there is a theoretical possibility that some putative crypt ORNs from our experiments were confounded with microvillous ORNs whose dendrites retracted during the dissociation process. Yet, this seems highly unlikely, since average crypt ORNs are at least twice as large as microvillous ORNs in *T. symmetricus* [see Fig. 1F (Schmachtenberg, 2006)], and cells of doubtful identity were excluded from the analysis.

The present study also aimed at providing evidence for the components of the transduction pathway(s) operating in crypt ORNs. The most significant result is that crypt ORNs that responded to odorants were also excited by agonists of the cAMP cascade, 8Br-cAMP and IBMX plus forskolin. The latter compounds frequently caused initial spike suppression, followed by post-stimulus excitation. This might reflect a non-specific blocking

action of the agonists on ion channels (Sanhueza et al., 2000), or result from excessive depolarization of the cell ('excitation block'). A different phenomenon is post-stimulus inhibition, which was often observed after agonist or odorant stimulation (cell 1 in Fig. 1A) and appears to be an adaptational consequence (Schmachtenberg, 2006).

The patch clamp data were complemented by calcium imaging experiments testing the same compounds, altogether suggesting that a cAMP transduction cascade may operate in crypt ORNs to transduce chemosignals. Further evidence for this hypothesis is provided by the labeling of crypt ORNs with an antiserum against adenylate cyclase III. This enzyme is an integral component of the cAMP transduction pathway of ciliated ORNs of terrestrial vertebrates, and presumably also operates in the ciliated ORNs of fish (Hansen et al., 2003; Schmachtenberg and Bacigalupo, 2004). Crypt ORNs have both cilia and microvilli in their crypt, and may also express two different G-proteins, $G_{\alpha o}$ and $G_{\alpha q}$ (Hansen et al., 2004; Hansen and Zielinski, 2005). This opens the possibility that more than one odorant transduction pathway is operational in crypt ORNs. The fact that over 50% of the crypt ORNs were unresponsive to the applied cAMP pathway agonists suggests that these cells did not express that pathway, possibly because they were immature or because they expressed another transduction cascade instead. Alternatively, they might have been rendered unresponsive by the dissociation process. While the cells maintained a healthy aspect for up to 6 h in primary culture, tissue dissociation is clearly an invasive procedure that may affect the response properties of isolated cells. This problem could be overcome with the development of a genetic crypt ORN marker, that should allow the study of these cells in intact tissue or even in live animals, and thus help to resolve the mystery of these peculiar receptor neurons.

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