ATP activates both receptor and sustentacular supporting cells in the olfactory epithelium of Xenopus laevis tadpoles

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Abstract
Nucleotides and amino acids are acknowledged categories of water-borne olfactory stimuli. In previous studies it has been shown that larvae of Xenopus laevis are able to sense amino acids. Here we report on the effect of ATP in the olfactory epithelium (OE) of Xenopus laevis tadpoles. First, ATP activates a subpopulation of cells in the OE. The ATP-sensitive subset of cells is almost perfectly disjoint from the subset of amino acid-activated cells. Both responses are not mediated by the well-described cAMP transduction pathway as the two subpopulations of cells do not overlap with a third, forskolin-activated subpopulation. We further show that, in contrast to amino acids, which act exclusively as olfactory stimuli, ATP appears to feature a second role. Surprisingly it activated a large number of sustentacular supporting cells (SCs) and, to a much lower extent, olfactory receptor neurons. The cells of the amino acid- and ATP-responding subsets featured differences in shape, size and position in the OE. The latencies to activation upon stimulus application differed markedly in these subsets. To obtain these results two technical points were important. We used a novel dextran-tetramethylrhodamine-backfilled slice preparation of the OE and we found out that an antibody to calnexin, a known molecular chaperone, also labels SCs. Our findings thus show a strong effect of ATP in the OE and we discuss some of the possible physiological functions of nucleotides in the OE.

Introduction
Odorant recognition in vertebrates begins at the level of the olfactory epithelium (OE), in particular in olfactory receptor neurons (ORNs). The nature of the odorants detected depends on the animals’ habitat. While in terrestrial species olfactory stimuli are volatile compounds, in aquatic species they are water-soluble. Olfactory receptors (ORs) are predominantly situated on the cilia or microvilli of ORNs, the primary site of odorant transduction. In mammals, ORs are coded by gene families with up to 1000–1300 genes (Buck & Axel, 1991; Young et al., 2002; Zhang & Firestein, 2002), in fish there appear to be approximately 100 OR genes (Ngai et al., 1993a,b; Naito et al., 1998; Speca et al., 1999; Asano-Miyoshi et al., 2000).

ORNs in aquatic species respond to a variety of water-borne stimuli, such as amino acids (Caprio & Byrd, 1984; Kang & Caprio, 1995; Vogler & Schild, 1999; Manzini et al., 2002a,b; Manzini & Schild, 2003a, 2004; Schild & Manzini, 2004), bile salts (Kang & Caprio, 1995; Sato & Suzuki, 2001), gonadal steroids (Sorensen & Caprio, 1998), prostaglandins (Sorensen et al., 1988; Kitamura et al., 1994) and nucleotides (Derby et al., 1984; Carr et al., 1986; Kang & Caprio, 1995; Rolen et al., 2003). These classes of molecules have been identified as behaviourally relevant odorants involved in feeding, predator detection, social interaction and reproductive synchrony (Sorensen & Caprio, 1998). In contrast to some air-borne odorants the above-mentioned water-borne stimulants are chemically well-defined groups of molecules, many of which act primarily as neurotransmitters, neurotransmitter precursors, energy carriers or hormones.

In a recent study, Hegg and co-workers (Hegg et al., 2003) have shown that one of the above-mentioned classes of known water-borne odorants, namely nucleotides, acts via purinoceptors on sustentacular supporting cells (SCs) as well as on ORNs in mice (Hegg et al., 2003). These authors further report a nucleotide-mediated modulation of odor transduction, which they assume may act as a neuroprotective mechanism preventing overstimulation both in the OE and the olfactory bulb (OB) during exposure to noxious stimuli (Hegg et al., 2003). They also showed a similar modulatory effect of dopamine (Hegg & Lucero, 2004).

The intriguing evidence that nucleotides, important water-borne olfactory stimuli, elicit responses in SCs, at least in a terrestrial vertebrate, raises a number of basic and important questions. (i) Do nucleotides show similar effects also in the OE of aquatic species, where these molecules are known to be important odorants? (ii) Do other known potent water-borne odorants show similar direct effects on SCs in aquatic species? (iii) Is it always possible to clearly distinguish responses of ORNs and SCs when working with slice preparations of the OE? Many recent studies in the OE have been carried out using the calcium imaging technique, where it is inherently difficult to unambiguously identify the different cell types. Thus, the last question is of particular importance.

In the present work we measured responses of cells in an acute slice preparation of the OE of Xenopus laevis tadpoles to application of amino acids, ATP and forskolin. Forskolin is known to activate the adenylyl cyclase. In previous work we showed that amino acids and forskolin act on two almost distinct subpopulations of cells in the OE of Xenopus laevis tadpoles (Manzini et al., 2002b; Manzini & Schild, 2003a). Here we show that cells responsive to ATP form a third subpopulation of cells. With calcium imaging in a novel dextran-tetramethylrhodamine...
Materials and methods

Slice preparation for calcium imaging and patch-clamping

Tadpoles of *Xenopus laevis* (stages 51–54; Nieuwkoop & Faber, 1994) were chilled in a mixture of ice and water and decapitated, as approved by the Göttingen University Committee for Ethics in Animal Experimentation. A block of tissue containing the olfactory epithelium, the olfactory nerves and the brain was cut out and kept in bath solution (see below). The tissue was glued onto the stage of a vibrroslicer (VT 1000S, Leica, Bensheim, Germany) and cut horizontally into 130–150-μm-thick slices.

For calcium imaging the tissue slices were then transferred to a recording chamber, and 200 μL of bath solution (see below) containing 50 μM fluo-4 AM (Molecular Probes, Leiden, the Netherlands) and 50 μM MK571 (Alexis Biochemicals, Grünberg, Germany) was added. The fluorescence of fluo-4 increases with rising intracellular calcium concentration. Fluo-4 AM was dissolved in dimethylsulphoxide (DMSO; Sigma, Deisenhofen, Germany) and Pluronic F-127 (Molecular Probes). The final concentrations of DMSO and Pluronic F-127 did not exceed 0.5% and 0.1%, respectively. ORNs of *Xenopus laevis* tadpoles express multidrug resistance transporters (Manzini & Schild, 2003b), with a wide inhibitor of the multidrug resistance-associated proteins (MRP, Gekeler et al., 1995; Abrahamse & Rechkkermer, 2001) was added to the incubation solution. After incubation at room temperature for 35 min, the tissue slices were placed under a grid in a recording chamber (Edwards et al., 1989) and placed on the microscope stage of an Axiovert 100M (Zeiss, Jena, Germany) to which a laser scanning unit (LSM 510, Zeiss) was attached. Before starting the calcium-imaging experiments, the slices were rinsed with bath solution for at least 15 min. In most slices some of the cells showed a high fluorescence from the beginning, presumably because they did not survive the tissue slicing. Such cells were discarded from any further evaluation. To test the viability of the cells in the OE we applied a KCl solution (100 mM). All cells responding with a transient increase of fluorescence were considered healthy.

For filling the cells with biocytin through the patch-pipette the slices were placed under a grid in a recording chamber and viewed using Nomarski optics (Axioskop 2, Zeiss, Göttingen, Germany).

Calcium imaging of odor responses

Intracellular calcium was monitored using a laser-scanning confocal microscope (Zeiss). The confocal pinhole was about 100 μm and excluded fluorescence detection from more than one cell layer. Fluorescence images (excitation at 488 nm; emission >505 nm) of the olfactory mucosa were acquired at 0.25–1.27 Hz, with three to five images taken as control images before the onset of odor delivery. The fluorescence changes ΔF/F were calculated for individual ORNs, as ΔF/F = (F1 – F2)/F2, where F1 was the fluorescence averaged over the pixels of an ORN, while F2 was the average fluorescence of that ORN prior to stimulus application, averaged over three images.

A response was assumed if the following two criteria were met: (i) the first two intensity values after stimulus arrival at the mucosa, R(t1) and R(t2), had to be larger than the maximum of the prestimulus intensities; (ii) R(t2) > R(t1) with t2 > t1.

The images showing the maximum response of a stimulus application (see Figs 1, 3, 4 and 5; black cells on grey background) were obtained by calculating the fluorescence differences from the image with the maximum response and the first image of the sequence using the image processing software WinView32 (Princeton Instruments, Trenton, USA).

Staining single cells using the patch-clamp technique

Patch electrodes with a tip diameter of 1–2 μm and approximately 7–10 MΩ resistance were fabricated from borosilicate glass with 1.8 mm outer diameter (Hilgenberg, Malsfeld, Germany) using a two-stage electrode puller (Narishige, Tokyo, Japan) and fire-polished. To visualize cells, 0.5% biocytin (Molecular Probes) was added to the pipette solution (see below). After biocytin filling through the patch pipette the slices were left in bath solution for 20 min and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at 4 °C, rinsed in PBS, left overnight in PBS with 0.5% Triton X-100 (PBS-TX) and then immersed in a solution of avidin Alexa Fluor 488 conjugate (10 μg/mL, Molecular Probes) in PBS-TX overnight. The slices were then rinsed several times in PBS, placed under a grid in a recording chamber and examined using a laser scanning confocal microscope (LSM 510, Zeiss).

Solutions and stimulus application

The composition of the bath solution was (in mM): NaCl, 98; KCl, 2; CaCl2, 1; MgCl2, 2; glucose, 5; sodium pyruvate, 5; HEPES, 10. The pipette solution used for patch-clamp injections contained (in mM): NaCl, 2; KCl, 11; MgSO4, 2; K-gluconate, 80; HEPES, 10; EGTA, 0.2; Na2-ATP, 2; Na2-GTP, 0.1. The pH of all solutions was adjusted to 7.8. This is the physiological pH in this poikilothermic species (Howell et al., 1970). Osmolarities of the bath and pipette solutions were 230 mOsmol and 190 mOsmol, respectively. All of the chemicals necessary for the above solutions were purchased from Sigma.

As odorants, we used the same mixture of 19 amino acids as in our previous work (see Manzini et al., 2002b) and ATP. Furthermore, we used forskolin as an activator of the adenylate cyclase. The amino acids and ATP were dissolved in bath solution (stocks of 10 mM and 50 mM, respectively) and used at a final concentration of 200 μM in all of the experiments. Forskolin was dissolved in DMSO (stock of 20 mM) and used at a final concentration of 100 μM. The amino acids, ATP and forskolin were purchased from Sigma.

Stimulus solutions were prepared immediately before use by dissolving the respective stock solution in bath solution. The bath solution was applied by gravity feed from a storage syringe through a funnel drug applicator (Schild, 1985) to the recording chamber. The flow was 350 μL/min. Stimuli were pipetted directly into the funnel without stopping the flow. Outflow was through a syringe needle placed close to the mucosa to ensure that odorant molecules were removed rapidly. The minimum interstimulus interval between odorant applications was at least 2 min.
The dilution of the stimulus within the funnel was less than 1%. In the mucosa the dilution of the stimulus was determined by putting a confocal volume [approximately 1 fl (1 x 10^{-15} L)] of a laser-scanning confocal microscope (LSM 510/Axiovert 100M, Zeiss), firstly, in front of the funnel outlet and, secondly, in front of the epithelial surface and measuring the respective fluorescences. For this control measurement we used the fluorescent probe TMR (500 nm, Sigma) as a ‘dummy stimulus’. The dilution factor was 0.91 ± 0.02 (mean ± SD, n = 7). The delay between TMR leaving the funnel outlet and reaching the mucosal surface was less than 1 s and, after the end of stimulation, TMR was completely rinsed from the mucosa within 15 s.

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We defined the ‘latency of activation’ as the time between stimulus arrival at the OE and the beginning of the corresponding cellular response.

Preparation of d-TMR-backfilled slices

In order to backfill ORNs, OE slice preparations were stained by injection of d-TMR (3 kDa, Molecular Probes) into the cut olfactory nerve. To do so we put a small crystal of d-TMR into the cut nerve of chilled tadpoles and then closed the lesion with cyanoacrylate glue (Roti-Coll 1, Roth, Karlsruhe, Germany). Three hours later the tadpoles were decapitated and the acute slices were prepared and stained with fluo-4 as described above. The combination of ORN staining with d-TMR on the one hand and calcium imaging of odor responses on the other allowed us to identify responding cells morphologically as ORNs.

The following calcium imaging of odor responses was carried out as described above using a laser-scanning confocal microscope (Zeiss LSM 510/Axiovert 100M). In addition, we used the second channel of the confocal microscope (excitation at 543 nm; emission >585 nm) to visualize the d-TMR-stained ORNs.

Immunohistochemistry

ORNs of Xenopus laevis tadpoles (stages 51–54; Nieuwkoop & Faber, 1994) were backfilled with biocytin (Molecular Probes) through the olfactory nerve similarly as described above for d-TMR. Briefly, after cutting out a block of tissue containing the epithelium and the nerves, a crystal of biocytin was put into the nerve and we waited for at least 30 min. Then the block was put into bath solution (see above) for another 2 h before it was transferred into a solution of 4% paraformaldehyde in PBS, pH 7.4, and fixed overnight at 4 °C. Brains were then washed at least three times in PBS, embedded in 5% low melting point agarose (Agarose type II, Amresco, Solon, OH, USA) and sectioned approximately 70 μm on a vibroslicer (Leica). The slices were then immersed in a solution of avidin Alexa Fluor 488 conjugate (10 μg/mL, Molecular Probes) in PBS-TX overnight.

The OE slices were then double-stained with an antibody to calnexin, kindly provided by Ngyn van Phuc (Department of Cardiology and Pneumology, Centre for Internal Medicine, University of Göttingen, Germany). We obtained the best results when sections were preincubated (1 h at room temperature) in 0.1 m PBS-TX and 2% normal goat serum (NGS; ICN Biomedicals GmbH, Orsay, France). Background was reduced after preincubation in NGS. The tissue was then incubated overnight at 4 °C with rabbit anti-calnexin (1 : 500), diluted in PBS with 2% NGS. Sections were subsequently washed with PBS, and Alexa 546-conjugated anti-rabbit secondary antibody (Molecular Probes) was applied at a dilution of 1 : 250 in 1% NGS/PBS for 2 h at RT. The secondary antibody was washed off by five changes of PBS.

Finally, preparations were transferred into 60% glycerol/PBS for at least 1 h, and finally mounted on slides for confocal microscopy in 80% glycerol/PBS.

Preparations were viewed using a laser-scanning confocal microscope (Zeiss). Series of optical sections were imaged at intervals of 0.5–5 μm through the depth of the thick sections. They were saved as single optical images or three-dimensional stacks. Two-dimensional projections were generated for each channel and merged with the use of pseudocolours. Where needed, the digitized images were modified only to enhance contrast. Image processing was performed using either Zeiss imaging software (Zeiss) or GIMP (GNU Image Manipulation Program; website: http://www.gimp.org).

Results

Responses to ATP, amino acids and forskolin

Cells in 17 slices of the OE of Xenopus laevis tadpoles were tested for their responsiveness to ATP (200 μm), amino acids (200 μm) and forskolin (100 μm). After proper positioning of the slice on the microscope stage we applied the three stimuli one after another using interstimulus intervals of at least 2 min. Figure 1A shows a slice of the OE stained with the calcium-indicator dye fluo-4 (image acquired at rest). The three images in Fig. 1B–D show the responding cells of this slice upon application of ATP (Fig. 1B), amino acids (Fig. 1C) and forskolin (Fig. 1D). In this OE slice 39 cells showed a response to ATP, 16 to amino acids and 57 to forskolin. Among the responding cells, three cells showed an overlapping responsivity to amino acids and ATP, and one cell responded to ATP as well as to forskolin. This shows that the three groups of stimuli elicited responses in almost completely different subsets of cells of the OE. Figure 1E summarizes this result showing the ATP-sensitive cells in blue, the amino acid-sensitive cells in red and the forskolin-sensitive cells in green. The cells with overlapping sensitivities to amino acids/ATP and ATP/forskolin are drawn in magenta and cyan, respectively. In all slices recorded, 384 cells responded either to ATP, amino acids or forskolin (Fig. 1F). One hundred and ten responded only to ATP (blue), 72 only to amino acid (red), 179 only to forskolin (green), 11 responded to amino acids and ATP (magenta), four to amino acids and forskolin (yellow), six to ATP and forskolin (cyan), and two cells responded to all three of the stimuli (white). Figure 2 shows the selectivities of the cells marked with an arrow in Fig. 1B–D. Cell #1 responded to ATP, while it responded neither to amino acids nor to forskolin. Cells #2 and #3 responded only to amino acids and forskolin, respectively.

Correlation between cell type and responsiveness to ATP and amino acids

In order to attribute a responding cell to a specific cell type of the OE, in a set of experiments ORNs were backfilled with d-TMR previous to tissue slicing and calcium imaging (see Materials and methods). The images shown in Fig. 3A and B show the cells responsive to application of ATP and amino acids of two d-TMR-backfilled OE slices, respectively. Fifteen cells showed a response to ATP (Fig. 3A) and 20 cells were sensitive to amino acids (Fig. 3B). For the sake of clarity the responsive cells were encircled in both images. Figure 3C and D visualize the d-TMR-backfilled ORNs of the slices shown in Fig. 3A and B. The superposition of the contours of the responsive cells shows that none of the 15 ATP-sensitive cells is d-TMR-positive, while 12 out of the 20 cells sensitive to amino acids exhibit a d-TMR staining. Figure 3E summarizes the results for the 14 d-TMR-backfilled slices tested for their responsiveness to ATP or amino acids. In eight slices tested for ATP 168 cells responded, but only nine of them (~ 5%) were d-TMR backfilled. In the six slices tested for their sensitivity to amino acids, 52 cells responded, and 32 of them (~ 62%) were d-TMR-positive. The percentage of backfilled forskolin-sensitive cells was in the same range (50%; 12 backfilled cells, 24 responding cells in three slices; data not shown). Figure 3F shows one of the nine ATP-sensitive backfilled ORNs (upper part) and the respective time course of the calcium-dependent fluorescence change upon stimulation with ATP (lower part).
The markedly different ratio (backfilled responding cells/total responding cells) for ATP and amino acids together with their different location in the OE (see Figs 1, 3 and 5) strongly indicates that the two stimuli act mainly on two different cell types of the OE, i.e. ATP mostly on SCs and amino acids almost exclusively on ORNs.

Further evidence supporting this interpretation was obtained by combined biocytin backfilling/calnexin immunostaining experiments. Figure 4A–C shows a slice of the OE double-stained with biocytin/avidin (green fluorescence) and an antibody to calnexin (red fluorescence). Figure 4A shows calnexin-immunoreactivity in cells in the apical and basal part of the OE. In the medial part of the OE only a small number of cells showed a calnexin-staining. Figure 4B shows the biocytin/avidin fluorescence of the backfilled ORNs of the same slice. When merging both pictures (Fig. 4C), no overlap between calnexin-immunolabelling and biocytin–avidin fluorescence was found. Similar calnexin-immunoreactivity was observed in the absence of biocytin backfilling (data not shown). On the basis of their location and shape, together with the complete absence of overlap with biocytin-backfilled ORNs, the calnexin-immunoreactive cells in the apical part of the OE can be attributed to SCs. Figure 4D shows the ATP-responding cells of a fluorescently stained OE slice. In Fig. 4E, the cell encircled in Fig. 4D is shown at a higher magnification. Figure 4F gives a higher magnification of the area marked in Fig. 4C, while Fig. 4G gives, in comparison, a SC filled with biocytin through a patch pipette. The position in the OE, as well as the shape and size of the three cells shown in Fig. 4D–G clearly suggests that these cells belong to the same cell type of the OE, namely SCs.

**Latencies of activation upon application of ATP and amino acids**

Figure 5 shows the ATP- and amino acid-sensitive cells of an OE slice along with the corresponding time courses of intracellular calcium \([\text{Ca}^{2+}]\), transients of cell #1 (see Fig. 1B), evoked by the application of ATP, amino acids and forskolin. The traces show that this cell clearly responded to ATP, while it showed no response to amino acids and forskolin. (B) Cell #2 (see Fig. 1C) responded to amino acids. No response to ATP and forskolin. (C) Cell #3 (see Fig. 1D) responded to ATP and amino acids.

In all slices measured the cells responsive to amino acids were activated simultaneously and immediately after stimulus arrival to the OE (97 cells in 10 slices; average latency 0.28 s, response always occurring in the first or second post-stimulus confocal image taken). The cells sensitive to ATP (220 cells in 11 slices) showed markedly longer latencies (average 2.2 s; skewed distribution, longest latency 8 s). Importantly, repeated ATP applications to an individual cell led to the same latency.

Finally, to focus on the latency analysis in cells that clearly were ORNs, we analysed cells in d-TMR-backfilled slices (see Fig. 3). As stated above, the responses to amino acids always occurred within the first two post-stimulus confocal images taken, while the responses to ATP scattered over a larger range (up to 3.5 s) indicating either varying response dynamics in ATP olfactory responses or non-olfactory nucleotide receptors on ORNs.

**Discussion**

In the present study, we recorded calcium responses of cells in slices of the OE of *Xenopus laevis* tadpoles upon stimulation with ATP, amino acids and forskolin. By means of confocal calcium imaging in a novel d-TMR-backfilled slice preparation and the application of an antibody to calnexin to label SCs we were able to attribute responding cells to a specific cell type of the OE. In this work we concentrated on three major points, i.e. (i) a comparison of cells responding to ATP, amino acids and forskolin; (ii) a correlation of cell type and responsiveness to ATP and amino acids; and (iii) differences in the latency of activation upon application of ATP and amino acids.
Responses to ATP, amino acids and forskolin

Application of the different groups of molecules (nucleotides/ATP, amino acids and forskolin) elicited calcium-dependent fluorescence changes in three different subsets of OE cells (Figs 1 and 2). In previous work, we have already shown that amino acids and forskolin induce calcium responses in two subsets of cells in the OE of *Xenopus laevis* tadpoles (Manzini & Schild, 2003a). In other aquatic species it has already been shown that nucleotides are relevant olfactory stimuli.

![Image of calcium imaging](image-url)
Correlation between cell type and sensitivity to ATP and amino acids

It is inherently difficult to attribute a responding cell to one of the three cell types in the OE of vertebrates, namely basal cells, SCs and ORNs. Especially when working with slices of the OE and using the calcium-imaging technique the responding cells often cannot easily be assigned to one of the above-mentioned cell types. Because it has been shown that SCs of at least one vertebrate species are capable of detecting nucleotides, and that the activated cells form a third almost completely different subset of cells. As forskolin is a known activator of the cAMP-dependent transduction pathway, successfully used to the same end in a number of previous studies (Frings & Lindemann, 1991; Kashiwayanagi et al., 1996; Manzini et al., 2002b; Manzini & Schild, 2003a), it can be concluded that both amino acid and ATP stimuli are transduced in a cAMP-independent manner.

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**Fig. 4.** Calnexin labels sustentacular supporting cells (SCs). (A–C) Image showing calnexin immunoreactivity (red fluorescence, A and C) of a biocytin-backfilled/avidin-stained (green fluorescence, B and C) slice of the olfactory epithelium (OE) of a *Xenopus laevis* tadpole. Note the localization of calnexin immunoreactivity in the sustentacular cell layer and in cells in the basal cell layer (arrows). In the olfactory receptor cell layer only a few cells show positive calnexin immunoreactivity (asterisks). The dashed lines in (A and B) indicate the border of the OE, the continuous line the approximate termination of the sustentacular cell layer. (D) ATP-sensitive cells in an acute OE slice determined with fluo-4-calcium imaging. The dashed rectangle indicates one of the ATP-responding cells shown at a higher magnification in (E). (F) Higher magnification of the dashed rectangle drawn in (C). The arrow marks an olfactory receptor neuron (ORN) dendrite. The asterisk tags a typical calnexin-positive cell. (G) Typical sustentacular cell in the OE of *Xenopus laevis* tadpoles filled with biocytin through a patch pipette and then stained with avidin. PC, principal cavity; SCL, sustentacular cell layer. Scale bars, 50 μm (A and D); 5 μm in (E–G).

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certainly supports this interpretation (compare Figs 1B and C, 3A and B, and Fig. 5A and B). A similar tendency was noticed in all of the slices tested for ATP and amino acids.

Calnexin is highly expressed in SCs

In search of a marker for SCs we tested antibodies against various proteins that we thought could be important for the metabolism of this cell type. Eventually we succeeded in our attempt with an antibody against calnexin. Calnexin is a type I transmembrane endoplasmic reticulum lectin that binds to proteins containing monoglucosylated oligosaccharides (Hammond et al., 1994). Calnexin has been shown to function as a molecular chaperone by enhancing the assembly of a variety of cellular substrates. Molecular chaperones bind transiently to nascent polypeptide chains and function by preventing aggregation and by maintaining polypeptides in conformations competent for folding and subunit assembly. Nascent proteins that are unable to fold or assemble correctly are generally retained within the endoplasmic reticulum in association with chaperones and are ultimately degraded, a phenomenon known as ‘quality control’ (Ellgaard et al., 1999). Our results show that a high concentration of calnexin represents a fundamental feature of cells in the sustentacular and basal cell layer of the OE of Xenopus laevis tadpoles. Biocytin/avidin-stained

Fig. 5. Latencies of activation upon stimulus application of cells responsive to ATP and amino acids in a slice of the olfactory epithelium (OE) of Xenopus laevis tadpoles. (A) ATP-sensitive cells in an OE slice measured with fluo-4 calcium imaging. The dashed lines indicate the border of the mucosa. The responding cells are numbered. (B) Amino acid-sensitive cells in the same OE slice as in (A). The responding cells are numbered. (C) Time courses of [Ca²⁺]ᵢ transients of the cells marked in (A), ordered by increasing activation latencies. The straight vertical line going through all traces shows the arrival time of the stimulus to the OE. The dotted line indicates the response latencies to ATP of the different cells. (D) Time courses of [Ca²⁺]ᵢ transients of the cells marked in (B). The straight vertical line going through all traces shows the arrival time of the stimulus to the OE. PC, principal cavity. Scale bar, 50 µm.
biocytin-backfilled ORNs never showed calnexin immunoreactivity. This showed that SCs as well as basal cells but not mature ORNs express a high concentration of calnexin. The few calnexin-positive cells in the olfactory receptor cell layer of the OE were never biocytin/avidin stained. These cells could be immature ORNs not yet having the axon connected to the OB. In contrast to mature ORNs, SCs, basal cells and immature ORNs most probably have a higher metabolic activity and therefore a higher calnexin/chaperone activity. The morphological similarity of ATP-sensitive cells identified with calcium imaging and calnexin-positive cells of the sustentacular cell layer gives clear evidence that these cells are SCs (compare Fig. 4E and F). To further substantiate the above assertion we filled single SCs in an OE slice with biocytin through a patch pipette (see Fig. 4G). Again when comparing shape, size and location in the OE of these cells there is no doubt that they belong to the same cell type, namely SCs. To date we can only speculate about the physiological meaning(s) of the nucleotide effect on SCs. One possibility proposed in mice (Hegg et al., 2003) is the modulation of odor sensitivity in situations of olfactory stress. As it has been shown that tadpoles of *Xenopus laevis* have two types of SCs, ciliated and secretory (with small, secretory granules) (Hansen et al., 1998), another potential action of endogenously released ATP could therefore be a modulation of the vesicle release of secretory SCs. This could influence the composition of the mucus that covers the OE and this in turn could affect the entire mucosal odor detection.

**Differences in the latency of activation of ATP- and amino acid-sensitive cells**

A further fundamental difference between cells sensitive to ATP and amino acids is their different latency of activation upon stimulation. While the responses of amino acid-sensitive cells of a single OE slice initiated almost simultaneously and immediately after stimulus arrival to the OE, the cells responsive to ATP showed markedly different activation latencies, with the fastest activation times coinciding with those measured for the amino acid-sensitive cells. In addition we have shown that both groups of ATP-sensitive cells, the d-TMR-backfilled ORNs and d-TMR-negative cells, comprise cells responding immediately after stimulus arrival to the OE and cells with differing latencies of activation. At the moment it is not clear how the different activation latencies of the ATP-sensitive cells should be interpreted. First, fast responding ATP-sensitive cells could be ORNs, their responses being mediated by ORs sensitive to nucleotides, while slower responses could arise from ORNs and SCs, expressing purinoceptors coupled to slower intracellular transduction cascades. In mice, it has been shown that ORNs and SCs express purinoceptors (Hegg et al., 2003). As a second explanation ATP responses could be mediated by purinoceptors, the fast responses by ionotropic P2X-receptors and the slower responses by metabotropic P2Y-receptors. Of course these hypotheses are not mutually exclusive. Due to the experimental evidence showing ATP responses in the OB of various aquatic animals (Friedrich & Korsching, 1998; Nikonov & Caprio, 2001) we would tend to prefer the former explanation.

In summary, our results show that the OE of *Xenopus laevis* tadpoles possess ATP-sensitive cells and that they form a subset, almost completely non-overlapping with the previously described subsets of amino acid- and forskolin-sensitive cells. By means of a newly developed d-TMR-backfilled OE slice together with the finding that calnexin labels SCs, we were able to attribute the vast majority of ATP-sensitive cells to SCs. This shows that well-known water-borne olfactory stimuli may act also on non-sensory cells in the OE. This finding opens a number of interesting questions that were clearly beyond the scope of this study. First, what transduction pathways are behind the responses of ATP in the OE. Second, what are the physiological roles of ATP in the OE; and third, how are the differences in the latencies to activation of many ATP-sensitive cells brought about.

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**Abbreviations**

[Ca$^{2+}$], intracellular calcium; DMSO, dimethylsulphoxide; d-TMR, dextran-tetramethylrhodamine; NGS, normal goat serum; OB, olfactory bulb; OE, olfactory epithelium; OR, olfactory receptor; ORN, olfactory receptor neuron; PBS, phosphate-buffered saline; PBS-TX, PBS with 0.5% Triton X-100; SC, sustentacular supporting cell.

**References**


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