Nucleotide-Induced Ca^{2+} Signaling in Sustentacular Supporting Cells of the Olfactory Epithelium

THOMAS HASSENKLÖVER,¹ SILVIA KURTANSKA,¹ ILONKA BARTOSZEK,¹ STEPHAN JUNEK,¹ DETLEV SCHILD,^{1,2} AND IVAN MANZINI^{1,2*}

¹Department of Neurophysiology and Cellular Biophysics, University of Göttingen, Humboldtallee 23, 37073 Göttingen, Germany

²DFG Research Center for Molecular Physiology of the Brain (CMPB), University of Göttingen, Humboldtallee 23, 37073 Göttingen, Germany

KEY WORDS

purinergic receptors; ATP; Ca^{2+} imaging; Ca^{2+} wave; *Xenopus laevis*

ABSTRACT

Extracellular purines and pyrimidines are important signaling molecules acting via purinergic cell-surface receptors in neurons, glia, and glia-like cells such as sustentacular supporting cells (SCs) of the olfactory epithelium (OE). Here, we thoroughly characterize ATP-induced responses in SCs of the OE using functional Ca^{2+} imaging. The initial ATP-induced increase of the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ always occurred in the apical part of SCs and subsequently propagated toward the basal lamina, indicating the occurrence of purinergic receptors in the apical part of SCs. The mean propagation velocity of the Ca^{2+} signal within SCs was $17.10 \pm 1.02 \mu\text{m/s}$. ATP evoked increases in $[\text{Ca}^{2+}]_i$ in both the presence and absence of extracellular Ca^{2+} . Depletion of the intracellular Ca^{2+} stores abolished the responses. This shows that the ATP-induced $[\text{Ca}^{2+}]_i$ increases were in large part, if not entirely, due to the activation of G protein-coupled receptors followed by Ca^{2+} mobilization from intracellular stores, suggesting an involvement of P2Y receptors. The order of potency of the applied purinergic agonists was $\text{UTP} > \text{ATP} > \text{ATP}\gamma\text{S}$ (with all others being only weakly active or inactive). The ATP-induced $[\text{Ca}^{2+}]_i$ increases could be reduced by the purinergic antagonists PPADS and RB2, but not by suramin. Our findings suggest that extracellular nucleotides in the OE activate SCs via P2Y₂/P2Y₄-like receptors and initiate a characteristic intraepithelial Ca^{2+} wave. © 2008 Wiley-Liss, Inc.

INTRODUCTION

The olfactory sensory organ of vertebrates is made of a specialized neuroepithelium consisting of three main cell types: olfactory receptor neurons (ORNs), which transmit the olfactory information from the nose to the olfactory bulb in the brain, sustentacular supporting cells (SCs), which share common properties with glial and epithelial cells, and basal cells (BCs) including olfactory stem cells, which maintain the regenerative capacity of the olfactory epithelium (OE) (Graziadei, 1971, 1973; Graziadei and Metcalf, 1971). While innu-

merable studies focused on the physiology and function of ORNs (Getchell, 1986; Schild and Restrepo, 1998), much less attention has so far been given to SCs. Sustentacular supporting cells function very much like glia cells and share characteristics of both the macrophage-like microglia as well as macroglia (Getchell, 1977; Okano and Takagi, 1974). Their high resting potential and low input resistance are two typical features of glial cells (Masukawa et al., 1985). In many ways, they are morphologically and physiologically similar to the Müller glia of the retina (Newman and Reichenbach, 1996). It is known that cell bodies of SC form a tightly packed columnar monolayer on the apical surface of the OE, and that their basal processes expand through the whole OE and terminate in the BC layer (Rafols and Getchell, 1983). Sustentacular supporting cells have been shown to be involved in mucus secretion (Getchell and Mellert, 1991; Hansen et al., 1998), phagocytosis of dead and dying cells (Suzuki et al., 1996), elimination of noxious substances, and the regulation of the extracellular ionic environment (Breipohl et al., 1974; Getchell and Getchell, 1992). In addition, they have been shown to insulate ORNs both physically and chemically (Breipohl et al., 1974). Recent findings, however, suggest that, in addition to the above mentioned “maintenance functions,” SCs are likely to be involved in intraepithelial signaling mechanisms. It has been shown that cells in the mouse OE express purinergic receptors (Hegg et al., 2003), and it has been suggested that these receptors may play an important role in signaling acute damage in the OE (Hegg and Lucero, 2006; Hegg et al., 2003). Furthermore, it has been shown that application of ATP evokes strong increases in the $[\text{Ca}^{2+}]_i$ in SCs of mouse and larval *Xenopus laevis* (Czesnik et al., 2006; Hegg et al., 2003), and that nucleotides induce heat shock protein expression in SCs in the mouse (Hegg and Lucero, 2006). In other sensory systems, for example, in the visual, auditory, and gustatory system, extracellular nucleotides have long been known to have neuromodula-

Grant sponsor: DFG Research Center Molecular Physiology of the Brain.

*Correspondence to: Ivan Manzini, Department of Neurophysiology and Cellular Biophysics, Universität Göttingen, Humboldtallee 23, 37073 Göttingen, Germany. E-mail: imanzin@gwdg.de

Received 1 April 2008; Accepted 8 May 2008

DOI 10.1002/glia.20714

Published online 12 June 2008 in Wiley InterScience (www.interscience.wiley.com).

tory effects and to be involved in cellular signaling (Burnstock, 2007; Thorne and Housley, 1996).

Purines and pyrimidines are important extracellular signaling molecules that mediate various physiological effects via membrane-bound receptors termed purinergic receptors (Burnstock, 2007; Ralevic and Burnstock, 1998). There are two main families of purinergic receptors, adenosine or P1 receptors and P2 receptors recognizing primarily tri- and dinucleotides (Burnstock, 2007; Ralevic and Burnstock, 1998). The P1 receptors have been further subdivided into four subtypes (P1A₁, P1A_{2A}, P1A_{2B}, and P1A₃) all of which couple to G proteins. The P2 receptors are subdivided into two families, the ionotropic P2X receptors and the G protein coupled P2Y receptors. P2X receptors have been further subdivided into seven subtypes (P2X₁–P2X₇), the P2Y receptors have been subdivided into eight subtypes (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄). These subtypes differ in their molecular structure and their different selectivities to agonists and antagonists (Burnstock, 2007; Ralevic and Burnstock, 1998).

The present study was aimed to identify the purinergic receptor subtypes of SCs in the OE of larval *Xenopus laevis* and to characterize the effect of their activation. The conducted study suggests the expression of P2Y₂/P2Y₄-like purinoceptors on SCs. Activation of these purinergic receptors initiates characteristic spatiotemporal (wave-like) patterns of [Ca²⁺]_i increases in SCs that propagate from their cell body in the apical part of the OE along their basal processes toward the basal lamina.

MATERIALS AND METHODS

Preparation of the Slices of the Olfactory Epithelium

Tadpoles of *Xenopus laevis* (stages 51 to 54; staged after Nieuwkoop and Faber, 1994) were cooled to produce complete immobility, and then killed by transection of the brain at its transition to the spinal cord. All procedures for animal handling and tissue dissections were carried out according to the guidelines of the Göttingen University Committee for Ethics in Animal Experimentation. A block of tissue containing the olfactory mucosae, the olfactory nerves, and the brain was cut out and kept in bath solution (see later). The tissue was glued onto the stage of a vibroslicer (VT 1000S, Leica, Bensheim, Germany) and cut horizontally into 120–130 μm thick slices. The tissue slices were then transferred to a recording chamber, and 200 μl of bath solution (see later) containing 50 μM Fluo-4/AM (Molecular Probes, Leiden, The Netherlands) and 50 μM MK571 (Alexis Biochemicals, Grünberg, Germany) was added. Fluo-4/AM was dissolved in DMSO (Sigma, Deisenhofen, Germany) and Pluronic F-127 (Molecular Probes). ORNs of *Xenopus laevis* tadpoles express multidrug resistance transporters (Manzini and Schild, 2003; Manzini et al., 2008) with a wide substrate spectrum, including Ca²⁺-indicator dyes. To avoid transporter-mediated destaining of the slices, MK571, a specific inhibitor of the multi-

drug resistance-associated proteins (MRP, Abrahamse and Rechkemmer, 2001; Gekeler et al., 1995) was added to the incubation solution. After incubation on a shaker at room temperature for 35 min, the tissue slices were fixed with a grid in a recording chamber and placed either on the microscope stage of an Axiovert 100M (Zeiss, Jena, Germany) to which a laser scanning unit (LSM 510, Zeiss, Jena, Germany) was attached or on the microscope stage of a fast line scanning device, custom-built in our lab. Before starting the Ca²⁺ imaging experiments, the slices were rinsed with bath solution for at least 10 min.

Ca²⁺ Imaging and Data Evaluation

For our standard experiments, the intracellular Ca²⁺ was monitored using a laser-scanning confocal microscope (Zeiss LSM 510/Axiovert 100M, Jena, Germany). The thickness of the optical slice 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 and 786.4 ms exposure time per image with about 10 images taken as control images before the onset of stimulus delivery. The fluorescence changes $\Delta F/F$ were calculated for individual olfactory receptor neurons (ORNs) as $\Delta F/F = (F_1 - F_2)/F_2$, where F_1 was the fluorescence averaged over the pixels of an ORN, whereas F_2 was the average fluorescence of that ORN before 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, $I(t_1)$ and $I(t_2)$, had to be larger than the maximum of the prestimulus intensities; (ii) $I(t_2) > I(t_1)$ with $t_2 > t_1$. To characterize the spatiotemporal pattern of [Ca²⁺]_i increases in sustentacular supporting cells (SCs) additional Ca²⁺ imaging experiments were carried out using a fast line scanning device, custom-built in our lab. Images were acquired at a rate of up to 40 Hz. Slice preparation and staining procedures were the same as for the experiments performed at the commercial laser-scanning confocal microscope. The propagation velocity of the Ca²⁺ signal within SCs was calculated by monitoring the wave front of the intracellular Ca²⁺ signal. Thereby, the wave front of the Ca²⁺ signal was taken as the point where the amplitude of the steep rise of relative fluorescence ($\Delta F/F$) was half-maximum.

The data were analyzed using custom written programs in MATLAB (Mathworks, Natick). To facilitate selection of regions of interest, a “pixel correlation map” was obtained by calculating the cross-correlation between the fluorescence signals of a pixel to that of its immediate neighbors and then displaying the resulting value as a grayscale map. As physiological responses often give similar signals in adjacent pixels, this method specifically highlights those pixels. In contrast, pixels that contain only noise show uncorrelated traces and thus appear dark in the cross-correlation map.

Solutions and Stimulus Application

The composition of the standard bath solution was (in mM): 98 NaCl, 2 KCl, 1 CaCl₂, 2 MgCl₂, 5 glucose, 5 Na-pyruvate, 10 HEPES. The composition of the Ca²⁺-free bath solution was (in mM): 98 NaCl, 2 KCl, 2 MgCl₂, 5 glucose, 5 Na-pyruvate, 10 HEPES, 2 EGTA. All solutions were adjusted to pH 7.8. This is the physiological pH in this poikilothermal species (Howell et al., 1970). The osmolality of the bath solution was 230 mOsm/l. All of the chemicals used for the preparation of the bath solutions were purchased from Sigma and were of the highest purity available. The purinergic agonists (see later), antagonists (see later), ARL 67156 and cyclopiazonic acid were dissolved in concentrated stock solutions (10 or 50 mM), which were aliquoted and frozen. Aliquots were thawed only once and the working solutions were made fresh before every experiment.

The following substances were all purchased from Sigma and were of the highest purity available: uridine; uridine 5'-monophosphate (UMP); uridine 5'-diphosphate (UDP); uridine 5'-triphosphate (UTP); adenosine; adenosine 5'-monophosphate (AMP); adenosine 5'-diphosphate (ADP); adenosine 5'-triphosphate (ATP); cytidine-5'-triphosphate (CTP); guanosine-5'-triphosphate (GTP); α,β -methylene ATP (α,β -meATP); β,γ -methylene ATP (β,γ -meATP); 2-methylthio ATP (2MeSATP); 3'-O-(4-benzoyl)-benzoyl ATP (BzATP); adenosine 5'-O-(3-thiotriphosphate) (ATP γ S); P¹,P⁴-diadenosine tetraphosphate (Ap₄A); 2-(Methylthio)adenosine 5'-diphosphate (2MeSADP); adenosine 5'-[β -thio]diphosphate (ADP β S); ARL 67156; pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS); reactive blue 2 (RB2); suramin; cyclopiazonic acid (CPA).

The bath solution was applied by gravity feed from a storage syringe through a funnel drug applicator to the recording chamber. The tip of the applicator was placed directly above the olfactory epithelium (OE). The purinergic agonists were pipetted directly into the funnel without stopping the flow. Outflow was through a syringe needle placed close to the OE. ARL 67156, the purinergic antagonists and cyclopiazonic acid were dissolved in the bath solution as indicated in the text.

Immunocytochemistry

For immunocytochemistry, larval *Xenopus laevis* were cooled to produce complete immobility in iced water, and then their olfactory nerves were cut through. To stain olfactory receptor neurons biocytin (ϵ -biotinoyl-L-lysine, Molecular Probes, Leiden, The Netherlands) crystals were inserted into the lesioned nerve, and the wound was closed with histoacryl glue (Braun, Melsungen, Germany). After 2 h the animals were sacrificed, and a tissue block containing both noses and the anterior part of the brain was cut out. Tissue blocks were fixed in 4% formaldehyde, washed in PBS, embedded in 5% low melting point agarose (Sigma, Deisenhofen, Germany), and subsequently sectioned on a vibratome (Leica VT 1000S, Bensheim, Germany) at 70 μ m. Sections were

then washed in PBS containing 0.2% Triton X-100 (PBST), and nonspecific binding was blocked with 2% normal goat serum (NGS; ICN, Aurora, Ohio, USA) in PBST for 1 h at room temperature. Tissue was then incubated overnight at 4°C with primary antibody against *Xenopus laevis* cytokeratin II (1:1,000) or with primary antibody against mouse Keratin 5 (1:1,000) diluted in 2% NGS/PBST. The *Xenopus laevis* cytokeratin type II antibody (1h5, monoclonal, derived from mouse) developed by Michael Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The mouse Keratin 5 antibody (AF 138, polyclonal, derived from rabbit) was from Covance, Princeton, NJ. Primary antibody was washed off with PBS, and Alexa 546 conjugated streptavidin (Molecular Probes, Leiden, The Netherlands) was applied at a final concentration of 5 μ g/mL in PBST for 5 h at room temperature. Slices were repeatedly rinsed in PBS, and Alexa 488 conjugated goat anti mouse secondary antibody (Molecular Probes, Leiden, The Netherlands) or Alexa 488 conjugated goat anti rabbit secondary antibody (Molecular Probes, Leiden, The Netherlands) was applied at a dilution of 1:250 in 1% NGS/PBS for 1 h 30 min at room temperature. The secondary antibody was washed off in several changes of PBS. Slices were then transferred to microscopy slides and mounted in mounting medium (Dako, Hamburg, Germany).

RESULTS

Morphology of Cells in the Olfactory Epithelium and Responses to ATP

We visualized mature ORNs and SCs in the OE of larval *Xenopus laevis* by combined biocytin backfilling (Fig. 1A) and cytokeratin type II immunostaining (Fig. 1B). When merging both pictures (Fig. 1C), there was no overlap between the backfilled ORNs and the cytokeratin-like immunoreactivity (CKLI). This indicates a complete absence of CKLI in ORNs thereby allowing a clear distinction between ORNs and the cells surrounding them. On the other hand, the CKLI-positive cells formed a tightly packed columnar monolayer on the apical surface of the same OE slice (see arrows). They sent their basal processes (see filled arrowheads) across the entire OE terminating in endfeet-like structures (see open arrowheads) at the level of the basal lamina. Virtually identical results were obtained with an antibody against keratin 5 (see Materials and Methods; data not shown). On the basis of its location and morphology, the CKLI can clearly be attributed to SC, although an additional but faint staining of basal cells cannot entirely be excluded.

ATP (200 μ M) applied to an acute Fluo-4-stained slice preparation of the OE led to specific cellular response patterns (Fig. 1D). The response time courses of two ATP-sensitive cells are plotted in Figure 1E. The overall shape and duration of the intracellular Ca²⁺ transients

Fig. 1. ATP-induced $[Ca^{2+}]_i$ increases in individual sustentacular supporting cells in a slice of the olfactory epithelium of larval *Xenopus laevis*. **A**: Slice of an OE with biocytin-streptavidin stained ORNs (stage 52; PC, principal cavity; LP, lamina propria; SCL, sustentacular cell layer; ORNL, olfactory receptor cell layer; BCL basal cell layer). **B**: Immunoreactivity to cytokeratin type II antibody of the same slice. Note the localization of the cytokeratin-like-immunoreactivity. The cytokeratin-positive cells form a tightly packed columnar monolayer in the SCL on the apical surface of the OE (see arrows) and send basal processes (see filled arrowheads) across the width of the OE that terminate in endfeet-like structures (see open arrowheads) at the level of the LP. **C**: Overlay of ORNs (**A**, red fluorescence) and cytokeratin-positive cells (**B**, green fluorescence). **D**: (**d**₁) slice of the OE stained with the Ca^{2+} indicator dye Fluo-4 (stage 54, image acquired at rest). Cells that

responded with an $[Ca^{2+}]_i$ increase upon application of ATP (200 μ M) are superimposed to the OE slice (grey shaded area). The ATP-induced $[Ca^{2+}]_i$ transients of the two cells labelled red and blue are shown in **E** (red and blue traces). (**d**₂–**d**₄) Sequence of three pseudocolored images of the same OE slice showing that application of ATP (200 μ M) transiently increases Ca^{2+} -dependent fluorescence in individual SCs. (**d**₂) Image taken before the application of ATP. (**d**₃) Image taken at the peak of the response. (**d**₄) Image taken after return to the base line fluorescence. The $[Ca^{2+}]_i$ increases clearly occurred in the SCL of the slice (cell bodies of SCs, see arrows in **d**₁). **E**, the time course and amplitude of ATP-induced $[Ca^{2+}]_i$ increases in individual SCs were highly reproducible if ATP was applied repeatedly. **F**, mean intracellular Ca^{2+} transients $\pm$ SEM of three successive ATP applications of all responsive cells of this slice (superposition in **d**₁).

were highly reproducible when ATP was applied repeatedly (Fig. 1E). Figure 1F shows the mean intracellular Ca^{2+} transients $\pm$ SEM of three successive ATP applications of all ATP-responsive cells of this slice [superposition in Fig. 1D (**d**₁)]. Similar results were obtained with all of the OE slices tested for their responsiveness to ATP. On the basis of their location and shape [see arrows in Fig. 1B,D (**d**₁)] the ATP-responsive cells can be clearly identified as SCs.

Characterization of the ATP-Induced Ca^{2+} Signaling in SCs

To describe the ATP-induced $[Ca^{2+}]_i$ increases in SCs in detail we performed high frequency Ca^{2+} imaging experiments in acute slice preparation of the OE stained with Fluo-4. Figure 2A shows a slice of the OE with a highlighted individual SC spanning most of the OE. Application of ATP (200 μ M) always initiated an $[Ca^{2+}]_i$ increase in the

Fig. 2. Characterization of the ATP-induced spatiotemporal pattern of $[Ca^{2+}]_i$ increases in SCs. **A:** Horizontal slice of the OE (pseudocolored illustration) stained with the Ca^{2+} indicator dye Fluo-4 (stage 52; image acquired at rest; PC, principal cavity; LP, lamina propria). An individual SC spanning the OE is highlighted. **B:** Higher magnification of the SC encircled in A. Detailed visualization of the ATP-induced spatiotemporal pattern of $[Ca^{2+}]_i$ increase. After application of ATP (200 μ M) the $[Ca^{2+}]_i$ increase initiates in the apical part of the SC and then spreads along its basal process towards the LP (see successive pictures, from $t = 0$ s to $t = 5$ s). Intracellular Ca^{2+} concentration returned to basal level after 25 s. **C:** Ca^{2+} transients taken from three out of a total of 50 regions of interest (ROIs) that were put on the SC

(ROI #2, apical, black; ROI #22, middle, blue; ROI #43, basal, red). The position on the SC of these three ROIs (ROI #2, ROI #22, and ROI #42) are indicated by white squares (see arrows) on the SC in B [SC ($t = 0$ s)]. **D:** Fluorescence intensity values ($\Delta F/F$) of 50 adjacent square ROIs (each $1.08 \times 1.08 \mu$ m) placed over the whole length of the SC plotted over time. Each of the 50 horizontal lines represents the color-coded intensity values of one ROI. The dotted line in Figure 2D highlights the virtually linear progression of the Ca^{2+} signal from the apical to the basal region of the SC. In this SC the Ca^{2+} transient advanced with 17.03μ m/s from the apical to the basal region of the SC. Note that the time scale is the same in C and D.

apical part of the SCs, even if ATP was applied to the basal region of the olfactory epithelium. Subsequently the Ca^{2+} signal migrated along the basal process of the SC toward the basal lamina (Fig. 2B; from $t = 2$ s to $t = 5$ s). The ATP-induced $[Ca^{2+}]_i$ transients of three selected regions lying in an apical, medial, and basal part of the SC are shown in Figure 2C. The distance between the selected regions was $\sim 23 \mu$ m. Thereby, the maximum amplitude of the $[Ca^{2+}]_i$ transient occurred in the medial region of the SC. The $[Ca^{2+}]_i$ returned to prestimulus levels after about 25 s. To visualize the progression of the Ca^{2+} signal over the entire length of the SC ($\sim 57 \mu$ m), we plotted the intracellular Ca^{2+} transient of 50 adjacent regions against the time (Fig. 2D). The dotted line in Figure 2D highlights the virtually linear progression of the Ca^{2+} signal from the apical to the basal region of the SC. In this SC, the progression velocity of the Ca^{2+} signal was 17.03μ m/s. The calculated mean progression velocity of the Ca^{2+} signals in 34 SCs was $17.10 \pm 1.02 \mu$ m/s.

Pharmacological Characterization of the Purinergic Receptor Subtype(s) on SCs

To determine which subtype(s) of purinergic receptors are involved in the ATP-induced $[Ca^{2+}]_i$ increases in

SCs, we first examined whether the presence of extracellular Ca^{2+} was essential for the responses to ATP. Responses to ATP in Ca^{2+} -free bath solution were the same or larger than those in standard bath solution (Fig. 3A). Repeated applications of ATP in Ca^{2+} -free bath solution inevitably resulted in a rundown of the Ca^{2+} responses (Fig. 3A), presumably due to a gradual depletion of the intracellular Ca^{2+} stores. After returning to standard conditions the responses recovered (Fig. 3A). Similar results were obtained in all of the 66 SCs (three OE slices) tested (Fig. 3B). Furthermore, depleting intracellular Ca^{2+} stores with CPA eliminated the intracellular Ca^{2+} responses to ATP (Fig. 3C). Virtually identical results were obtained in all of the 56 SCs (three OE slices) tested (Fig. 3D). These data indicate that the $[Ca^{2+}]_i$ increases evoked by ATP were mediated by release of Ca^{2+} from CPA-sensitive intracellular stores, and that Ca^{2+} influx through the plasma membrane contributed little, if at all. This suggested the involvement of metabotropic P2Y receptors. To further substantiate the above results and to determine which P2Y receptor subtype(s) are expressed on SCs, we examined the potency of a variety of purinergic agonists known to differentially activate different purinergic receptor subtypes.

Fig. 3. Responses to purinergic agonists do not require the presence of extracellular Ca²⁺ but do require intact intracellular Ca²⁺ stores. **A**: [Ca²⁺]_i increases in response to ATP (200 μ M; mean response $\pm$ SEM of all ATP-responsive cells of an OE slice) persisted in Ca²⁺-free bath solution (grey-shaded rectangle; ATP applications after 1, 5, 10, 20, and 25 min in Ca²⁺-free bath solution, respectively). Repeated applications of ATP in Ca²⁺-free bath solution eventually led to a rundown of the Ca²⁺ response. The responses completely recovered after switching back to standard bath solution. **B**: Mean responses $\pm$ SEM, expressed as percent of control response to ATP, of 66 SCs (three OE slices) in

standard (dark grey columns) and Ca²⁺-free bath solution (light grey columns; #1 to #5 = 1, 5, 10, 20, and 25 min in Ca²⁺-free bath solution, respectively). **C**: [Ca²⁺]_i increases in response to ATP (200 μ M; mean response $\pm$ SEM of all ATP-responsive cells of an OE slice) were irreversibly inhibited by depletion of intracellular Ca²⁺ stores with CPA (25 μ M, grey-shaded rectangle; ATP applications after 4 and 8 min in bath solution with CPA, respectively). **D**: Mean responses $\pm$ SEM, expressed as percent of control response to ATP, of 56 SCs (three OE slices) in control (dark grey columns) and bath solution with CPA (light grey columns; #1 and #2 = 4 and 8 min in bath solution with CPA, respectively).

[Ca²⁺]_i responses, averaged over all responsive cells of a slice, on mucosal application of a selection of purinergic agonists (each 200 μ M) are shown in Figure 4A–C (one slice per subfigure). Figure 4D summarizes the results obtained from 323 SCs (21 OE slices). In addition to ATP, SCs responded to UTP and ATP γ S. Thereby, the order of agonist potency was UTP > ATP > ATP γ S. All other agonists tested were inactive or very weakly active. All of the individual nucleotide responsive SCs tested had the above mentioned response profiles to the applied agonists.

As a last point we examined the effects of three non-selective P2-antagonists on the ATP-induced [Ca²⁺]_i increases in SCs (see Fig. 5). The mean ATP-induced [Ca²⁺]_i increase in the presence of PPADS (200 μ M; Fig. 5A,B) and RB2 (200 μ M; Fig. 5C,D) was reduced to about 70% and 50%, respectively, if compared to the [Ca²⁺]_i increase in absence of these antagonists. Suramin (200 μ M), in turn, did not affect the ATP-induced [Ca²⁺]_i increase (Fig. 5E,F).

Finally, Figure 6A and B show the dose dependency of the ATP- and UTP-induced increases in the [Ca²⁺]_i in

Fig. 4. Responsiveness of SCs to purinergic agonists. **A–C:** $[Ca^{2+}]_i$ increases of SCs of three different OE slices in response to application of various purinergic agonists (each 200 μ M; mean response $\pm$ SEM of all ATP-responsive cells of the OE slice). **D:** Mean responses $\pm$ SEM, expressed as percent of control response to ATP (UTP, data from 189

SCs from 13 OE slices; β,γ -meATP, 139/9; ATP γ S, 129/8; 2MeSATP, 75/6; Ap4A, 114/7; α,β -meATP, 124/8; BzATP, 90/5; 2MeSADP, 10/1; ADP β S, 23/2; ADP, 124/7; AMP, 78/5; adenosine, 78/5; UDP, 24/2; UMP, 24/2; uridine, 24/2; CTP, 36/3; GTP, 36/3).

SCs. The averaged dose-response relationships for ATP- and UTP-induced $[Ca^{2+}]_i$ increases in SCs of acute OE slices gave EC_{50} values of 82.6 μ M and 56.3 μ M, respectively (Fig. 6C). ATP degradation by ectonucleotidases in the extracellular space can considerably reduce the effective concentration of endogenously released or bath applied ATP. We therefore tested the influence of the ectonucleotidase inhibitor ARL 67156 on ATP-induced responses of SCs. ARL 67156 (100 μ M) clearly increased the ATP-induced $[Ca^{2+}]_i$ responses (Fig. 6D). In the SCs of this OE slice ARL 67156 increased the $[Ca^{2+}]_i$ response by more than 70%. In 84 SCs (four OE slices) ARL 67156 enhanced the mean increase of the $[Ca^{2+}]_i$ by more than 100% (Fig. 6E).

DISCUSSION

The OE as a whole has not been studied so far. Although there are a host of papers on olfactory receptors (Mombaerts, 1999) and ORNs (Getchell, 1986; Schild and Restrepo, 1998), very little is known about the SCs (Getchell and Getchell, 1992; Trotier, 1998) and nothing about interactions among the cell types within the OE. To put it the other way round, our view of how olfaction works would not change markedly if there were no SCs in the OE. In a sense this was exactly the fate of glia cells up to two decades ago. Today, neuronal and, in particular, synaptic function is unimaginable

without the glial players. With the present manuscript we intend to contribute to a more thorough comprehension of the tissue olfactory epithelium. Specifically, we concentrate on three points: that is, (i) the cellular localization of purinergic receptors on SCs, (ii) a precise analysis of the characteristic spatiotemporal pattern of nucleotide-induced $[Ca^{2+}]_i$ increases in SCs, and (iii) the pharmacological characterization of the purinergic receptor subtype(s) involved in SC Ca^{2+} signaling.

To understand the localization of purinergic receptors and their effects, it was extremely helpful that antibodies against cytokeratin type II turned out to be excellent markers for SCs of the OE of larval *Xenopus laevis*. While ORNs are clearly cytokeratin type II negative we cannot completely exclude that some BCs are labeled. These findings are in contrast with results obtained in the murine OE, where antibodies to type II cytokeratins have been shown to specifically label BCs (Calof and Chikaraishi, 1989; Schwartz Levey et al., 1991; Holbrook et al., 1995). On the other hand, Cytokeratin 18, a type I cytokeratin, has been reported to be specifically expressed in SCs of the rat (Holbrook et al., 1995). A comparison of the location and shape of cytokeratin-positive cells and ATP-responsive cells (see Fig. 1) clearly shows that the ATP-induced $[Ca^{2+}]_i$ increases occur in SCs and initiate in the cell bodies of SC in the most apical part of the OE. These data show that mucosal application of ATP induces strong $[Ca^{2+}]_i$ increases in the vast majority, if not in all SCs of the OE

Fig. 5. Effects of purinergic antagonists on ATP-induced $[Ca^{2+}]_i$ increases of SCs. **A:** $[Ca^{2+}]_i$ increases in response to ATP (200 μ M; mean response $\pm$ SEM of all ATP-responsive cells of an OE slice) were attenuated, but not completely blocked by PPADS (200 μ M, grey-shaded rectangle; ATP applications 10 and 15 min after PPADS application, respectively). After a washout time of 10 min the responses recovered almost completely. **B:** Mean responses $\pm$ SEM, expressed as percent of control response to ATP, of 63 SCs (four OE slices) in control (dark grey columns) and bath solution with PPADS (light grey columns; #1 and #2 = 10 and 15 min in bath solution with PPADS, respectively). **C:** $[Ca^{2+}]_i$ increases in response to ATP (200 μ M; mean response $\pm$ SEM of all ATP-responsive cells of an OE slice) were attenuated, but not completely blocked by RB2 (200 μ M, grey-shaded rectangle; ATP applica-

tions 10 and 15 min after RB2 application, respectively). After a wash-out time of 10 min the responses recovered almost completely. **D:** Mean responses $\pm$ SEM, expressed as percent of control response to ATP, of 66 SCs (four OE slices) in control (dark grey columns) and bath solution with RB2 added (light grey columns; #1 and #2 = 10 and 15 min in bath solution with RB2, respectively). **E:** $[Ca^{2+}]_i$ increases in response to ATP (200 μ M; mean response $\pm$ SEM of all ATP-responsive cells of an OE slice) were not affected by suramin (200 μ M, grey-shaded rectangle; ATP applications 10 and 15 min after suramin application, respectively). **F:** mean responses $\pm$ SEM, expressed as percent of control response to ATP, of 37 SCs (four OE slices) in control (dark grey columns) and bath solution with suramin added (light grey columns; #1 and #2 = 10 and 15 min in bath solution with suramin, respectively).

of larval *Xenopus laevis*. The few mature ORNs (approximately 5% of ATP-responsive cells in the OE of larval *Xenopus laevis*), that have been shown to be sensitive to ATP (Czesnik et al., 2006), most probably express olfactory receptors sensitive to nucleotides. As nucleotides can act as water-borne odorants (Carr et al., 1986; Derby et al., 1984; Kang and Caprio, 1995; Rolen et al., 2003) this speculation appears rather obvious.

As to our second goal, we characterized the ATP-induced $[Ca^{2+}]_i$ increases in SCs. Acquisition of images

at a rate of 40 Hz allowed to clearly identify and characterize a wave-like propagation of the $[Ca^{2+}]_i$ increases within individual SCs. The onset of the $[Ca^{2+}]_i$ increase always occurred in the apical part of the SCs and subsequently propagated along their basal processes toward the basal lamina. This strongly suggests that the purinergic receptors may be localized on the soma of the SCs. Thereby, the calculated mean intracellular advance velocity of the Ca^{2+} signal (17.10 ± 1.02 μ m/s) is comparable with other known intracellular fast Ca^{2+} wave

Fig. 6. Dose-response relationships of Ca^{2+} transients induced by ATP and UTP. **A:** $[\text{Ca}^{2+}]_i$ increase in response to various concentrations of ATP (0.2–2,000 μM ; mean responses $\pm$ SEM of all ATP-responsive cells of an individual OE slice). **B:** $[\text{Ca}^{2+}]_i$ increase in response to various concentrations of UTP (0.2–2,000 μM ; mean responses $\pm$ SEM of all UTP-responsive cells of an individual OE slice). **C:** Dose-response curves for ATP and UTP (mean $\pm$ SEM, ATP, 32 SCs from four slices; UTP, 36 SCs from three slices). Response amplitudes in each SC were normalized to the maximum response of each SC. The curve was fitted by a Boltzmann equation. The EC_{50} values are 82.6 μM for ATP and

56.3 μM for UTP. **D:** $[\text{Ca}^{2+}]_i$ increases in response to ATP (200 μM ; mean response $\pm$ SEM of all ATP-responsive cells of an individual OE slice) were enhanced in bath solution with ARL 67156 (100 μM) added (grey-shaded rectangle; ATP application after 5 min in bath solution with ARL 67156 added). The responses returned to control levels after switching back to standard bath solution. **E:** Mean responses $\pm$ SEM, expressed as percent of control response to ATP, of 84 SCs (four OE slices) in control (dark grey columns) and bath solution with ARL 67156 added (light grey column).

velocities [mean velocity = 15 $\mu\text{m/s}$ at 20°C (Jaffe, 2002)].

The ATP-induced $[\text{Ca}^{2+}]_i$ increases in SCs were similar in the presence and absence of extracellular Ca^{2+} . Thereby, the first response to ATP in absence of extracellular Ca^{2+} was always larger than the responses in the presence of extracellular Ca^{2+} . Similar enhanced $[\text{Ca}^{2+}]_i$ increases in absence of extracellular Ca^{2+} have been reported in a similar study conducted with Müller cells, the radial glia cells in the retina (Saltzberg et al., 2003). On the other hand, depletion of the intracellular Ca^{2+} stores via application of CPA, a specific inhibitor of the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (Seidler et al., 1989), completely abolished the ATP-induced $[\text{Ca}^{2+}]_i$ increases. Together, these two experiments strongly suggest that the ATP-induced $[\text{Ca}^{2+}]_i$ increases were due, in large part, if not entirely, to the activation of G protein-coupled receptors followed by Ca^{2+} mobilization from intracellular stores. This in turn

strongly suggested an involvement of metabotropic purinergic receptors coupled to Ca^{2+} release from intracellular stores, most probably P2Y receptors.

To substantiate this hypothesis and to determine the receptor subtype(s) involved, we compared the observed effect of ATP with those of other putative agonists. Among these only UTP and ATP γS showed an effect. UTP was slightly more effective in triggering Ca^{2+} responses than ATP, which in turn was more effective than ATP γS . The relatively narrow rank order of purinergic agonist potency in SCs therefore was: UTP > ATP > ATP γS . In this context, the calculated dose-response relationships for UTP- and ATP-induced $[\text{Ca}^{2+}]_i$ increases (see Fig. 6) are in line with this agonist potency rank order, showing that UTP has a slightly lower EC_{50} value than ATP. The other 15 agonist tested were only very weakly and inconsistently active or completely inactive. Thereby, it is important to notice that all of the tested individual SCs had the aforementioned

agonist profile, manifestly suggesting that individual SCs do not appear to express varying purinergic receptors. The whole population of SCs rather expresses exactly the same subtype(s) of purinergic receptors. According to current established pharmacological studies, the determined agonist rank order is not consistent with that reported for P2Y₁ (2MeSATP = 2MeSADP > ADP > ATP), P2Y₆ (UDP ≫ UTP ≫ ATP), P2Y₁₁ (BzATP = ATPγS > ATP, UTP), P2Y₁₂ (2-MeSADP > ADP > ATP), P2Y₁₃ (2-MeSADP = ADP ≫ ATP), or P2Y₁₄ (UDP-glucose) (Burnstock, 2007; von Kügelen, 2006). Only P2Y₂ and P2Y₄ receptors have been reported to be activated preferentially and almost equipotently by UTP and ATP (Burnstock, 2007; von Kügelen, 2006). The third effective agonist, ATPγS, has also been reported to activate P2Y₂ and P2Y₄ receptors, but with a lower potency than UTP and ATP (Brunschweiler and Müller, 2006; Wildman et al., 2003). The fact that purinergic agonists such as α,β-meATP and β,γ-meATP, which are known to preferentially activate P2X receptors (Burnstock, 2007; Ralevic and Burnstock, 1998) and adenosine which is known to activate P1 receptors (Burnstock, 2007; Ralevic and Burnstock, 1998) were ineffective is a further indication against the involvement of P2X and P1 receptors in SC Ca²⁺ signaling.

Out of the tested nonselective P2 receptor antagonists PPADS, RB2, and suramin (Ralevic and Burnstock, 1998) all applied at a relatively high concentration (200 μM), only PPADS and RB2 moderately attenuated ATP-induced [Ca²⁺]_i increases in SCs. These antagonists have been successfully used for several years to block P2 receptor responses (Jacobson et al., 2004; Lambrecht et al., 2002; Ralevic and Burnstock, 1998; von Kügelen, 2006). Concerning their effectiveness on P2Y receptors, it has been shown that suramin, if applied at high micromolar concentrations, affects all P2Y subtypes except the P2Y₄ receptor (von Kügelen, 2006). PPADS and RB2 also inhibit several P2Y receptors with the strongest effect on the P2Y₁ and the P2Y₆ receptor. Only at a higher micromolar concentration they affect P2Y₂ and P2Y₄ receptors (von Kügelen, 2006). Therefore, our antagonist experiments provide a somewhat stronger evidence for the functional expression of P2Y₄ than P2Y₂ receptors on SCs.

The EC₅₀ values for ATP and UTP (82.6 μM and 56.3 μM, respectively) are considerably higher than those reported for heterologously expressed P2Y₂ and P2Y₄ receptors (Wildman et al., 2003) possible due to high ectonucleotidase activity in the OE of larval *Xenopus laevis*. Ectonucleotidases are enzymes of the plasma membrane with an extracellularly oriented catalytic site that hydrolyze extracellular nucleotides (Zimmermann, 2000, 2006). It is known that nucleotide degradation by ectonucleotidases in the extracellular space can considerably reduce the effective concentrations of endogenously released or bath-applied ATP and UTP (Zimmermann, 2000, 2006). Experiments with the ectonucleotidase inhibitor ARL 67156 (Westfall et al., 1996) confirmed the above hypothesis clearly showing that ATP-induced [Ca²⁺]_i increases were substantially enhanced in the presence of the inhibitor. This finding shows that the high EC₅₀ values for ATP

and UTP had to be expected and were at least in part due to ectonucleotidase activity in the OE.

At present, we can only speculate about the physiological role of the characteristic purinergic signaling in SCs in the OE of larval *Xenopus laevis*. Sustentacular supporting cells have been shown to be involved in a number of "maintenance functions" of the OE. Until very recently there was no evidence for a direct involvement of SC in intraepithelial signaling and modulation mechanisms. Three recent studies have, however, shown that application of nucleotides induce strong [Ca²⁺]_i increases in SCs of mouse and larval *Xenopus laevis* (Czesnik et al., 2006; Hegg et al., 2003), and that nucleotides induce heat shock protein expression in SCs in the mouse (Hegg and Lucero, 2006). In the OE of mouse, SCs express solely P2Y receptors (Hegg et al., 2003), whereas ORNs have been shown to express both, P2X and P2Y receptors (Hegg et al., 2003). In this context, Hegg et al. could nicely show that coapplication of nucleotides and odorants suppressed odorant-induced [Ca²⁺]_i increases of mouse ORNs via activation of P2X and P2Y receptors (Hegg et al., 2003). They argued that purinergic receptors may play a role in signaling acute damage to the OE and that ATP release of damaged cells in the OE may, therefore, prevent overstimulation of both, the OE and the olfactory bulb. The induction of heat-shock proteins in SCs, in turn, apparently initiates a form of neuroprotection in the OE (Hegg and Lucero, 2006). In contrast, the results from the present study and data of an earlier work (Czesnik et al., 2006) convincingly show that ORNs in the OE of larval *Xenopus laevis* do not express purinergic receptors. We have shown that in larval *Xenopus laevis* only SCs but not ORNs are activated by extracellular nucleotides. In addition, and for the first time here we show that nucleotides induce a characteristic wave-like Ca²⁺ signal within individual SCs. This distinctive spatiotemporal pattern of [Ca²⁺]_i increases in SCs allows to speculate about a novel form of intraepithelial communication path from the very apical part of the OE to the basal lamina. The fact that ORNs in the OE of larval *Xenopus laevis*, different from ORNs in the mouse OE, do not express purinergic receptors shows that there are differences in the purinergic system in the OE of mouse and *Xenopus*. Whether this difference is functionally crucial or whether a common denominator of both will emerge, future studies must show. It will also be critical for our understanding of the OE to determine the physiological role of the intraepithelial signaling, to determine possible physiological sources of extracellular ATP and to investigate a possible involvement of the spatiotemporal pattern of [Ca²⁺]_i increases in SCs in triggering a crosstalk between different cell types of the OE.

REFERENCES

- Abrahamse SL, Rechkemern G. 2001. Identification of an organic anion transport system in the human colon carcinoma cell line HT29 clone 19A. *Pflügers Arch* 441:529–537.

- Breipohl W, Laugwitz HJ, Bornfeld N. 1974. Topological relations between the dendrites of olfactory sensory cells and sustentacular cells in different vertebrates. An ultrastructural study. *J Anat* 117:89–94.
- Brunschweiler A, Müller CE. 2006. P2 receptors activated by uracil nucleotides—an update. *Curr Med Chem* 13:289–312.
- Burnstock G. 2007. Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* 87:659–797.
- Calof AL, Chikaraishi DM. 1989. Analysis of neurogenesis in a mammalian neuroepithelium: Proliferation and differentiation of an olfactory neuron precursor in vitro. *Neuron* 3:115–127.
- Carr WES, Gleeson RA, Ache BW, Milstead ML. 1986. Olfactory receptors of the spiny lobster: ATP-sensitive cells with similarities to P2-type purinoceptors of vertebrates. *J Comp Physiol A* 158:331–338.
- Czesnik D, Kuduz J, Schild D, Manzini I. 2006. ATP activates both receptor and sustentacular supporting cells in the olfactory epithelium of *Xenopus laevis* tadpoles. *Eur J Neurosci* 23:119–128.
- Derby CD, Carr WES, Ache BW. 1984. Purinergic olfactory cells of crustaceans: Response characteristics and similarities to internal purinergic cells of vertebrates. *J Comp Physiol A* 155:341–349.
- Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J. 1995. The leukotriene LTD4 antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* 208:345–352.
- Graziadei PPC. 1971. The olfactory mucosa of vertebrates. In: Beidler LM, editor. *Handbook of sensory physiology*, Vol. 4. New York: Springer-Verlag, p 27–58.
- Graziadei PPC. 1973. Cell dynamics in the olfactory mucosa. *Tissue Cell* 5:113–131.
- Graziadei PPC, Metcalf JF. 1971. Autoradiographic and ultrastructural observations of the frog's olfactory mucosa. *Z Zellforsch Mikrosk Anat* 116:305–318.
- Getchell TV. 1977. Analysis of intracellular recordings from salamander olfactory epithelium. *Brain Res* 123:275–286.
- Getchell TV. 1986. Functional properties of vertebrate olfactory receptor neurons. *Physiol Rev* 66:772–818.
- Getchell ML, Getchell TV. 1992. Fine structural aspects of secretion and extrinsic innervation in the olfactory mucosa. *Microsc Res Tech* 23:111–127.
- Getchell ML, Mellert TK. 1991. Olfactory mucus secretion. In: Getchell TV, Doty R, Bartoshuk L, Snow JB, editors. *Smell and taste in health and disease*. New York: Raven Press, pp 83–95.
- Hansen A, Reiss JO, Gentry CL, Burd GD. 1998. Ultrastructure of the olfactory organ in the clawed frog, *Xenopus laevis*, during larval development and metamorphosis. *J Comp Neurol* 398:273–288.
- Hegg CC, Greenwood D, Huang W, Han P, Lucero MT. 2003. Activation of purinergic receptor subtypes modulates odor sensitivity. *J Neurosci* 23:8291–8301.
- Hegg CC, Lucero MT. 2006. Purinergic receptor antagonists inhibit odorant-induced heat shock protein 25 induction in mouse olfactory epithelium. *Glia* 53:182–190.
- Holbrook EH, Szumowski KE, Schwob JE. 1995. An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. *J Comp Neurol* 363:129–146.
- Howell BJ, Baumgardner FW, Bondi K, Rahn H. 1970. Acid-base balance in cold-blooded vertebrates as a function of body temperature. *Am J Physiol* 218:600–606.
- Jacobson KA, Costanzi S, Ohno M, Joshi BV, Besada P, Xu B, Tchilibon S. 2004. Molecular recognition at purine and pyrimidine nucleotide (P2) receptors. *Curr Top Med Chem* 4:805–819.
- Jaffe L. 2002. On the conservation of fast calcium wave speeds. *Cell Calcium* 32:217–229.
- Kang J, Caprio J. 1995. In vivo responses of single olfactory receptor neurons in the channel catfish, *Ictalurus punctatus*. *J Neurophysiol* 73:172–177.
- Lambrech G, Braun K, Damer M, Ganso M, Hildebrandt C, Ullmann H, Kassack MU, Nickel P. 2002. Structure-activity relationships of suramin and pyridoxal-5'-phosphate derivatives as P2 receptor antagonists. *Curr Pharm Des* 8:2371–2399.
- Manzini I, Schild D. 2003. Multidrug resistance transporters in the olfactory receptor neurons of *Xenopus laevis* tadpoles. *J Physiol (Lond)* 546:375–385.
- Manzini I, Schweer TS, Schild D. 2008. Improved fluorescent (calcium indicator) dye uptake in brain slices by blocking multidrug resistance transporters. *J Neurosci Methods* 167:140–147.
- Masukawa LM, Hedlund B, Shepherd GM. 1985. Electrophysiological properties of identified cells in the in vitro olfactory epithelium of the tiger salamander. *J Neurosci* 5:128–135.
- Mombaerts P. 1999. Seven-transmembrane proteins as odorant and chemosensory receptors. *Science* 286:707–711.
- Newman E, Reichenbach A. 1996. The Müller cell: A functional element of the retina. *Trends Neurosci* 19:307–312.
- Nieuwkoop PD, Faber J. 1994. *Normal table of Xenopus laevis* (Daudin). New York and London: Garland Publishing, p 252.
- Okano M, Takagi SF. 1974. Secretion and electrogenesis of the supporting cell in the olfactory epithelium. *J Physiol (Lond)* 242:353–370.
- Ralevic V, Burnstock G. 1998. Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413–492.
- Rafols JA, Getchell TV. 1983. Morphological relations between the receptor neurons, sustentacular cells and Schwann cells in the olfactory mucosa of the salamander. *Anat Rec* 206:87–101.
- Rolen SH, Sorensen PW, Mattson D, Caprio J. 2003. Polyamines as olfactory stimuli in the goldfish *Carassius auratus*. *J Exp Biol* 206:1683–1696.
- Saltzberg JMR, Garvey KA, Keirstead SA. 2003. Pharmacological characterization of P2Y receptor subtypes on isolated tiger salamander Müller cells. *Glia* 42:149–159.
- Schild D, Restrepo D. 1998. Transduction mechanisms in vertebrate olfactory receptor cells. *Physiol Rev* 78:429–466.
- Schwartz Levey M, Chikaraishi DM, Kauer JS. 1991. Characterization of potential precursor populations in the mouse olfactory epithelium using immunocytochemistry and autoradiography. *J Neurosci* 11:3556–3564.
- Seidler NW, Jona I, Vegh M, Martonosi A. 1989. Cyclopiazonic acid is a specific inhibitor of the Ca²⁺-ATPase of sarcoplasmic reticulum. *J Biol Chem* 264:17816–17823.
- Suzuki Y, Takeda M, Farbman AI. 1996. Supporting cells as phagocytes in the olfactory epithelium after bullectomy. *J Comp Neurol* 376:509–517.
- Thorne PR, Housley GD. 1996. Purinergic signalling in sensory systems. *Semin Neurosci* 8:233–246.
- Trotier D. 1998. Electrophysiological properties of frog olfactory supporting cells. *Chem Senses* 23:363–369.
- von Kügelgen I. 2006. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol Ther* 110:415–432.
- Westfall TD, Kennedy C, Sneddon P. 1996. Enhancement of sympathetic purinergic neurotransmission in the guinea-pig isolated vas deferens by the novel ecto-ATPase inhibitor ARL 67156. *Br J Pharmacol* 117:867–872.
- Wildman SS, Unwin RJ, King BF. 2003. Extended pharmacological profiles of rat P2Y₂ and rat P2Y₄ receptors and their sensitivity to extracellular H⁺ and Zn²⁺ ions. *Br J Pharmacol* 140:1177–1186.
- Zimmermann H. 2000. Extracellular metabolism of ATP, other nucleotides. *Naunyn-Schmiedeberg Arch Pharmacol* 362:299–309.
- Zimmermann H. 2006. Ectonucleotidases in the nervous system. *Novartis Found Symp* 276:113–130.