European Journal of Neuroscience, Vol. 19, pp. 2497–2510, 2004

Subunit-specific P2X-receptor expression defines chemosensory properties of trigeminal neurons

Jennifer Spehr, Marc Spehr, Hanns Hatt and Christian H. Wetzel
Lehrstuhl fuer Zellphysiologie, Ruhr-Universitaet Bochum, Universitaetsstr. 150, 44780 Bochum, Germany

Keywords: ATP, chemosensation, odorants, purine receptors, rat

Abstract

The facial innervation pattern of trigeminal nerve fibres comprises the innervation of the nasal epithelium, where free trigeminal nerve endings contribute to detection and discrimination of chemical stimuli including odourants. The signal transduction mechanisms in sensory nerve endings underlying perception of chemical stimuli remain widely uncovered. Here, we characterized trigeminal ATP-activated P2X receptors in cultured rat trigeminal neurons and investigated their role in chemoperception. We identified a new subpopulation of neurons lacking typical nociceptive characteristics and expressing homomeric $P2X_2$ receptors. Using a certain group of chemicals known as trigeminal stimuli we found no direct activation of trigeminal neurons, but a modulation of $P2X_2$ receptor mediated currents. In contrast, $P2X_3$ receptor mediated currents of nociceptive trigeminal neurons remained unaffected by the tested chemicals. Therefore, we assume a functional role for the newly identified subpopulation in chemodetection of certain trigeminal stimuli.

Introduction

Most of the facial sensory innervation is provided by the trigeminal (V cranial) nerve, including the innervation of the mucous membranes of the oral and nasal cavities, the cornea and the conjunctiva of the eye. The trigeminal system mainly functions to protect the organism from potentially harmful stimuli and comprises neurons that transduce mechanical, thermal and chemical stimuli probably including odourant molecules (Silver & Finger, 1991). Psychophysical investigations on anosmics revealed that the trigeminal system can mediate different sensations to different odourants (Laska *et al*., 1997). In contrast to the olfactory system the expression of olfactory receptors in the trigeminal system has not been demonstrated until now. The molecular mechanisms used by trigeminal neurons to detect odourant molecules remain to be uncovered. It is well established that both the olfactory and the trigeminal system contribute to the perception of the majority of odourants and much research has been performed to investigate the interaction between both systems (reviewed in Hummel & Livermore, 2002).

A recent study revealed that the odour sensitivity of olfactory receptor neurons (ORN) is influenced by the neuromodulator adenosine 5′-triphosphate (ATP) acting via various ATP receptors expressed in ORN (Hegg *et al*., 2003). Additionally, two types of ATP-induced currents with distinct desensitization kinetics (Cook *et al*., 1997) have been recorded from trigeminal nociceptive neurons. Both findings suggest a yet undefined role for ATP in the olfactory and trigeminal system.

In general, the neurotransmitter/neuromodulator ATP can activate metabotropic P2Y or ionotropic P2X receptors. Until now, seven mammalian P2X receptor subunits ($P2X_{1-7}$) have been cloned and functionally characterized (North & Barnard, 1997; Ralevic & Burnstock, 1998). P2X receptors build oligomeric complexes of uncertain stoichiometry, but are possibly composed of either three (Nicke *et al*., 1998; Stoop *et al*., 1999) or four subunits (Kim *et al*., 1997; Ding & Sachs, 2000). In various sensory neurons (nodose, DRG, trigeminal) transcripts for six subunits ($P2X_{1-6}$) have been found (Chen *et al*., 1995; Collo *et al*., 1996; Cook *et al*., 1997). Immunohistochemical studies reveal a predominance of $P2X_3$-like protein, which is frequently colocalized with $P2X_2$-like immunoreactivity in sensory ganglia (Cook *et al*., 1997; Vulchanova *et al*., 1997; Bradbury *et al*., 1998; Xiang *et al*., 1998), giving rise to the formation of both homooligomeric $P2X_2$ or $P2X_3$ receptors as well as heterooligomeric $P2X_{2/3}$ receptors (Lewis *et al*., 1995; Thomas *et al*., 1998; Grubb & Evans, 1999). The specific expression of $P2X_3$ receptors by a subset of putative nociceptive neurons (peripherin expression, capsaicin sensitivity) introduces a possible mechanism for ATP-evoked nociceptive signalling (Chen *et al*., 1995). Indeed, mice lacking the $P2X_3$ gene show a significant decrease in pain-related behaviours in several models of injury (Cockayne *et al*., 2000; Souslova *et al*., 2000).

The complete expression pattern and subunit composition of P2X receptors on trigeminal neurons as well as their function is not known. In the present study, we investigated the P2X receptor subunit expression in cultured dissociated neurons of the rat gasserian ganglion using an electrophysiological, immunohistological and pharmacological approach. We classified certain subpopulations of trigeminal neurons and tried to elucidate their possible function in trigeminal odourant detection.

Data presented in this paper led to the identification of a novel population of trigeminal neurons that exhibits ATP responses different in time course and pharmacology from nociceptive trigeminal neurons. This population could play an important physiological role in chemodetection as a certain group of trigeminal and olfactory stimuli selectively modulates ATP-induced currents in these neurons. Such a

Correspondence: Dr Christian H. Wetzel, as above.
E-mail: christian.h.wetzel@rub.de

Received 8 December 2003, revised 17 February 2004, accepted 19 February 2004

doi:10.1111/j.1460-9568.2004.03329.x

detection mechanism would require stimulus-induced ATP release in the nasal epithelium. Indeed, our experimental data suggest that ORNs could act as an activity-dependent source of ATP in the olfactory epithelium.

Materials and methods

Primary culture of trigeminal sensory neurons

Animals were cared for in accordance with European Union Community Council guidelines. The primary cell culture of trigeminal neurons has been described previously (Viana *et al*., 2001) and was only slightly altered. New-born Wistar rats (P0–P5) were decapitated and both trigeminal ganglia (ganglion gasseri) were excised under a binocular. The ganglia were washed in phosphate buffered saline solution (PBS, Invitrogen, Karlsruhe, Germany) and collected in cold Leibovitz Medium (L15, Invitrogen, Karlsruhe, Germany). Ganglia were cut into small pieces and incubated for 45 min in warm Dulbecco's modified essential medium (DMEM) (37 °C, 95% air, 5% CO_2) containing 0.025% collagenase (type IA, Sigma, Deisenhofen, Germany).

The tissue was gently titrurated with a fire-polished glass pipette and the suspension was centrifugated at 200 ***g*** for 8 min. The obtained pellet was resuspended in culture medium with the following composition: DMEM/F-12 (1 : 1) with Glutamax (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany), 100 μg/mL penicillin/streptomycin and 100 ng/mL nerve growth factor (NGF, mouse-7s, Alomone laboratories, Jerusalem, Israel).

Cells were plated on the central region of poly L-lysine (0.01%) coated Petri dishes (Falcon, BD Biosciences, Heidelberg, Germany, 140 μL cell solution/dish) and kept in a humidified atmosphere (37 °C, 95% air, 5% CO_2). One hour after plating, 2 mL culture medium was added to each dish. Neurons were grown for 4 h up to 5 days before being used.

Olfactory receptor neuron preparation

Olfactory receptor neurons (ORN) were prepared as described previously (Spehr *et al*., 2002). Wistar rats (P20–P30) were killed by decapitation after CO_2 anaesthesia. The intact olfactory epithelium was dissected from the septal bone and placed in papain solution (in mM: 140 NaCl, 10 HEPES, 10 glucose, plus 10 μg/mL papain (EC 3.4.22.2 from papaya latex, pH 7.4) to obtain acutely dissociated ORNs after gentle trituration.

HEK293 cell culture and transfection

HEK293 cells were grown at 37 °C in MEM medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum under a 5% CO_2 atmosphere. The cDNA clones of the rat ATP-receptor subunits $P2X_2$ and $P2X_3$ were kindly provided by Dr R. A. North (University of Sheffield, UK), the cDNA clone of the rat TRPM8-receptor (CMR1) was kindly provided by Dr D. Julius (University of California, San Francisco, USA).

Semiconfluent cells were transiently transfected in 35 mm dishes (Falcon, BD Biosciences, Heidelberg, Germany) by using the CaP-precipitation technique and the plasmids $pP2X_2$ (2 μg), $pP2X_3$ (10 μg) or pTRPM8 (8 μg), as described previously (Zufall *et al*., 1993).

Electrophysiological recordings

Recordings were made using the whole cell mode of the patch clamp technique. Cells were maintained in an extracellular recording solution consisting of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM HEPES (pH 7.4). Patch electrodes were pulled from borosilicate glass (1.2 mm O.D. × 1.17 mm I.D., Harvard apparatus, Edenbridge, Kent, UK) and fire polished to 6–8 MΩ tip resistance using a horizontal pipette puller (Zeitz Instr., Munich, Germany). The pipette solution contained 140 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES and 2 mM ATP, pH 7.4 for recordings from trigeminal neurons or 150 mM KF, 10 mM EGTA, 10 mM HEPES and 30 mM KCl, pH 7.4 for HEK293 cell recordings. Patch clamp recordings were carried out at room temperature using a HEKA EPC9 amplifier. Adjustment of the capacity and series resistance was made by using the built in compensation algorithm of the amplifier. Membrane potential was held at −60 mV. Data were acquired using Pulse software.

Drug application

Cells were exposed to drugs using a custom-made application system that could transiently superfuse all the cells in the field of view from one of five user-selected capillary tubes. A suction pipette is placed opposed to the capillary tubes and guaranties the complete removal of the applied drug solution avoiding contamination of the bath. Switching time between test solutions essentially was instantaneous, as was the delay to onset after switching due to the close proximity of the tube tips to the optical field. This drug application system was used for electrophysiological and imaging experiments (Spehr *et al*., 2002).

The stimuli benzaldehyde, citral, toluene, 3-phenylpropionaldehyde (3-PPA), 5-phenylvaleraldehyde (5-PVA), cyclohexanone, furfural and pyridine were dissolved in ethanol before being diluted to final concentration in extracellular solution. The resulting concentration of ethanol had no effect on the cells. The used odourant mixture Henkel50 was a gift from Henkel, Düsseldorf, Germany.

In some experiments the generation of action potentials was prevented by using the voltage activated sodium channel blocker QX 314 (2 nM, Tocris, Cologne, Germany) in the intracellular solution.

To test for the sensitivity of the trigeminal neurons to tetrodotoxin (TTX, Alomone Laboratories, Jerusalem, Israel) the ability to generate an action potential was examined before and during the application of the drug. Unless otherwise noted, all drugs were purchased from Sigma Chemical, Deisenhofen, Germany.

The data were tested for significance with Student's *t*-test.

Immunocytochemistry

For the immunohistochemical experiments cultured trigeminal neurons were washed with PBS and fixed with paraformaldehyde (4% in PBS, 60 °C) for 20 min. After fixation the cells were washed in PBS (3 × 5 min each) before exposure to a blocking solution containing 10% goat serum and 0.2% Triton in PBS for 1 h at 37 °C, followed by overnight incubation at 4 °C in a cocktail of the primary antisera. Afterwards cells were washed in PBS (5 × 5 min each) and then incubated with the appropriate secondary antibody for 30 min at 37 °C and again washed in PBS (5 × 5 min each). Primary and secondary antibodies were diluted in PBS with 0.2% Triton. Immunostaining against specific P2X receptor subunits was performed using primary antibodies: (i) anti-$P2X_2$ (1 : 800 dilution), a rabbit polyclonal antibody raised against a highly purified peptide corresponding to amino acid residues 457–472 of rat $P2X_2$ (Alomone Laboratories, Jerusalem, Israel); and (ii) anti-$P2X_3$ (1 : 500 dilution), a guinea pig polyclonal antiserum raised against amino acid residues 383–397 of rat $P2X_3$ (Neuromics, Minneapolis, MN, USA). The secondary antibodies were IgGAlexa Fluor® 488 goat anti-rabbit IgG and Alexa Fluor® 546 goat anti-guinea pig (1 : 1000 dilution, Molecular Probes, Leiden, the Netherlands). As a control, cultured neurons were incubated with

primary antibodies preabsorbed with their respective cognate peptide for one hour resulting in abolition of immunopositive cells.

Cells were imaged using a laser scanning confocal microscope (Zeiss LSM 510 meta, Zeiss, Jena, Germany). Images were obtained with an Achroplan 40×/0.8 W objective for reconstructing individual neurons and fibres. Alexa dye 546 was excited with the 543 nm line of an argon-laser and detected using a LP 560 nm emission filter. Alexa dye 488 was excited with the 488 nm line of an argon laser and detected using a BP 500–530 nm emission filter. We acquired z-series projections using Zeiss LSM software. All shown pictures (including the control pictures) were obtained with identical settings. The proportion of neurons showing specific immunoreactivity was determined by counting the cells in four defined sections of two dishes.

The representative picture of a trigeminal explant with outgrowing fibres was made from an animal of a mouseline with ubiquitous eGFP (enhanced green fluorescent protein) expression (Hadjantonakis *et al*., 1998). Images were obtained with the confocal microscope using 488 nm line of an argon-laser for excitation of the GFP. The fluorescence was detected using a LP 505 nm emission filter.

Imaging

For the calcium imaging experiments the cultured trigeminal neurons were loaded with the calcium sensitive dye FURA-2 AM (6 μM, Molecular Probes, Leiden, the Netherlands) for 60 min (at 37 °C gassed with 95% air and 5%CO_2). After washing, the dish was transferred to the stage of a Zeiss inverted microscope equipped for ratiometric imaging (Tillvision, Munich, Germany) and viewed with 5× and 32× magnification. All the cells in a field of vision were illuminated every second for 150 ms at 340 nm and 150 ms at 380 nm. The average pixel intensity within each region of interest was digitized and stored on a PC. The Ca^{2+}-dependent fluorescence signal was expressed as the f_{340}/f_{380} ratio and viewed as a function of time.

The same set up was used to examine putative ATP stores in olfactory receptor neurons using quinacrine (Sigma, Deisenhofen, Germany). Freshly dissociated cells were loaded with 5 μM quinacrine for 15 min. Fluorescence was detected after 150 ms pulses at 468 nm excitation using a 490–540 nm bandpass filter.

mRNA isolation and cDNA synthesis

Total RNA was isolated from collected gasserian ganglion or the olfactory epithelia of three adult rats by the TRIZOL reagent (Invitrogen, Carlsbad, CA). After DNaseI digestion, mRNA was isolated by oligo-dT coated paramagnetic particles (Dynal, Norway). cDNA was constructed by using the Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT_{12-18}) primer.

RT-PCR

A pair of sequence degenerated PCR primers,
P1: ATGGCITA(TC)GA(TC)(AC)GITA(TC)GTIGCIATITG
and P2: CCIATG(CT)TIAA(CT)CC(GC)TT(TC)ATITA,
(I = inositol) was derived from highly conserved regions of olfactory receptors (Buck & Axel, 1991). PCR-amplification was performed for 40 cycles (94 °C 1 min, 50 °C 1 min, 72 °C 1 min, 2.5 units of *Taq*-polymerase) with 100 pmols of P1 and P2 and ≈ 1 ng cDNA according to the manufacturer's recommendation (Invitrogen, Carlsbad, CA). The cDNA synthesis was checked by PCR detection of GAPDH cDNA using 30 pmols of the primers

P3: AGGGGCCATCCACAGTCTTCTG and
P4: CATCACCATCTTCCAGGAGCGA

and 30 cycles (94 °C 1 min, 60 °C 1 min, 72 °C 1 min, 2.5 units of *Taq*-polymerase).

Results

ATP induced currents

To investigate trigeminal chemoperception at a single cell level we used a culture system of the rat gasserian ganglion (Viana *et al*., 2001), allowing the survival of dissociated cells for up to one week. An electrophysiological classification of the P2X-receptor expression profile of trigeminal neurons was examined by ultrarapid application of various doses of ATP in whole-cell patch-clamp experiments.

Ninety eight per cent of all cells tested (230/234) were sensitive to ATP (30 μM). This concentration is within the physiological range as extracellular ATP degrades quickly *in vivo* (Cook *et al*., 1997). According to the characteristic kinetics of ATP evoked currents three different neuronal populations could be distinguished. These populations revealed no difference in ATP sensitivity and were therefore pooled for evaluation of dose–response correlation. The resulting curve revealed an EC_{50} of 30 ± 5.5 μM ATP and a Hill-coefficient of 0.8 ± 0.04. As not all concentration could be tested at each cell, the dose–response curve was composed of 74 experiments with a minimum of seven independent measurements for each concentration.

One prominent population of neurons (57%, 130/230) showed slowly activating inward currents (risetime: 84 ± 7 ms, time from 10 to 90% of peak amplitude), that weakly desensitized during 1 s application of ATP (30 μM) ('persistent current', Fig. 1a). The amplitude at the end of ATP application declined to 83 ± 3% of the initial peak amplitude ($n = 33$). Peak currents varied considerably from 30 to 1079 pA (average: 383 ± 52 pA) (Table 1).

The second type of neurons (18%, 42/230) showed phasic inward currents, which were characterized by a rapid current onset (33 ± 7 ms), amplitudes from 94 to 605 pA (average: 315 ± 37 pA) and fast desensitization with a biexponential kinetic ($\tau_1 = 48 \pm 10$ ms, $\tau_2 = 231 \pm 59$ ms). These currents desensitized almost completely to approximately 4 ± 1% of the peak amplitude during 1 s ATP application ($n = 17$) ('transient current', Fig. 1b). Both risetime and desensitization rate of this second neuronal population differed significantly from the first group ($P \leq 0.001$).

In the third group of neurons (25%, 58/230) the ATP-induced response consisted of two components. A fast and quickly desensitizing inward current was followed by a second component showing a sustained response in presence of ATP ('mixed current', Fig. 1c). Application of 30 μM ATP induced currents ranging from 107 to 620 pA (average: 335 ± 32 pA) with a risetime of 51 ± 11 ms. The amplitude of the nondesensitizing component at the end of 1 s ATP application was 52 ± 5% of the peak amplitude ($n = 25$). Therefore, the desensitization rate of these neurons significantly differed from the other groups of neurons ($P \leq 0.001$ for both). Furthermore, the risetime of mixed currents was significantly different from persistent currents observed in the first group of neurons ($P \leq 0.05$), but not from transient currents of the second group.

The three neuronal populations described above could also be distinguished in current clamp recordings. A one second application of ATP induced depolarization and a steady train of action potentials (APs) in neurons of the first group (Fig. 1d), which is related to the nondesensitizing current observed in voltage clamp recordings. However, neurons of the second population generated only a few APs at the beginning of ATP application and the membrane potential returned to resting values in presence of ATP (Fig. 1e). Current clamp recordings from members of the third neuronal population revealed a response pattern different from the other two groups. These neurons only responded with a few APs at the beginning of ATP application but showed a sustained depolarization in the presence of ATP (Fig. 1f).

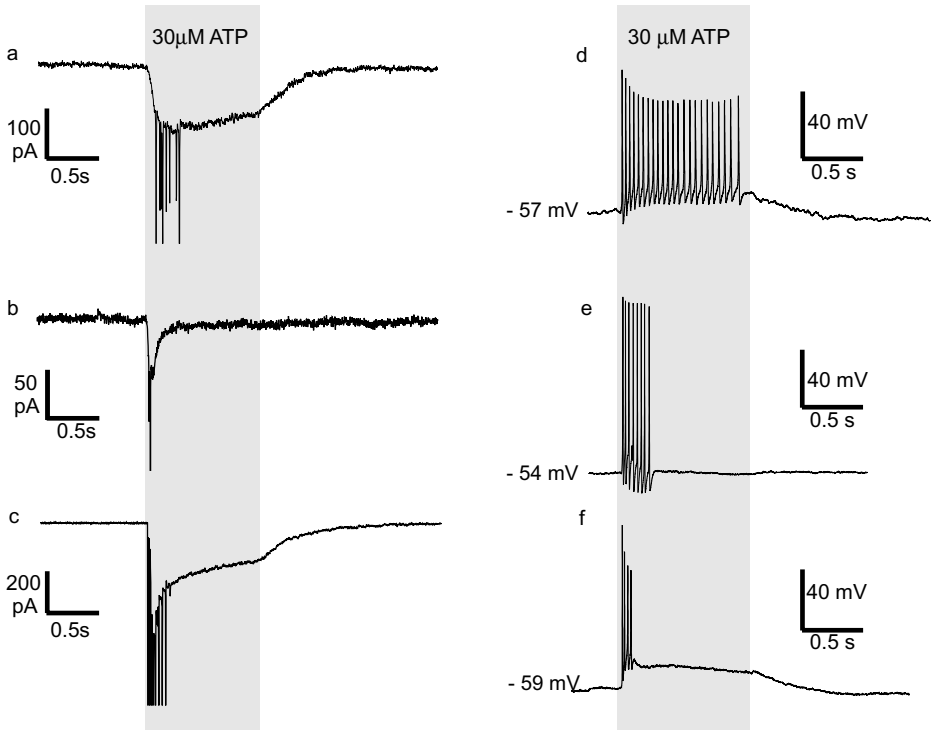

FIG. 1. Different ATP responses of cultured trigeminal neurons to ATP application. ATP (30 μM) was applied for 1 s. (a, b and c) Whole cell voltage clamp recordings, holding potential −60 mV. (d, e and f) Whole cell current clamp recordings, no current was injected, the resting membrane potential is indicated. (a and d) sustained current; (b and e) transient current; (c and f) mixed current.

Pharmacological characterization of different ATP responses

Subunit-specific pharmacological tools were used to investigate the subunit composition of P2X receptors expressed by the different populations of cultured trigeminal neurons (reviewed in Dunn *et al*., 2001).

Various P2X receptors differ in their sensitivity to the ATP analogue α,β methylene ATP (α,β meATP). Whereas α,β meATP activates homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors in very low concentrations (EC_{50} 1 μM and 9 μM, respectively) (Lewis *et al*., 1995; Robertson *et al*., 1996), homomeric $P2X_2$ receptors should not be activated by agonist at concentrations up to 100 μM (Dunn *et al*., 2001). Diinosine pentaphosphate (Ip_5I) is a potent antagonist at homomeric $P2X_3$ receptors (IC_{50} 3 μM), but should be ineffective at heteromeric $P2X_{2/3}$ receptors or homomeric $P2X_2$ receptors (King *et al*., 1999; Dunn *et al*., 2000). Finally, Cibacron blue potently inhibits an ATP-induced activation of homomeric $P2X_2$ receptors (IC_{50} 10 μM) (Zhong *et al*., 1998), whereas activation of homomeric $P2X_3$ receptors is potentiated (EC_{50} 10 μM) (Alexander *et al*., 1999). The effect of the drug on heteromeric $P2X_{2/3}$ receptors changes dose-dependently. Up to 30 μM cibacron blue enhances while higher concentrations antagonize the activation of $P2X_{2/3}$ receptors (Jarvis *et al*., 2001).

In the following experiments all neurons were stimulated with an interstimulus interval of at least 90 s, which has been tested to be sufficient for full recovery of the current.

We found that neurons showing the persistent ATP current could not be activated by low concentrations of α,β meATP (15 μM) ($n = 31$). Application of Ip_5I (50 μM, 60 s before and during ATP-application) had no effect, whereas application of cibacron blue (20 μM; 120 s before and during the ATP application) inhibits the persistent current (Fig. 2a). Lower concentrations of cibacron blue (10 μM) led to partial inhibition of the persistent current (data not shown). This pharmacological profile indicated the expression of homomeric $P2X_2$ receptors. The second group of neurons, which responds to ATP with transient currents, could be activated by low concentrations of α,β meATP (15 μM) and was blocked by the antagonist Ip_5I ($n = 8$, Fig. 2b). These currents were likely mediated by homomeric $P2X_3$ receptors. The third group's pharmacological profile revealed interesting properties. Low concentrations of α,β meATP (15 μM) activated only the rapid rising and fast desensitizing component of the mixed current and the antagonist Ip_5I blocked only this current component ($n = 13$, Fig. 2c). These properties are not in accordance with the pharmacological profile of heteromeric $P2X_{2/3}$ receptors, but indicate the expression of two separate populations of homomeric $P2X_2$ and homomeric $P2X_3$ receptors that were independently activated or inhibited.

TABLE 1. Properties of the 30 μM ATP-induced currents

	Risetime to peak (ms)	Desensitization rate (%) after 1 s	Peak amplitude (pA)
Persistent currents	84 ± 7	17 ± 3	383 ± 52 (range 30– 1079)
Transient currents	$33 \pm 7^{**}$	$96 \pm 1^{**}$	315 ± 37 (range 94– 605)
Mixed currents	$51 \pm 11^{*}$	$52 \pm 5^{**,\#\#}$	335 ± 35 (range 107– 620)

Holding potential −60 mV; mean ± SEM; $^{*}P \leq 0.05$ and $^{**}P \leq 0.001$, compared with persistent currents; $^{\#\#}P \leq 0.001$, compared with transient currents.

Immunostaining

We investigated the P2X-receptor subunit expression by immunostaining with specific antibodies against $P2X_2$ and $P2X_3$ receptor protein. A small amount of cultured trigeminal neurons (22%, 11/50) showed immunoreactivity for $P2X_3$ subunits on the somata and dendrites (red

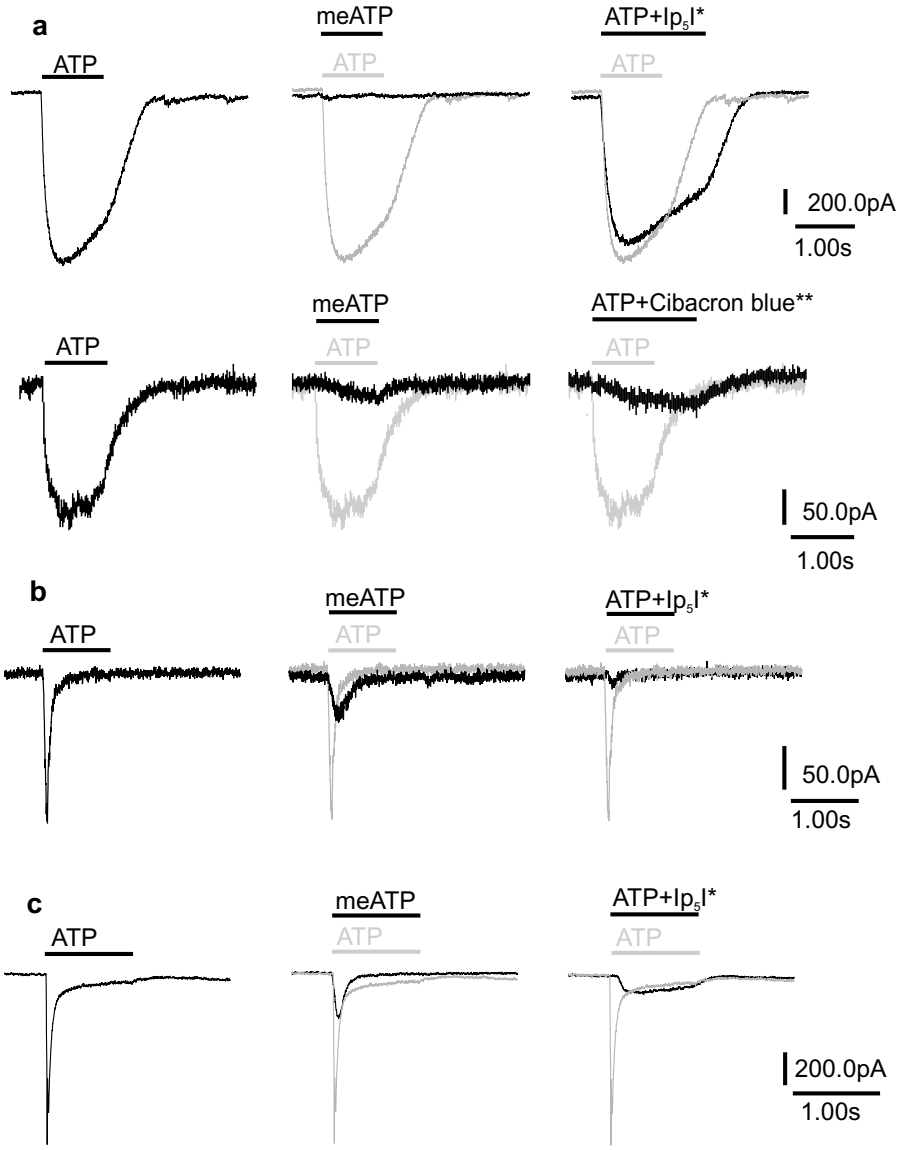

FIG. 2. Pharmacological identification of the P2X subunit composition of native P2X receptors. Applications were 30 μM ATP, 15 μM meATP, 50 μM Ip_5I + ATP and 20 μM cibacron blue + ATP, each for 1 s. (a, b and c) *60 s preincubation with Ip_5I before application of ATP + Ip_5I. (a) **120 s preincubation with cibacron blue before application of ATP + cibacron blue. (a) ATP induced sustained current, no effect of meATP or Ip_5I, but effective block of cibacron blue – pharmacological profile of homomeric $P2X_2$ receptors. (b) ATP induced transient current, meATP induced transient current, Ip_5I blocked the transient current – pharmacological profile of homomeric $P2X_3$ receptors. (c) ATP induced mixed current, meATP induced only the transient component of the mixed current, Ip_5I blocked only the transient component of the ATP induced transient current – pharmacological profile of homomeric $P2X_2$ and $P2X_3$ receptors.

staining) (Fig. 3a–c). Thirty six per cent (18/50) of the cells were immunopositive for both $P2X_2$ and $P2X_3$ subunits showing red and green (or yellow = merged) staining on the somata. The dendritic staining pattern revealed that both subunits, $P2X_2$ and $P2X_3$, are located on the neurites as well (Fig. 3d–f). The remaining neurons (42%, 21/50) were immunopositive only for the $P2X_2$ subunit. These immunocytochemical results correspond to the quantitative analysis of P2X subunit expression deduced from patch clamp experiments.

Functional proof of dendritic P2X expression

The spatial distribution of the P2X receptors could be important for their physiological function. Immunohistochemical studies (see above) revealed the existence of $P2X_3$ and $P2X_2$ receptor subunits on both somata and neurites. To investigate the functionality of dendritic P2X receptors we performed calcium imaging recordings from small, undissociated tissue-explants of the trigeminal ganglion. In cell culture these explants send out long neurites (Fig. 4a) allowing an exclusive stimulation of peripheral receptors. Membrane depolarization induced by distal P2X receptor activation should be transferred to the somata. Here, depolarization leads to an increase of the intracellular calcium concentration, which can be measured using calcium-sensitive dyes (fura-2).

We used focal application of ATP to stimulate the most distal parts of the trigeminal neurites (Fig. 4b). Diffusion of ATP to the somata in the explant could be excluded by an effective suction mechanism, which completely removed the applied ATP-solution (see Materials and methods). The focal ATP-application on distal neurites led to an increase of intracellular calcium in several somata located within the tissue-explants hundreds of micrometers away from the stimulation side (Fig. 4c). This indicates that beside their likely expression on the

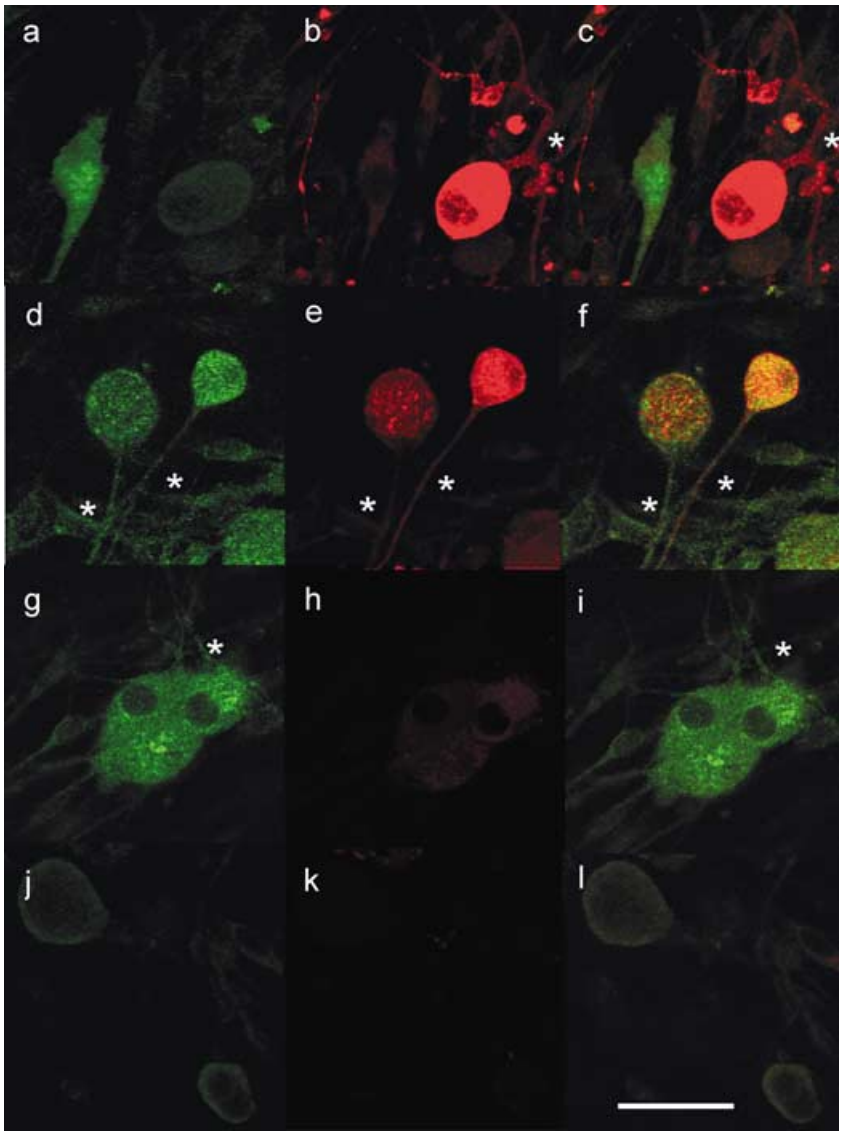

FIG. 3. Immunostaining of cultured trigeminal neurons (2 DIV) with antibodies against $P2X_2$ and $P2X_3$ receptor subunits. (a, d, g, and j) Staining against $P2X_2$ receptor subunits with Alexa dye 546 (green). (b e, h and k). Staining against $P2X_3$ receptor subunits with Alexa dye 488 (red). (c, f, i and l) Costaining against $P2X_2$ and $P2X_3$ receptor subunits (red and green). (a–c) Show a neuron only expressing $P2X_3$ receptor subunits, note the stained dendrites. (d–f) Show two neurons expressing $P2X_2$ and $P2X_3$ receptor subunits resulting in a red/green or yellow staining. The dendrite of one neuron is stained in red and green, the dendrite of the other neuron is only stained in green. (g–i) Show neurons only expressing $P2X_2$ receptor subunits (three neurons lying together). (j–l) Control staining, both primary antibodies were preabsorbed with specific blocking peptides. The scaling bar is the same for all pictures and represents 50 μm. The coimmunostaining reveals the existence of three different types of neurons. Note the specific staining of the dendrites (marked by *).

somata P2X-receptors are also functionally expressed on the distal ends of neurites.

Comparison nociceptors/non-nociceptors

To correlate the P2X subunit expression pattern with nociceptive or non-nociceptive function of the respective trigeminal neurons we used the presence of TTX-insensitive sodium currents as a marker for nociceptors (Pearce & Duchen, 1994; Djouhri *et al*., 1998; Lopez de Armentia *et al*., 2000). In whole cell patch clamp recordings 67% (69/103) of the neurons still generated APs in response to current injection in the presence of TTX (1 μM). Most of these neurons (87%) were pharmacologically characterized as expressing $P2X_3$ subunits (solely or in combination with $P2X_2$ subunits). In contrast, neurons showing $P2X_2$-mediated persistent currents (31/34, 91%) were TTX sensitive and might therefore possess non-nociceptive functions.

Modulation of ATP currents

The discovery of a new, non-nociceptive, $P2X_2$-expressing subpopulation of trigeminal neurons within the gasserian ganglion led to speculation about its physiological role. Therefore, we tested for the ability of known trigeminal stimuli to modulate ATP currents and revealed a subpopulation-specific effect of the odourant benzaldehyde, which is described as detectable by the trigeminal system (Doty *et al*., 1978; Bryant & Silver, 2000; Hummel *et al*., 2003). To examine, if olfactory receptors are involved in trigeminal odourant detection, we investigated their expression in the gasserian ganglion by RT-PCR assay. PCR with a sequence degenerated primer pair yielded no detectable PCR

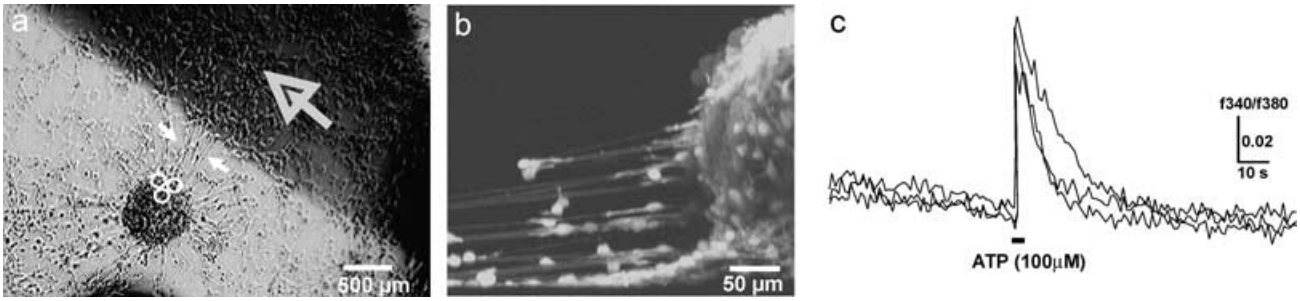

FIG. 4. Stimulation of trigeminal neurites with ATP. (a) Picture of a cultured trigeminal tissue sending out long neurites (see small arrows), the dark colouring in the upper right corner is the stained ATP solution superfusing only the neurites and not the cell somata located in the tissue. The ellipses mark the regions of interest marked for the Ca-imaging experiments. (b) A 3D reconstruction (projection for z-stack) of a GFP-mouse trigeminal explant with neuritis protruding outwards at various degrees from the source tissue. Only the end of the neurites sticks to the ground. The small cells attached to the neurites seem to be glia cells. This confocal picture is only shown for clarity; experiments were made with rat tissues at the Ca-imaging system as described in the Materials and methods. (c) Kinetic of the relative changes of the calcium concentration in the tissue induced by ATP stimulation of neurites. Data are f_{340}/f_{380} ratios.

product with ganglion cDNA but a strong band with cDNA from the olfactory epithelium (Fig. 5a).

Benzaldehyde (up to 4.5 mM) did not affect cultured trigeminal neurons directly, either in patch clamp ($n = 15$) or in calcium-imaging experiments ($n = 16$).

Interestingly, coapplication of benzaldehyde and ATP revealed a modulatory effect on ATP-induced currents. Further investigations found this benzaldehyde effect to be specific for nondesensitizing $P2X_2$-receptor mediated currents. Coapplication of benzaldehyde (2.25 mM) and ATP (10 µM) reduced the peak amplitude significantly

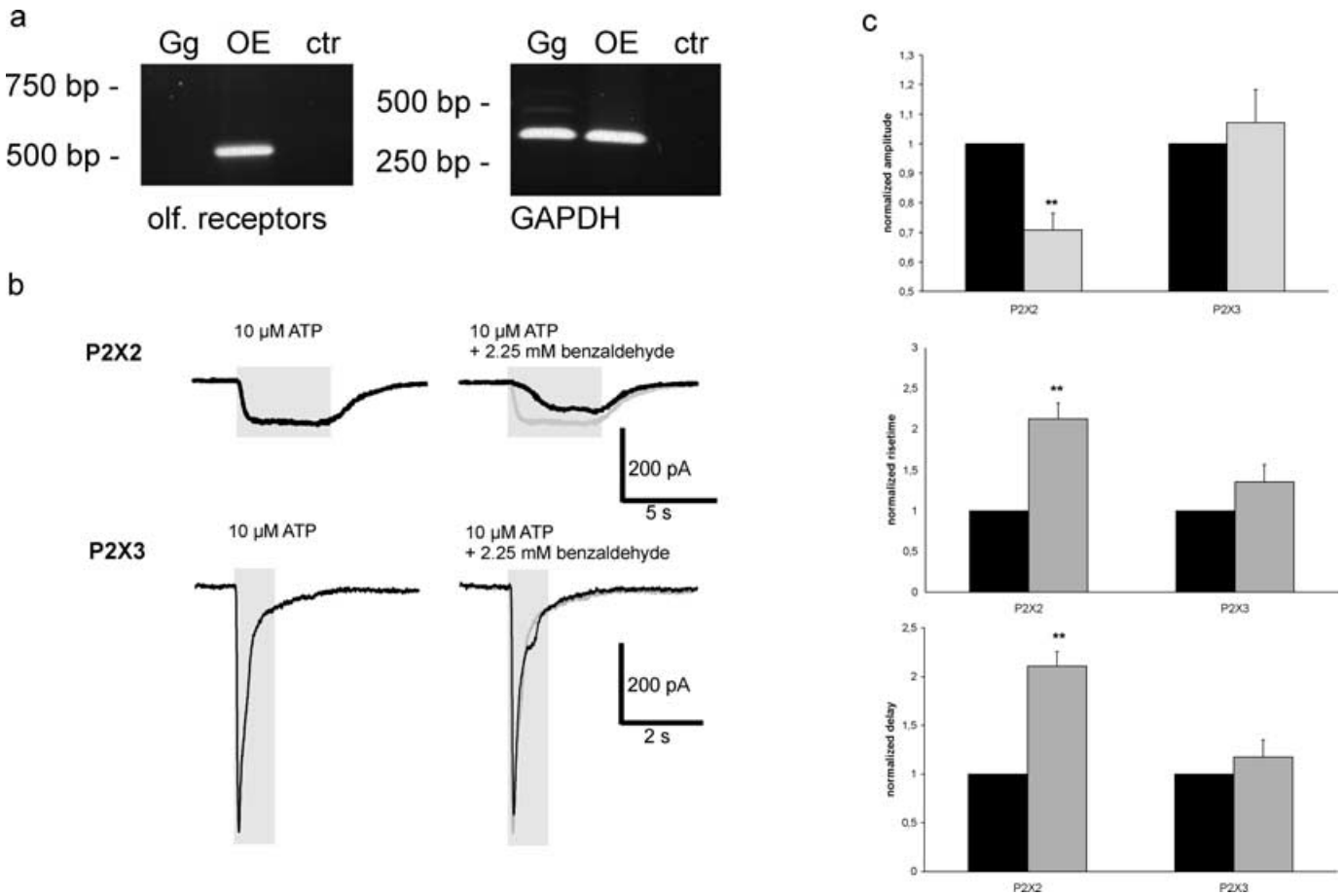

FIG. 5. Mechanisms of benzaldehyde detection. (a) PCR detection of odourant receptor expression in the trigeminal ganglion and the olfactory epithelium. PCR with a pair of sequence degenerated primers derived from conserved regions of odourant receptor proteins detected transcripts in RNA of the olfactory epithelium but not in the trigeminal ganglion of rats. The quality of cDNA synthesis was controlled by detection of GADPH cDNA (glyceraldehyde-3-phosphate dehydrogenase). (Gg, gasserian ganglion; OE, olfactory epithelium; ctr, water control). (b and c) Different effects of benzaldehyde on $P2X_2$ and $P2X_3$-receptor mediated currents. (b) Representative currents mediated by heterologously expressed $P2X_2$ and $P2X_3$ receptors. $P2X_2$ receptor mediated currents were modulated, while $P2X_3$ receptor mediated currents were unaffected by benzaldehyde. (Whole-cell recordings, holding potential −60 mV.). (c) Comparison of benzaldehyde modulation on $P2X_2$ and $P2X_3$ receptor mediated currents. Maximal amplitude, risetime and delay were normalized to the ATP induced response at each receptor type. Each property was significantly altered by benzaldehyde regarding $P2X_2$ receptors, no significant change could be observed in $P2X_3$ receptor mediated currents (mean ± SEM, $^{**}P \leq 0.001$).

to 63 ± 11% compared to the response to ATP alone ($P = 0.05$, $n = 10$). The risetime (time from 10% to 90% of peak amplitude) and the delay (time from the start of application to 50% of peak amplitude) of the currents were also modulated. They significantly increased to 293 ± 71% and 257 ± 37%, respectively ($P = 0.05$, $n = 10$ for both).

We also tested for an effect of benzaldehyde preincubation (30 s, 2.25 mM) on ATP currents. This procedure further decreased the peak amplitude significantly to 34 ± 8% ($P = 0.001$, $n = 10$) and enhanced the risetime significantly to 385 ± 88% ($P = 0.05$, $n = 10$). Prolonged preincubation did not lead to further augmentation of the effects. The delay was not significantly changed by benzaldehyde preincubation.

Fast desensitizing $P2X_3$-receptor currents were not affected by benzaldehyde, regardless of current induction by ATP or α,β meATP (each 10 μM). Preincubation did not have an effect either (data not shown).

A possible direct effect of benzaldehyde on $P2X_2$-receptors was tested in a recombinant expression system (HEK293 cells). Benzaldehyde alone (up to 4.5 mM) did not induce a detectable response in

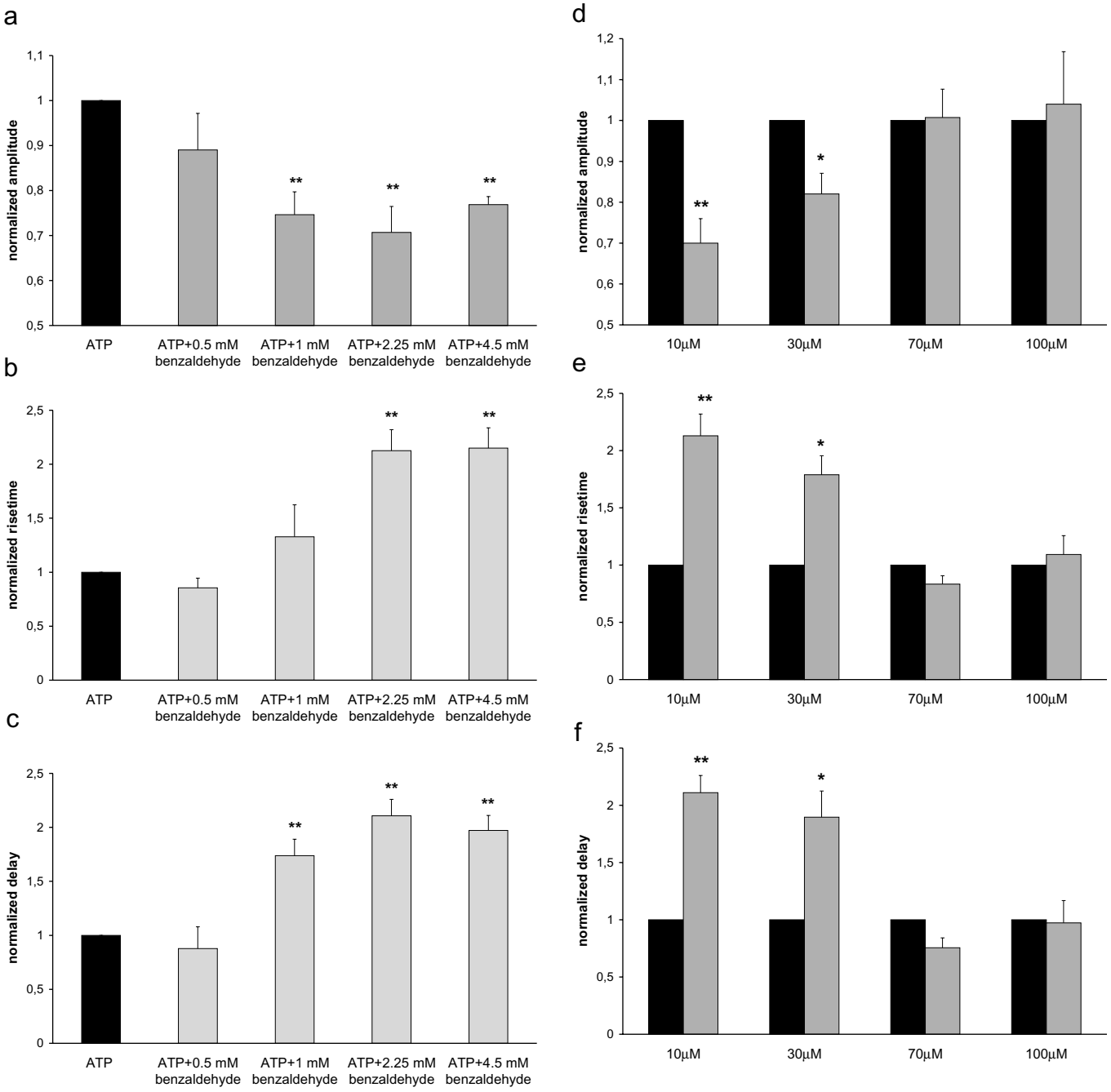

FIG. 6. Concentration dependency of the $P2X_2$-receptor modulation. (a–c) Dose-dependency of the benzaldehyde modulation of ATP induced currents. Peak amplitude and delay were affected by concentrations of 1 mM benzaldehyde or higher, while the risetime was only affected by 2.25 mM benzaldehyde or higher. (d–f) ATP concentration dependent modulation of heterologously expressed receptors by benzaldehyde: peak amplitude, risetime and delay of the ATP induced response. Data are normalized to the relative ATP induced response (black bars). The modulatory effect of benzaldehyde is abolished by the increase in ATP concentration to or higher than 70 μM ATP, where the maximal response is elicited. All data are mean ± SEM, $^{*}P \leq 0.05$, $^{**}P \leq 0.001$.

TABLE 2. Benzaldehyde modulates $P2X_2$-receptor mediated currents

	n	Maximum amplitude (%)	Risetime (%)	Delay (%)
10 μM ATP + 0.5 mM benzaldehyde	8	89 ± 8	86 ± 9	88 ± 20
10 μM ATP + 1.0 mM benzaldehyde	11	75 ± 5**	132 ± 30	173 ± 15**
10 μM ATP + 2.25 mM benzaldehyde	36	70 ± 6**	213 ± 19**	211 ± 15**
10 μM ATP + 4.5 mM benzaldehyde	13	77 ± 2**	215 ± 18**	197 ± 14**
2.25 mM benzaldehyde + 10 μM ATP	36	70 ± 6**	213 ± 19**	211 ± 5**
2.25 mM benzaldehyde + 30 μM ATP	8	82 ± 5*	178 ± 17*	189 ± 23*
2.25 mM benzaldehyde + 70 μM ATP	9	100 ± 7	83 ± 7	76 ± 8
2.25 mM benzaldehyde + 100 μM ATP	7	103 ± 13	109 ± 16	97 ± 19

Data were normalized to the response induced by ATP application, different benzaldehyde and different ATP concentrations were tested (mean ± SEM, $^{*}P \leq 0.05$, $^{**}P \leq 0.001$).

whole cell patch clamp experiments. However, coapplication of benzaldehyde (2.25 mM) and ATP (10 μM), which is the described half-maximally activating ATP concentration (EC_{50}) for recombinant $P2X_2$-receptors (Spelta *et al*., 2003), led to significant modulations of the ATP currents, similar to the effect described in trigeminal neurons (Fig. 5a). Benzaldehyde significantly decreased the peak amplitude to 70 ± 6% and increased the risetime to 213 ± 19% ($P = 0.001$, $n = 8$ for both). The delay of the ATP induced response is also significantly prolonged to 211 ± 15% by benzaldehyde ($P = 0.001$, $n = 8$) (Fig. 5a). All effects recovered after washing.

To verify the receptor specificity of the inhibitory benzaldehyde effect, we investigated heterologously expressed $P2X_3$-receptors. ATP-induced currents mediated by $P2X_3$-receptors were not significantly altered by coapplication of ATP (10 μM) and benzaldehyde (2.25 mM) (Fig. 5a and b). Moreover, activation of recombinantly expressed TRPM8 (100 μM menthol; McKemy *et al*., 2002), another ligand-gated ion channel found in trigeminal neurons, was not modulated by benzaldehyde either (data not shown). These results indicate the specificity of benzaldehyde modulation at $P2X_2$-receptors.

The investigation of the dose–response relationship of the inhibitory benzaldehyde effect on heterologously expressed $P2X_2$-receptors revealed a steep correlation: the peak amplitude and the delay of the ATP induced current was not affected at 0.5 mM benzaldehyde but maximally affected at 2.25 mM. The effect on the risetime of the current was even more confined with no effect at 1 mM and a maximal effect at 2.25 mM benzaldehyde (Fig. 6a–c, Table 2).

To elucidate the pharmacological mechanism by which benzaldehyde modulates $P2X_2$-mediated currents, we coapplied various ATP concentrations with a saturating concentration of benzaldehyde (2.25 mM) (Fig. 6d–f, Table 2). The modulatory affects of benzaldehyde on the peak amplitude, the risetime and the delay of the ATP induced currents are decreased by ATP concentrations higher than 10 μM. Significant benzaldehyde effects could be observed at currents induced by 10 and 30 μM ATP ($P = 0.001$ and $P = 0.05$, respectively). Stimulation with ATP concentrations higher than 70 μM (≥ maximal concentration) prevented the benzaldehyde effect, indicating a competitive mechanism.

To investigate further the specificity of the measured effect on $P2X_2$-receptors we also tested other chemical trigeminal and olfactory stimuli structurally related to benzaldehyde (aldehyde-group connected to an aromatic ring structure) by coapplication with ATP (10 μM) (Fig. 7). As previously described for benzaldehyde, possible effects were examined regarding the maximal amplitude (Fig. 7a), the risetime (Fig. 7b) and the delay (Fig. 7c) of the induced currents. Shortening the side chain at the aromatic ring to a methyl-group (toluene) was tolerated. Toluene (2 mM) showed a similar effect as benzaldehyde ($n = 17$). Addition of a methyl-group to the side chain was also tolerated, the resulting ketone acetophenone (2 mM) was effective as well ($n = 17$). We then tested the impact of elongating the carbon chain. An aldehyde-group connected to the aromatic ring via a carbon chain of three carbons, 3-phenylpropionaldehyde (3-PPA, 2 mM), still showed a benzaldehyde-like effect ($n = 9$), whereas further elongation to five carbons, 5-phenylvaleraldehyde (5-PVA, 3 mM), led to an inactive compound ($n = 12$). Though slight modulations at the side chain of the aromatic ring were tolerated, modulations concerning the ring structure were not. Citral (3 mM), an aldehyde lacking the aromatic ring, had no effect, indicating the significance of the ring structure ($n = 7$). Substitution of one carbon within the aromatic ring by nitrogen, pyridin (3 mM), also abolished the effect ($n = 7$). The nonaromatic ketone cyclohexanone (2.5 mM) had no effect either ($n = 15$). The same ineffectiveness could be observed for furfural (2.5 mM), a cyclic structure of five carbons with one carbon substituted by an oxygen and an aldehyde-group connected to the ring ($n = 7$). None of the tested effective chemicals had a direct measurable effect on trigeminal neurons (patch clamp and calcium-imaging experiments, $n = 10$ and $n = 14$, respectively). These results indicated that a small group of closely structurally related trigeminal olfactory stimuli have the same modulatory effect on $P2X_2$-receptors and thereby on the activity of expressing neurons.

A possible source of ATP in the nasal epithelium

In the nasal epithelium, the source of extracellular ATP acting on trigeminal P2X receptors is not known. It is well established that ATP can be released by many neuronal and non-neuronal cells (reviewed in Bodin & Burnstock, 2001b). To test the hypothesis that ATP is released from activated ORNs we used the fluorescent acridine derivative quinacrine. Its binding affinity to adenine nucleotides in general and especially to ATP make it a preferred marker for intracellular ATP stores (White *et al*., 1995; Mitchell *et al*., 1998; Bodin & Burnstock, 2001a; Sorensen & Novak, 2001). Quinacrine labels putative purinergic central and peripheral neurons, which presumably store the dye because of a high vesicular content of ATP. Thus, quinacrine allows for investigations at the single cell level.

Freshly dissociated ORN quickly accumulated quinacrine and displayed a green fluorescence, which was stable over time in unstimulated ORN (Fig. 8a, grey line). It has been reported that membrane depolarization by high potassium concentrations promoted ATP release from neurons (White, 1978). Therefore, we chose a highly concentrated potassium solution (50 mM K^+) as a reference stimulus. Alternatively, we stimulated the ORN with a mixture of 50 different odourants (Henkel 50, 1 : 5000) or the specific adenylyl cyclase activator forskolin (50 μM) to mimic odour stimulation (Spehr *et al*., 2002; Ma *et al*., 2003). In all tested ORNs ($n = 15$) stimulation with 50 mM K^+ led to a strong decrease of fluorescence intensity (Fig. 8b), possibly due to ATP release. This robust fluorescence reduction was individually used as the standard value (100%) for normalization. In all cells

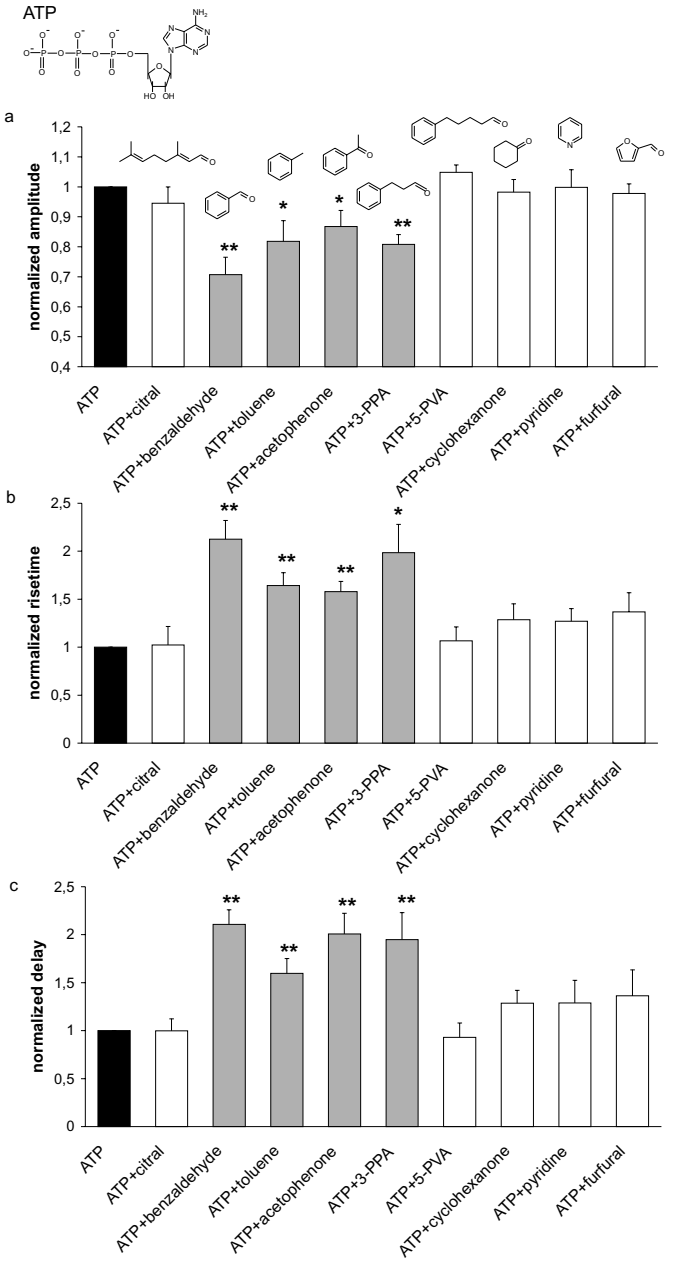

FIG. 7. Effect of structural modifications on the benzaldehyde-effect. (a) Amplitude; (b) risetime; (c) delay of the induced currents. Currents induced by coapplication of ATP, various odourants were normalized to ATP induced responses (mean ± SEM, $^{*}P \leq 0.05$, $^{**}P \leq 0.001$).The aromatic ring structure seems to be of central importance, slight modulations of the side chain were tolerated, those odourants displayed the same effect as benzaldehyde. Modulations of the aromatic ring structure abolished the effect.

tested ($n = 10$), forskolin also induced a significant decrease in fluorescence intensity ($57 \pm 22\%$ of the K^+ induced reduction). As expected, the odourant mixture Henkel 50 only induced a decrease in fluorescence intensity in a subset of cells due to the diversity of the expressed olfactory receptors. Eight out of 15 cells tested showed a significant decrease, which reached $34 \pm 22\%$ of the K^+ induced reduction. Fluorescence intensity in the remaining seven cells was unchanged in response to Henkel 50. However, these cells showed a significant reduction in the presence of forskolin and K^+, indicating the ability of the neurons to respond and thereby elucidating the specificity of the induced decrease of fluorescence intensity.

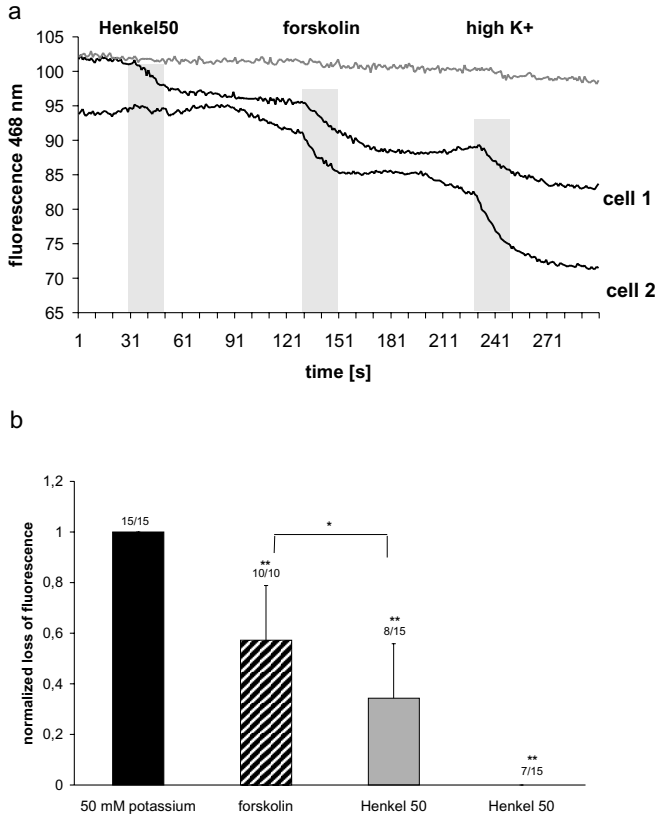

FIG. 8. Stimulus induced ATP release from ORN. (a) Representative data of two different ORN, the reduction of the fluorescence after stimulation could be seen in the life kinetic. Cell 1 lost fluorescence after each stimulation, while cell 2 lost no fluorescence after odour stimulation. (b) comparison of the fluorescence reduction to each stimuli. Data were normalized to the reduction after high potassium stimulation (mean ± SEM, $^{**}P \leq 0.001$).

Discussion

In the present study, we investigated functional P2X receptor expression in cultured dissociated trigeminal neurons from neonatal rats by means of electrophysiological recordings, immunohistochemistry and ratiofluometric imaging. Based on the kinetics of ATP-induced currents we identified three populations of cultured neurons. One population responded to ATP application with rapidly desensitizing currents, which were very similar to $P2X_3$ receptor responses seen in heterologous expression systems (Cook *et al*., 1998). The pharmacological profile of these receptors indicates that these currents are mediated by homomeric $P2X_3$ receptors. King *et al*. (1999) reported that homomeric $P2X_1$ receptor mediated currents also reveal a similar kinetic and pharmacological profile. However, $P2X_1$ receptor mediated currents show a single exponential desensitization kinetic (Collo *et al*., 1996), while the desensitization kinetic of the ATP-induced currents measured in this work could be fitted biexponentially as reported typically for $P2X_3$ receptor mediated currents (Lewis *et al*., 1995). Additionally, expression of homomeric $P2X_1$ receptors in trigeminal neurons is unlikely, because only low mRNA levels (in comparison with $P2X_3$ mRNA) and no immunoreactivity of $P2X_1$ receptors have been found in trigeminal ganglion neurons (Collo *et al*., 1996; Cook *et al*., 1997; Xiang *et al*., 1998).

We also tested for the functional expression of $P2X_2$ subunits in the cultured trigeminal neurons. The pharmacological profile of neurons responding to application of ATP with a nondesensitizing current

showed that these neurons express $P2X_2$ subunits and build homomeric $P2X_2$ receptors.

The third population of cultured trigeminal neurons responded with mixed currents to the application of ATP. The fast desensitizing component of these currents revealed the same pharmacological properties as currents induced by ATP activation of homomeric $P2X_3$ receptors, while the nondesensitizing component equalled the properties of $P2X_2$ receptor mediated currents. Most probably, these neurons express distinct populations of homooligomeric $P2X_2$ and $P2X_3$ receptors. An expression of multiple P2X receptor subtypes in one neuron resulting in a mixed current was reported for DRG neurons and in heterologous expression systems (Burgard *et al*., 1999; Grubb & Evans, 1999; Dunn *et al*., 2000).

None of the investigated cultured trigeminal neurons revealed a pharmacological profile fitting heteromeric $P2X_{2/3}$ receptors, although their occurrence has been suggested for nociceptive trigeminal fibres innervating the teeth (Cook *et al*., 1997). The reason might be a low proportion of these neurons in the trigeminal ganglion. Thus, in a culture preparation from the whole ganglion these few cells might have escaped the investigations.

In our experiments all three populations have the same EC_{50} value for ATP, although there are different EC_{50} values reported for $P2X_2$ and $P2X_3$ receptors (Ueno *et al*., 1999; Liu *et al*., 2001). Especially for $P2X_3$ receptors, the measured EC_{50} values are higher than previously reported. This might be due to our application system, which assures an instant complete removal of the ATP solution without contamination of the bath solution. This might be of certain relevance especially for examination of the fast desensitizing $P2X_3$ receptor and might also be the reason for the relative shortness of the required interstimulus intervals of 90 s. P2X receptors display a wide range of sensitivities depending on the expression site and other laboratories reported even higher EC_{50} values for $P2X_2$ receptors in other types of neurons (Zhong *et al*., 1998; Ueno *et al*., 1999; Zhong *et al*., 2000).

Electrophysiological results were supported by immunohistochemical studies using specific antibodies against $P2X_3$ and $P2X_2$ receptor subunits. We identified neurons that were immunopositive only for $P2X_3$ receptor protein on somata and neurites. This is in agreement with other groups, who recently described $P2X_3$ receptor immunoreactivity (IR) occurring in slice preparations of trigeminal ganglia (Cook *et al*., 1997; Llewellyn-Smith & Burnstock, 1998). These authors also report immunostaining on nerve cell bodies and their processes, which indicates that the cellular distribution of expressed receptors *in vivo* and *in vitro* is similar.

Our immunohistochemical experiments further revealed that a subpopulation of cultured trigeminal neurons is indeed immunoreactive for both, $P2X_3$ and $P2X_2$ receptor subunits. However it was not possible to identify the exact receptor subunit assembly from these studies.

Interestingly, we also found a population of neurons that was immunopositive exclusively for $P2X_2$ subunits. The immunohistochemical studies therefore support the existence of trigeminal neurons expressing $P2X_2$ subunits with no or undetectable of $P2X_3$ subunits.

The physiological function of sensory neurons is determined by the spatial distribution of the expressed receptors. Our immunohistochemical results indicate that both $P2X_2$ and $P2X_3$ receptor subunits are located on somata as well as neurites of cultured trigeminal neurons. Recent work revealed the occurrence of $P2X_2$-subunit mRNA in the olfactory epithelium by RT-PCR analysis (Hegg *et al*., 2003). The authors were unable to detect cellular $P2X_2$-IR in the sliced epithelium, but they found a punctate staining on the blood vessels below. Problems in detecting $P2X_2$-IR could be due to the small diameter of the fine trigeminal nerve endings within the epithelium (0.25 μm, Finger *et al*., 2003) and the potential low level of expressed receptors on these endings. The punctate staining of the blood vessels could be of trigeminal origin, because the fibres also wrap around the blood vessels (Silver & Finger, 1991).

Our calcium imaging investigations revealed that neurons were activated by stimulation of the distal parts of the neurites. The calcium increase in the somata could be explained by the activation of peripheral P2X receptors by ATP leading to a depolarization of the fibre. The signal is then actively propagated towards the somata (Gover *et al*., 2003) and activates voltage-gated calcium channels allowing calcium influx. The increase of the intracellular calcium concentration in cell somata following ATP stimulation of distal parts of neurites provides evidence for the functional expression of P2X receptors on dendritic ends. The expression of G protein-coupled P2Y receptors in trigeminal neurons of rodents is still controversially discussed (Ruan & Burnstock, 2003; Weick *et al*., 2003). The involvement of probably expressed P2Y receptors in trigeminal neurites cannot be fully excluded, but appear unlikely, as the pharmacological inhibition of ionotropic P2X receptors completely eliminated ATP-induced currents in our patch-clamp experiments. It is unclear how a possible G protein-mediated calcium increase in distal parts of neurites could then be transmitted to the somata.

Consequently, our electrophysiological, immunohistochemical and optical imaging data suggest the existence of a previously undescribed subpopulation of trigeminal neurons, whose ATP sensitivity is mediated by homomeric $P2X_2$ receptors located on somata and peripheral nerve fibres.

Cook and colleagues demonstrated that nociceptive trigeminal neurons innervating tooth pulps exhibit currents that are probably carried by homomeric $P2X_3$ or heteromeric $P2X_{2/3}$ receptors (Cook *et al*., 1997). We identified nociceptive neurons in culture by their described TTX-insensitivity (Pearce & Duchen, 1994; Djouhri *et al*., 1998; Lopez de Armentia *et al*., 2000). In accordance with Cook and colleagues nearly all TTX-insensitive neurons in our culture system exhibited pharmacological properties of $P2X_3$-receptor subunits. In contrast, almost all neurons solely expressing $P2X_2$ receptor subunit were TTX sensitive. Therefore, we suggest that these neurons do not primarily have nociceptive function. This has already been discussed for dorsal root ganglion neurons, where neurons expressing $P2X_2$-receptor subunits are thought not to be related to nociception (Ueno *et al*., 1999).

Here, we were able to show a possible involvement of the $P2X_2$ receptor expressing subpopulation in the detection of a certain group of trigeminal and olfactory stimuli. Coapplication of ATP and benzaldehyde or structurally related chemicals specifically modulated $P2X_2$ receptors in a concentration-dependent manner. The concurrent decrease of the maximal amplitude, the increase in risetime and the increased delay of the induced current lead to an overall reduction of the signal. Such a mechanism could work as a low pass filter and could decrease or even inhibit response to short ATP signals.

The aromatic ring structure seems to be a basic requirement for the effect, small changes in the side chain being tolerated. By reducing ATP induced currents, the modulated activity pattern of these fibres could contribute to the central recognition of this group of odourants. The absence of olfactory receptors that might detect odourants as well as the lack of any detectable direct effect of benzaldehyde, toluene, acetophenone or 3-PPA on cultured trigeminal neurons underlines the importance of the described modulatory mechanism.

The used concentrations of ATP (10 μM) and odourants (2–3 mM) are in the physiological range. Extracellular ATP is quickly degraded by ectonucleotidases, the extracellular concentration is therefore much lower than inside cells, where it can be found in the millimolar range

(Sperlagh & Vizi, 1996; Cook *et al*., 1997). The psychometric detection threshold of the trigeminal system for odourant molecules like benzaldehyde is much higher than the threshold of the olfactory system (Cometto-Muniz *et al*., 1997, 1998a, 1998b, 1998c). Therefore, stimulus concentrations in the millimolar range are required for trigeminal chemodetection (Hummel *et al*., 1992).

The modulatory effect of benzaldehyde depends on ATP concentrations. In combination with the increased risetime, this strongly suggests a competitive mechanism at the ATP binding site of $P2X_2$-receptors (Clements & Westbrook, 1994). This competitive block is subunit-dependent, because $P2X_3$-receptor subunits are not affected by benzaldehyde. The lack of a benzaldehyde effect on $P2X_3$-receptors excludes a direct chemical interaction of ATP and benzaldehyde.

If modulation of $P2X_2$-receptor currents by benzaldehyde or related odourants is part of trigeminal odourant detection, ATP release in the vicinity of trigeminal fibres is a crucial prerequisite. A close spatial relationship between ORN and trigeminal fibres has been postulated (Silverman & Krüger, 1989; Biffo *et al*., 1990; Finger *et al*., 1990; Silver & Finger, 1991). Therefore, ORNs could be one possible source of ATP in the olfactory epithelium. We used the fluorescent dye quinacrine in our ATP studies, as quinacrine specifically binds to ATP accumulated in vesicles (Bodin & Burnstock, 2001a). The most common method to measure ATP release is the determination of the ATP amount in the supernatant after stimulation. Such measurement of ATP release bears considerable risks, because it is almost impossible to distinguish between stimulated ATP release and ATP release by cell lysis (Sorensen & Novak, 2001).

Stimulation of freshly dissociated ORN with a complex odourant mixture led to a significant decrease of quinacrine fluorescence in eight out of 15 cells, probably due to stimulus-dependent ATP release. The loss of quinacrine fluorescence as indicator for ATP release has already been used for other cell types (Bodin & Burnstock, 2001a; Sorensen & Novak, 2001; Knight *et al*., 2002). Cells showing no reduction in fluorescence likely expressed olfactory receptors that were not activated by the chosen odourant mixture. By-passing the receptor by activation of the cyclic nucleotide pathway with forskolin (Spehr *et al*., 2002) also reduced fluorescence in these neurons, indicating the ability of ORN to release ATP subsequent to odour stimulation. The exact mechanism of ATP release by ORN remains unclear. Several cases of intracellular ATP release are described (vesicular release, carrier-mediated release via ABC-proteins (ATP-binding cassette proteins), cytolysis), but the precise mechanisms are still subject to debate (reviewed in Bodin & Burnstock, 2001b).

Other sources for ATP in the nasal epithelium are also possible. Hegg *et al*. (2003) suggested a constant low level of ATP in the olfactory epithelium, which could induce a tonic stimulation of trigeminal fibres. Moreover, stimulation of sympathetic nerve fibres innervating the nasal epithelium could increase the extracellular ATP concentration by corelease of ATP and acetylcholine (Liang & Vizi, 1997). Stimulus induced ATP release has been also discussed for trigeminal fibres (Finger *et al*., 1990; Getchell & Getchell, 1992), giving rise to the possibility of an autocrine activation mechanism. A further possibility could be slight cell damage by highly concentrated trigeminal stimuli resulting in ATP release. In the respiratory epithelium Finger *et al*. (2003) found solitary chemoreceptor cells innervated by trigeminal nerve fibres. The transmitter used for synaptic transmission is still unclear. Expressing many molecules reminiscent of the bitter taste transduction cascade, these cells might also use ATP as transmitter as it has been proposed for taste buds (Bo *et al*., 1999).

The trigeminal system is known to transduce different sensory modalities and contributes to the perception of mechanical, thermal and chemical stimuli. This work presents for the first time a molecular mechanism used for the recognition of benzaldehyde and structurally related chemicals by trigeminal neurons. Interestingly, this small group of chemicals affects the neurons indirectly by blocking ATP activated $P2X_2$ channels. A prerequisite for such a mechanism is either the permanent presence of extracellular ATP in the mucosa or stimulus-induced ATP release, e.g. by activated ORNs. ATP would then lead to a tonic activation of $P2X_2$ receptors driving the activity of trigeminal neurons. Under such conditions the block of ATP-induced currents would lead to modulation of the AP frequency, which can be analysed in higher levels of the central nervous system and could contribute to odourant perception.

In general, perception of chemical stimuli is very complex. The vertebrate nose utilizes two systems for detection of volatile stimuli: the olfactory and the trigeminal system. While the molecular detection mechanisms used by olfactory neurons have been extensively studied in the last decade, the mechanisms underlying trigeminal chemoperception remain widely unclear. In this work we could show that trigeminal neurons do not express typical odourant receptors as used by olfactory neurons to detect odourant molecules. Instead we provide a specific mechanism that involves modulation of $P2X_2$-receptors by benzaldehyde and chemically related odourants and that could be relevant for trigeminal perception of these molecules. Alternative detection mechanisms different from those of olfactory neurons have already been reported for other odourants: eugenol is shown to stimulate trigeminal neurons via activation of the previously described heat- and capsaicin-receptor TRPV1 (Yang *et al*., 2003), and menthol and eucalyptol directly activate the trigeminal cold- and menthol-receptor TRPM8 (McKemy *et al*., 2002).

These findings imply that trigeminal neurons are equipped with a repertoire of receptors and ion channels that fulfil multiple tasks affecting different sensory modalities. Therefore odourants can modulate excitability of trigeminal neurons by directly activating or modulating receptors displaying sensitivity to e.g. temperature or ATP.

Acknowledgements

We thank Dr G. Gisselmann for support in molecular biology and Mr H. Bartel, Mrs A. Stoeck, Mrs J. Zwoycyk for technical assistance. We are grateful to Dr R. A. North (Sheffield, UK) for providing the cDNA clone of the $P2X_2$- and $P2X_3$-receptor subunits, to Dr D. Julius (San Francisco, USA) for providing the cDNA clone of the TRPM8-receptor. The work was supported by the Deutsche Forschungsgemeinschaft.

Abbreviations

AP, action potential; ATP, adenosine triphosphate; α,β, me ATP, α,β methylene ATP; Ip_5I, diinosine pentaphosphate; ORN, olfactory receptor neuron; PBS, phosphate buffered saline; TRP, transient receptor potential channel; TTX, tetrodotoxin; 3-PPA, 3-phenylpropionaldehyde; 5-PVA, 5-phenylvaleraldehyde.

References

Alexander, K., Niforatos, W., Bianchi, B., Burgard, E.C., Lynch, K.J., Kowaluk, E.A., Jarvis, M.F. & van Biesen, T. (1999) Allosteric modulation and accelerated resensitization of human P2X(3) receptors by cibacron blue. *J. Pharmacol. Expl. Ther.*, **291**, 1135–1142.

Biffo, S., DeLucia, R., Mulatero, B., Margolis, F. & Fasolo, A. (1990) Carnosine-, calcitonin gene-related peptide- and tyrosine hydroxylase-immunoreactivity in the mouse olfactory bulb following peripheral denervation. *Brain Res.*, **528**, 353–357.

Bo, X., Alavi, A., Xiang, Z., Oglesby, I., Ford, A. & Burnstock, G. (1999) Localization of ATP-gated P2X2 and P2X3 receptor immunoreactive nerves in rat taste buds. *Neuroreport*, **10**, 1107–1111.

Bodin, P. & Burnstock, G. (2001a) Purinergic signalling: ATP release. *Neurochem. Res.*, **26**, 959–969.

Bodin, P. & Burnstock, G. (2001b) Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. *J. Cardiovasc. Pharmacol.*, **38**, 900–908.

Bradbury, E.J., Burnstock, G. & McMahon, S.B. (1998) The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Mol. Cell. Neurosci.*, **12**, 256–268.

Bryant, B. & Silver, W.L. (2000) Chemesthesis: The Common Chemical Sense. In Finger, T.E., Silver, W.L. & Restrepo, D. (Eds), *The Neurobiology of Taste and Smell*. Wiley-Liss, pp. 74–100.

Buck, L. & Axel, R. (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell*, **65**, 175–187.

Burgard, E.C., Niforatos, W., van Biesen, T., Lynch, K.J., Touma, E., Metzger, R.E., Kowaluk, E.A. & Jarvis, M.F. (1999) P2X receptor-mediated ionic currents in dorsal root ganglion neurons. *J. Neurophysiol.*, **82**, 1590–1598.

Chen, C.C., Akopian, A.N., Sivilotti, L., Colquhoun, D., Burnstock, G. & Wood, J.N. (1995) A P2X purinoceptor expressed by a subset of sensory neurons. *Nature*, **377**, 428–431.

Clements, J.D. & Westbrook, G.L. (1994) Kinetics of AP5 dissociation from NMDA receptors: evidence for two identical cooperative binding sites. *J. Neurophysiol.*, **71**, 2566–2569.

Cockayne, D.A., Hamilton, S.G., Zhu, Q.M., Dunn, P.M., Zhong, Y., Novakovic, S., Malmberg, A.B., Cain, G., Berson, A., Kassotakis, L., Hedley, L., Lachnit, W.G., Burnstock, G., McMahon, S.B. & Ford, A.P. (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature*, **407**, 1011–1015.

Collo, G., North, R.A., Kawashima, E., Merlo-Pich, E., Neidhart, S., Surprenant, A. & Buell, G. (1996) Cloning of P2X5 and P2X6 receptors and the distribution and properties of an extended family of ATP-gated ion channels. *J. Neurosci.*, **16**, 2495–2507.

Cometto-Muniz, J.E., Cain, W.S. & Abraham, M.H. (1998c) Nasal pungency and odor of homologous aldehydes and carboxylic acids. *Exp. Brain Res.*, **118**, 180–188.

Cometto-Muniz, J.E., Cain, W.S., Abraham, M.H. & Kumarsingh, R. (1998a) Sensory properties of selected terpenes. Thresholds for odor, nasal pungency, nasal localization, and eye irritation. *Ann. N.Y. Acad. Sci.*, **855**, 648–651.

Cometto-Muniz, J.E., Cain, W.S., Abraham, M.H. & Kumarsingh, R. (1998b) Trigeminal and olfactory chemosensory impact of selected terpenes. *Pharmacol. Biochem. Behav.*, **60**, 765–770.

Cometto-Muniz, J.E., Cain, W.S. & Hudnell, H.K. (1997) Agonistic sensory effects of airborne chemicals in mixtures: odor, nasal pungency, and eye irritation. *Percept. Psychophys.*, **59**, 665–674.

Cook, S.P., Rodland, K.D. & McCleskey, E.W. (1998) A memory for extracellular Ca^{2+} by speeding recovery of P2X receptors from desensitization. *J. Neurosci.*, **18**, 9238–9244.

Cook, S.P., Vulchanova, L., Hargreaves, K.M., Elde, R., McCleskey & E.W. (1997) Distinct ATP receptors on pain-sensing and stretch-sensing neurons. *Nature*, **387**, 505–508.

Ding, S. & Sachs, F. (2000) Inactivation of P2X2 purinoceptors by divalent cations. *J. Physiol. (Lond.)*, **522**, 199–214.

Djouhri, L., Bleazard, L. & Lawson, S.N. (1998) Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. *J. Physiol. (Lond.)*, **513**, 857–872.

Doty, R.L., Brugger, W.E., Jurs, P.C., Orndorff, M.A., Snyder, P.J. & Lowry, L.D. (1978) Intranasal trigeminal stimulation from odorous volatiles: psychometric responses from anosmic and normal humans. *Physiol. Behav.*, **20**, 175–185.

Dunn, P.M., Liu, M., Zhong, Y., King, B.F. & Burnstock, G. (2000) Diinosine pentaphosphate: an antagonist which discriminates between recombinant P2X(3) and P2X(2/3) receptors and between two P2X receptors in rat sensory neurones. *Br. J. Pharmacol.*, **130**, 1378–1384.

Dunn, P.M., Zhong, Y. & Burnstock, G. (2001) P2X receptors in peripheral neurons. *Prog. Neurobiol.*, **65**, 107–134.

Finger, T.E., Bottger, B., Hansen, A., Anderson, K.T., Alimohammadi, H. & Silver, W.L. (2003) Solitary chemoreceptor cells in the nasal cavity serve as sentinels of respiration. *Proc. Natl Acad. Sci. USA*, **100**, 8981–8986.

Finger, T.E., St Jeor, V.L., Kinnamon, J.C. & Silver, W.L. (1990) Ultrastructure of substance P- and CGRP-immunoreactive nerve fibers in the nasal epithelium of rodents. *J. Comp. Neurol.*, **294**, 293–305.

Getchell, M.L. & Getchell, T.V. (1992) Fine structural aspects of secretion and extrinsic innervation in the olfactory mucosa. *Microsc. Res. Techn.*, **23**, 111–127.

Gover, T.D., Kao, J.P. & Weinreich, D. (2003) Calcium signaling in single peripheral sensory nerve terminals. *J. Neurosci.*, **23**, 4793–4797.

Grubb, B.D. & Evans, R.J. (1999) Characterization of cultured dorsal root ganglion neuron P2X receptors. *Eur. J. Neurosci.*, **11**, 149–154.

Hadjantonakis, A.K., Gertsenstein, M., Ikawa, M., Okabe, M., Nagy & A. (1998) Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech. Dev.*, **76**, 79–90.

Hegg, C.C., Greenwood, D., Huang, W., Han, P. & Lucero, M.T. (2003) Activation of purinergic receptor subtypes modulates odor sensitivity. *J. Neurosci.*, **23**, 8291–8301.

Hummel, T., Futschik, T., Frasnelli, J. & Huttenbrink, K.B. (2003) Effects of olfactory function, age, and gender on trigeminally mediated sensations: a study based on the lateralization of chemosensory stimuli. *Toxicol. Lett.*, **140–141**, 273–280.

Hummel, T. & Livermore, A. (2002) Intranasal chemosensory function of the trigeminal nerve and aspects of its relation to olfaction. *Int. Arch. Occup. Environ. Health*, **75**, 305–313.

Hummel, T., Livermore, A., Hummel, C. & Kobal, G. (1992) Chemosensory event-related potentials in man: relation to olfactory and painful sensations elicited by nicotine. *Electroencephalogr. Clin. Neurophysiol.*, **84**, 192–195.

Jarvis, M.F., Wismer, C.T., Schweitzer, E., van Yu, H., Biesen, T., Lynch, K.J., Burgard, E.C. & Kowaluk, E.A. (2001) Modulation of BzATP and formalin induced nociception: attenuation by the P2X receptor antagonist, TNP-ATP and enhancement by the P2X(3) allosteric modulator, cibacron blue. *Br. J. Pharmacol.*, **132**, 259–269.

Kim, M., Yoo, O.J. & Choe, S. (1997) Molecular assembly of the extracellular domain of P2X2, an ATP-gated ion channel. *Biochem. Biophys. Res. Commun.*, **240**, 618–622.

King, B.F., Liu, M., Pintor, J., Gualix, J., Miras-Portugal, M.T. & Burnstock, G. (1999) Diinosine pentaphosphate (IP5I) is a potent antagonist at recombinant rat P2X1 receptors. *Br. J. Pharmacol.*, **128**, 981–988.

Knight, G.E., Bodin, P., De Groat, W.C. & Burnstock, G. (2002) ATP is released from guinea pig ureter epithelium on distension. *Am. J. Physiol. Renal Physiol.*, **282**, F281–F288.

Laska, M., Distel, H. & Hudson, R. (1997) Trigeminal perception of odorant quality in congenitally anosmic subjects. *Chem. Senses*, **22**, 447–456.

Lewis, C., Neidhart, S., Holy, C., North, R.A., Buell, G. & Surprenant, A. (1995) Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. *Nature*, **377**, 432–435.

Liang, S.D. & Vizi, E.S. (1997) Positive feedback modulation of acetylcholine release from isolated rat superior cervical ganglion. *J. Pharmacol. Exp. Ther.*, **280**, 650–655.

Liu, M., King, B.F., Dunn, P.M., Rong, W., Townsend-Nicholson, A. & Burnstock, G. (2001) Coexpression of P2X(3) and P2X(2) receptor subunits in varying amounts generates heterogeneous populations of P2X receptors that evoke a spectrum of agonist responses comparable to that seen in sensory neurons. *J. Pharmacol. Exp. Ther.*, **296**, 1043–1050.

Llewellyn-Smith, I.J. & Burnstock, G. (1998) Ultrastructural localization of P2X3 receptors in rat sensory neurons. *Neuroreport*, **9**, 2545–2550.

Lopez de Armentia, M., Cabanes, C. & Belmonte, C. (2000) Electrophysiological properties of identified trigeminal ganglion neurons innervating the cornea of the mouse. *Neuroscience*, **101**, 1109–1115.

Ma, M., Grosmaitre, X., Iwema, C.L., Baker, H., Greer, C.A. & Shepherd, G.M. (2003) Olfactory signal transduction in the mouse septal organ. *J. Neurosci.*, **23**, 317–324.

McKemy, D.D., Neuhausser, W.M. & Julius, D. (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature*, **416**, 52–58.

Mitchell, C.H., Carre, D.A., McGlinn, A.M., Stone, R.A. & Civan, M.M. (1998) A release mechanism for stored ATP in ocular ciliary epithelial cells. *Proc. Natl Acad. Sci. USA*, **95**, 7174–7178.

Nicke, A., Baumert, H.G., Rettinger, J., Eichele, A., Lambrecht, G., Mutschler, E. & Schmalzing, G. (1998) P2X1 and P2X3 receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO J.*, **17**, 3016–3028.

North, R.A. & Barnard, E.A. (1997) Nucleotide receptors. *Curr. Opin. Neurobiol.*, **7**, 346–357.

Pearce, R.J. & Duchen, M.R. (1994) Differential expression of membrane currents in dissociated mouse primary sensory neurons. *Neuroscience*, **63**, 1041–1056.

Ralevic, V. & Burnstock, G. (1998) Receptors for purines and pyrimidines. *Pharmacol. Rev.*, **50**, 413–492.

Robertson, S.J., Rae, M.G., Rowan, E.G. & Kennedy, C. (1996) Characterization of a P2X-purinoceptor in cultured neurones of the rat dorsal root ganglia. *Br. J. Pharmacol.*, **118**, 951–956.

Ruan, H.Z. & Burnstock, G. (2003) Localization of P2Y1 and P2Y4 receptors in dorsal root, nodose and trigeminal ganglia of the rat. *Histochem. Cell Biol.*, **120**, 415–426.

Silver, W.L. & Finger, T.E. (1991) The Trigeminal System. In Getchell, T.V. (Eds), *Smell and Taste in Health and Disease*. Raven Press, New York, pp. 97–108.

Silverman, J.D. & Krüger, L. (1989) Calcitonin-gene-related-peptide-immunoreactive innervation of the rat head with emphasis on specialized sensory structures. *J. Comp. Neurol.*, **280**, 303–330.

Sorensen, C.E. & Novak, I. (2001) Visualization of ATP release in pancreatic acini in response to cholinergic stimulus. Use of fluorescent probes and confocal microscopy. *J. Biol. Chem.*, **276**, 32925–32932.

Souslova, V., Cesare, P., Ding, Y., Akopian, A.N., Stanfa, L., Suzuki, R., Carpenter, K., Dickenson, A., Boyce, S., Hill, R., Nebenuis, O.D., Smith, A.J., Kidd, E.J. & Wood, J.N. (2000) Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. *Nature*, **407**, 1015–1017.

Spehr, M., Wetzel, C.H., Hatt, H. & Ache, B.W. (2002) 3-Phosphoinositides modulate cyclic nucleotide signaling in olfactory receptor neurons. *Neuron*, **33**, 731–739.

Spelta, V., Jiang, L.H., Bailey, R.J., Surprenant, A. & North, R.A. (2003) Interaction between cysteines introduced into each transmembrane domain of the rat P2X2 receptor. *Br. J. Pharmacol.*, **138**, 131–136.

Sperlagh, B. & Vizi, E.S. (1996) Neuronal synthesis, storage and release of ATP. *Seminars in Neurosciences*, **8**, 175–186.

Stoop, R., Thomas, S., Rassendren, F., Kawashima, E., Buell, G., Surprenant, A. & North, R.A. (1999) Contribution of individual subunits to the multimeric P2X(2) receptor: estimates based on methanethiosulfonate block at T336C. *Mol. Pharmacol.*, **56**, 973–981.

Thomas, S., Virginio, C., North, R.A. & Surprenant, A. (1998) The antagonist trinitrophenyl-ATP reveals co-existence of distinct P2X receptor channels in rat nodose neurones. *J. Physiol. (Lond.)*, **509**, 411–417.

Ueno, S., Tsuda, M., Iwanaga, T. & Inoue, K. (1999) Cell type-specific ATP-activated responses in rat dorsal root ganglion neurons. *Br. J. Pharmacol.*, **126**, 429–436.

de la Viana, F.P.E., Pecson, B., Schmidt, R.F. & Belmonte, C. (2001) Swelling-activated calcium signalling in cultured mouse primary sensory neurons. *Eur. J. Neurosci.*, **13**, 722–734.

Vulchanova, L., Riedl, M.S., Shuster, S.J., Buell, G., Surprenant, A., North, R.A. & Elde, R. (1997) Immunohistochemical study of the P2X2 and P2X3 receptor subunits in rat and monkey sensory neurons and their central terminals. *Neuropharmacology*, **36**, 1229–1242.

Weick, M., Cherkas, P.S., Hartig, W., Pannicke, T., Uckermann, O., Bringmann, A., Tal, M., Reichenbach, A. & Hanani, M. (2003) P2 receptors in satellite glial cells in trigeminal ganglia of mice. *Neuroscience*, **120**, 969–977.

White, T.D. (1978) Release of ATP from a synaptosomal preparation by elevated extracellular K^+ and by veratridine. *J. Neurochem.*, **30**, 329–336.

White, P.N., Thorne, P.R., Housley, G.D., Mockett, B., Billett, T.E. & Burnstock, G. (1995) Quinacrine staining of marginal cells in the stria vascularis of the guinea-pig cochlea: a possible source of extracellular ATP? *Hear. Res.*, **90**, 97–105.

Xiang, Z., Bo, X. & Burnstock, G. (1998) Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. *Neurosci. Lett.*, **256**, 105–108.

Yang, B.H., Piao, Z.G., Kim, Y.B., Lee, J.K., Park, K., Kim, J.S. & Oh, S.B. (2003) Activation of vanilloid receptor 1 (VR1) by eugenol. *J. Dent. Res.*, **82**, 781–785.

Zhong, Y., Dunn, P.M. & Burnstock, G. (2000) Pharmacological comparison of P2X receptors on rat coeliac, mouse coeliac and mouse pelvic ganglion neurons. *Neuropharmacology*, **39**, 172–180.

Zhong, Y., Dunn, P.M., Xiang, Z., Bo, X. & Burnstock, G. (1998) Pharmacological and molecular characterization of P2X receptors in rat pelvic ganglion neurons. *Br. J. Pharmacol.*, **125**, 771–781.

Zufall, F., Hatt, H. & Firestein, S. (1993) Rapid application and removal of second messengers to cyclic nucleotide-gated channels from olfactory epithelium. *Proc. Natl Acad. Sci. USA*, **90**, 9335–9339.