P2 Receptor–Mediated Signaling in Spherical Bushy Cells of the Mammalian Cochlear Nucleus

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Milenkovic I, Rinke I, Witte M, Dietz B, Rübsamen R. P2 receptor–mediated signaling in spherical bushy cells of the mammalian cochlear nucleus. J Neurophysiol 102: 1821–1833, 2009. First published July 1, 2009; doi:10.1152/jn.00186.2009. Purinoreceptors of the P2 family contribute strongly to signaling in the cochlea, but little is known about the effects of purinergic neurotransmission in the central auditory system. Here we examine P2 receptor–mediated signaling in the large spherical bushy cells (SBCs) of Mongolian gerbils around the onset of acoustically evoked signal processing (P9–P14). Brief adenosine 5′-O-(3-thiotriphosphate) (ATP[S]) application evoked inward current, membrane depolarization, and somatic Ca2+ signals. Moreover, ATP[S] changed the SBCs firing pattern from phasic to tonic, when the application was synchronized with depolarizing current injection. This bursting discharge activity was dependent on [Ca2+], and Ca2+-dependent protein kinase (PKC) activity and is presumably caused by modulation of low-threshold K+ conductance. Activation of P2Y1 receptors data showed that both P2X and P2Y1 receptors mediate Ca2+ signals in SBCs where P2Y1 receptors most likely activate the PLC–IP3 (inositol trisphosphate) pathway and release Ca2+ from internal stores. Immunohistochemical staining confirmed the expression of P2X2 and P2Y1 receptor proteins in SBCs, providing additional evidence for the involvement of both receptors in signal transduction in these neurons. Purinergic signaling might modulate excitability of SBCs and thereby contribute to regulation of synaptic strength. Functionally, the increase in firing rate mediated by P2 receptors could reduce temporal precision of the postsynaptic firing, e.g., phase locking, which has an immediate effect on signal processing related to sound localization. This might provide a mechanism for adaptation to the ambient acoustic environment.

INTRODUCTION

Extracellular nucleotides such as ATP accomplish important functional roles in the CNS by functioning as neurotransmitters, cotransmitters, neuromodulators, and growth factors, and by influencing processes such as proliferation, survival, and repair of neuronal and glial cells (Burnstock 2007; Fields and Burnstock 2006; Franke and Illes 2006; Illes and Ribeiro 2004; Milenkovic et al. 2003). ATP is localized in synaptic vesicles of purinergic, adrenergic, and cholinergic neurons, and the corelease with noradrenaline, acetylcholine, GABA, and glutamate has been observed as well (Burnstock 1972, 2004; Jo and Schlichter 1999; Mori et al. 2001; Nieber et al. 1997; Pankratov et al. 1998; Poelchen et al. 2001; Unsworth and Johnson 1990; von Kugelgen and Starke 1991; White 1977).

In the peripheral auditory system, P2 receptors are expressed by different cells in the cochlea (Housley et al. 1999; King et al. 1998b; Lagostena and Mammino 2001; Nakagawa et al. 1990; Raybould and Housley 1997; Robertson and Paki 2002; Sugasawa et al. 1996), where they seem to play a role in maintaining the potential in the endolymph of the scala media, affecting neurotransmission of the hair cells, and modifying cochlear micromechanics (Housley et al. 2002). Recently it has been shown that, before hearing onset, supporting cells within Kölliker’s organ release ATP and thereby excite hair cells, which in turn trigger bursts of electrical activity in spiral ganglion neurons (Tritsch et al. 2007). This early spontaneous activity is considered necessary for survival of target neurons, refinement of afferent connections, and adjustment of synaptic strength in the cochlear nucleus (CN) (Friauf and Lohmann 1999; Leake et al. 2006; McKay and Oleskevich 2007; Rubel and Fritzsch 2002). However, the functional role of purinergic signaling in the central auditory system is still poorly understood. In the medial nucleus of the trapezoid body (MNTB), presynaptic P2X1 and P2X3 receptors facilitate transmitter release from inhibitory (GABAergic) and excitatory (glutamatergic) synaptic terminals onto principal neurons (Watano et al. 2004). To date, it is still unknown if extracellular ATP affects synaptic transmission in cochlear nucleus second-order neurons of the ascending auditory pathway. Spherical bushy cells (SBCs) of the mammalian CN integrate excitatory inputs from few auditory nerve terminals (endbulbs of Held) (Brawer and Moster 1975; Ryujo and Sento 1991) and likewise acoustically driven GABA- and glycine-mediated inhibitory inputs (Backoff et al. 1999; Caspary et al. 1994; Gai and Carney 2008; Kopp-Scheinpflug et al. 2002). They project to the medial nucleus of the superior olivary complex, where inputs from both sides are integrated for the processing of spatial acoustic information based on interaural time differences (reviewed in Oertel 1999). In this study, we extend the current view on neurotransmission in developing anteroventral CN (AVCN) and provide compelling evidence that, around the time of hearing onset, postsynaptic P2 receptors mediate depolarization of SBCs accompanied by somatic calcium responses. Furthermore, we show that purinergic signaling can increase the action potential firing in SBCs, indicating the possible role in regulation of neuronal responsiveness around the time of hearing onset.

METHODS

All experimental procedures were approved by the Saxonian district Government (TVV 50/06), Leipzig, and were done according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).
Slice preparation and maintenance

Mongolian gerbils (Meriones unguiculatus) aged postnatal days 9–14 (P9–P14) were decapitated, and the brains were quickly removed. Acute brain stem slices (200 µm) containing the most rostral region of the anteroventral cochlear nucleus were cut by means of a vibratome (HM 650, Microm, Walldorf, Germany) as previously described (Milenkovic et al. 2007). The slices were cut in cold (3–4°C) low-calcium artificial cerebrospinal fluid (ACSF) solution containing (in mM) 125 NaCl, 2.5 KCl, 0.1 CaCl2, 3 MgCl2, 1.25 NaH2PO4, 25 NaHCO3, 25 glucose, 2 sodium pyruvate, 3 myoinositol, and 0.5 ascorbic acid, continuously equilibrated with 5% CO2-95% O2, pH 7.4. The slices were incubated in ACSF (same as for slicing, except for CaCl2 and MgCl2, which were changed to 2 and 1 mM, respectively) for 30 min at 37°C and stored at room temperature until whole cell recording. For fluorometric Ca2+ measurements, the slices were bulk labeled by incubation with Fura 2 acetylomethyl ester (10 µM Fura-2 AM in ACSF, Molecular Probes) and 0.025% (wt/vol) pluronic acid for 30 min at 37°C. Thereafter, the slices were washed and stored in ACSF at room temperature in the dark until Ca2+ measurement. Recordings were made at room temperature (21–23°C).

Whole cell recordings and post hoc visualization of recorded cells

Patch pipettes were pulled from filamented borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) to have resistances of 3–6 MΩ when filled with internal solution containing (mM) 130 potassium-glucuronate, 5 KCl, 1 NaCl, 1 MgCl2, and 10 HEPES (pH 7.3 with KOH). In some experiments, 0.2% biocytin was added to pipette solution. Whole cell recordings were made with a discontinuous single-electrode-amplifier (npi electronic, Tamm, Germany) in current-clamp or voltage-clamp mode. Switching frequency was 20 kHz and signals were filtered at 1 kHz and digitized at 2–5 kHz using npi electronic hardware and software (Cell Works 5.0). Data analysis was performed with pClamp 9.0 software (Axon Instruments, Union City, CA). Before Fura-2–labeled cells were selected for recordings, it was tested whether the intact cells respond to the focal pressure ejection of adenosine 5’-O-(3-thiotriphosphate) (ATPγS). This metabolically stable ATP analog (Chung et al. 1991) was administered (2 psi, 500 ms) over the somata of large cells through a wider-tip patch pipette mounted on a Picospipetor (General Valve, Fairfield, NJ). Pairs of images were acquired at 10 Hz, and the ratios (F340/F380) were calculated from the regions of interest (ROIs) enclosing the soma of the neuron selected for recordings and also from the ROIs enclosing neighboring cells up to a distance of ~50–100 µm. Recorded cells were characterized as SBCs according to their firing of one or just a few action potentials at the start of depolarizing current step and to the prominent sag in response to the hyperpolarizing current injection (Cao et al. 2007; Oertel 1983; Schwarz and Puil 1997). Puff application of ATPγS was repeated in whole cell mode, and the membrane potential was recorded under current clamp. All voltages were compensated on-line for ~11-mV junction potential. Further morphological characterization was assessed post hoc by Cy2-conjugated streptavidin labeling of biocytin-filled neurons (Bischofberger et al. 2006). After recording, slices were fixed by superfusion of paraformaldehyde (PFA; 4% in 0.1 M PBS, pH 7.3) and postfixed overnight at 4°C. The slices were incubated for 2.5 h at room temperature with Cy2-conjugated streptavidin (5 µg/ml; Jackson Immunoresearch Laboratory, Dianova, Hamburg, Germany), washed (3 times for 10 min each in PBS and 1 time for 10 min in dH2O), and embedded in glycerol-gelatin mounting medium. In some experiments, Fura-2 AM labeling was omitted, and the whole cell recordings were done in current clamp or voltage clamp as described above.

Fluorometric Ca2+ measurements

For measurements of intracellular Ca2+ signals, single slices were positioned in the recording chamber (volume ~0.25 ml) and superfused with ACSF by a peristaltic pump at the rate of 1.5 ml/min. Drugs were diluted in ACSF and applied by bath perfusion controlled by a custom-made six-port distribution valve connected to solution reservoirs. For low Ca2+ extracellular solution (low Ca2+-ACSF), Ca2+ was reduced to 0.1 mM and Mg2+ was increased to 2.9 mM. Measurements were done using an inverted microscope (Axiovert 200, Zeiss); the cells facing the glass coverslip were visualized with a ×40 oil immersion objective (Zeiss Fluar). Pairs of images were obtained by an alternative excitation with light of 340- and 380-nm wavelengths generated from a monochromator (Polychrome V, TILL Photonics, Gräfeling, Germany) and the fluorescence emission was long pass filtered (D510, Fura-2 filter set, Chroma Technology, Brattleboro, VT). Images were acquired with full spatial resolution at 1 Hz with a cooled interline transfer CCD camera (IMAGO Typ VGA, TILL Photonics). Background fluorescence was subtracted using appropriate software (TILLvision, TILL Photonics). Data obtained in single ROIs defined around the somata of large Fura-2–labeled cells (10–15 cells within a single full image) are presented as ratio (R = F340/F380) or ratio changes relative to baseline (ΔR given as percentage). Average values are given as means ± SE unless noted otherwise. In the range of physiological Ca2+ concentrations, the ratios are linearly related to the changes in [Ca2+]i (Grynkiewicz et al. 1985). Ca2+ transients were considered significant when the maximal amplitude exceeded three times the root mean square value of the baseline fluorescence (average of 10 images before stimulation) of the respective ROI (c > 3, z-test). Cells with high initial ratio values and cells that did not return to baseline after stimulation were excluded from analysis. Groups of data were compared by Student’s t-test or one-way ANOVA followed by pairwise multiple comparisons where appropriate.

Immunofluorescence labeling

Four mongolian gerbils, P10–P14, and two gerbils, P27, were deeply anesthetized with pentobarbital sodium (10 mg/kg body weight, ip) and transcardially perfused with 0.9% sodium chloride (5 min), followed by 4% PFA (25 min) in 0.1 M PBS, pH 7.4. The brains were removed and postfixed overnight in 4% PFA at 4°C. Coronal brain stem sections (30 µm) containing AVCN were cut using a vibratome (HM 650, Microm). All immunofluorescence procedures were applied to free-floating sections at room temperature except noted otherwise. After blocking of nonspecific binding sites with 5% donkey normal serum (DNS) in PBS/0.3% Triton X-100 (30 min at 37°C), the sections were incubated overnight with rabbit anti-P2Y1 directed to the intracellular C terminus (1:200, APR-10, APR-006, and APR-011, respectively, Alomone). The P2Y1 antibody was raised against a 17-amino-acid peptide, corresponding to residues 242–258 of rat and human P2Y1 receptor, corresponding to residues 242–258 of rat and human P2Y1 receptor, between the TM5 and TM6 domains. Alternatively, the slices were stained with rabbit anti-P2X1, directed to the intracellular C terminus of the human receptor (1:500, AB9263, Chemicon). Both antibodies have been used extensively to localize P2Y1 by immunohistochemistry in various regions of the CNS (Amadio et al. 2007; Bowser and Studeny 2005; Florenzano et al. 2002; Rubio and Soto 2001; Studeny et al. 2005). The P2Y1 antibody was raised against a 17-amino-acid peptide, corresponding to residues 242–258 of rat and human P2Y1 receptor, i.e., the epitope location is in the presumed third intracellular loop (3) between the TM5 and TM6 domains. Alternatively, the slices were stained with rabbit anti-P2Y1, directed to the intracellular C terminus of the human receptor (1:500, AB9263, Chemicon). Both antibodies have been used extensively to localize P2Y1 by immunohistochemistry in various regions of the CNS (Amadio et al. 2007; Bowser and Khakh 2004; Franke et al. 2006; Fries et al. 2004; Ruan and Burnstock 2003; Sergeeva et al. 2006; Tonazzini et al. 2007). Additional experiments were done with rabbit anti-P2Y1, -P2Y4, and -P2Y6 antibodies (1:200, APR-10, APR-006, and APR-011, respectively, Alomone).
Immunofluorescence double labeling of large presynaptic calyceal inputs (endbulbs of Held), which terminate on SBC somata, was performed with goat anti-calretinin antibody as previously published (1:1,000, Swant) (Bazwinsky et al. 2008; Hartig et al. 2001). In some specimens, double labeling was done with antibody against the vesicular glutamate transporter 1 (1:1,500, guinea pig anti-VGLUT1, AB5905, Chemicon) (Hartig et al. 2003) to visualize the endbulbs of Held (Zhou et al. 2007). The secondary donkey anti-rabbit Cy2-, donkey anti-rabbit Cy3-, donkey anti-goat Cy3-, or donkey anti-guinea pig Cy3-tagged antibodies (20 μg/ml in blocking solution; Jackson Immunoresearch Laboratory) were incubated where appropriate for 2.5 h at room temperature. After rinsing with PBS and dH2O, the sections were dehydrated in xylol, dried, and coverslipped with entellan. The specificity of antisera for P2X, and P2Y receptors (Alomone) was analyzed by achieving a block of immunoreactivity by preadsorption of antisera with the respective peptide antigen for 1 h before use (1 μg of peptide for 1 μg of antibody). Additional control experiments were performed by omitting primary antibodies and the subsequent identical processing of a few sections. No labeling was seen under this condition in slices of the brain stem (data not shown).

Furthermore, the fluorophores related to the relevant markers were switched; for example, P2Y1 was also shown by donkey anti-rabbit Cy3 and calretinin by donkey anti-goat Cy2 antibodies. Biocytin labeling and immunohistochemistry images were acquired using a confocal laser scanning microscope (LSM 510, Zeiss).

RESULTS

ATPγS changes the firing properties of SBCs

To confirm that Fura-2 AM labels SBCs in P9–P14 slices, cells at the rostral pole of the AVCN were characterized as follows: ATPγS was pressure ejected in the vicinity of large Fura-2–labeled cells, and the calcium responses were measured (Fig. 1Aa). In each slice, one of the cells showing Ca2+ transients was subsequently recorded with biocytin-filled pipettes, electrophysiologically characterized (Cao et al. 2007; Schwarz and Puil 1997; Wu and Oertel 1984) (Fig. 1, Ac and Bc), and the effect of ATPγS on the membrane potential was recorded (Fig. 1, Ab and Bb).

ATPγS was applied by pressure ejection in 14 slices, where it evoked transient increases in [Ca2+]i in n = 34 cells (P < 0.01, z-test). Whole cell recordings were thereafter obtained from 14 SBCs, each selected from a distinct slice. Moderate membrane depolarization evoked by ATPγS puff was regularly observed in these recordings (13.4 ± 2.6 mM from Vm = −60 mV, n = 9). In five cells, we also monitored fast action potentials riding on the depolarization (Fig. 1Ab). When TTX was added in these recordings, the amplitudes of ATPγS-evoked depolarization were unchanged (14.2 ± 4.7 mV, n = 3, P = 0.9, t-test). All 14 cells initially fired a single or at most three action potentials at the onset of depolarizing current pulse (Fig. 1, Ac and Bc), consistent with type II discharge pattern of AVCN bushy cells (Francis and Manis 2000; Oertel 1983; Wu and Oertel 1984). As in previous studies on bushy cells in gerbil and mice (Cao et al. 2007; Leao et al. 2005; Schwarz and Puil 1997), hyperpolarizing current pulses produced voltage changes that sagged back toward rest caused by hyperpolarization-activated conductances, and anode-break action potentials occurred after the pulse offset. Biocytin labeling of recorded cells showed the morphological features of large spher- ical bushy cells which occupy the anterior pole of the AVCN in low frequency hearing animals (Bazwinsky et al. 2008; Brawer et al. 1974; Hackney et al. 1990; Morest et al. 1990; Ostapoff et al. 1994; Rouiller and Ryugo 1984; Ryugo and Sento 1991). These data suggest that, in slices from the rostral pole of the AVCN, large Fura-2 AM–labeled SBCs show responses to ATPγS application. In contrary, stellate cells (n = 3) characterized by the type I firing pattern did not respond to ATPγS (Fig. 1C), which excludes purinergic action on these cells.

We next examined whether the activation of P2 receptors affects the discharge pattern of SBCs. Consistent with our observation from biocytin-labeled SBCs, only a single or at most two action potentials were elicited with depolarizing square current pulse (100–200 pA). Thereafter, the pressure ejection (150 ms) of ATPγS or P2Y1 receptor agonist adenosine 5′-[β-thio]diphosphate (ADPβS) was set to begin 100 ms before the onset of depolarizing current injection (200 ms). The number of action potentials generated during depolarizing steps was significantly increased after application of ATPγS (Fig. 2, A and B) but not ADPβS (Fig. 2A; control = 1.0 ± 0.1, n = 14; ATPγS = 9.3 ± 1.0, n = 10, P < 0.05; ADPβS (100 μM) = 1.2 ± 0.2, n = 7; ADPβS (250 μM) = 1.2 ± 0.2, n = 7, P = 0.54, 1-way ANOVA on ranks followed by pairwise comparisons, Dunn’s test). The bursting discharge activity evoked by ATPγS, possibly indicating a reduction in low threshold K+ conductance (ik), persisted even after the end of depolarizing current (Fig. 2C, postpulse). Although the application of ADPβS induced membrane depolarization and Ca2+ signals, these responses did not change the firing pattern even at higher agonist concentrations (250 μM). To determine whether the increase in spike number induced by ATPγS was solely caused by depolarization, SBCs were depolarized by a prepulse to the same level as with ATPγS puffs, followed by the standard depolarizing current step. This did not change the cell’s firing properties (control = 1.0 ± 0.1, prepulse = 1.2 ± 0.6; n = 4, P = 0.98, t-test; Fig. 2B). These data suggest that the mere activation of P2Y1 receptors is not sufficient to increase action potential firing and implicate the requirement of P2X receptor activation.

To explore the signaling pathway involved in regulation of SBCs excitability downstream of P2 receptors, the effects of the fast Ca2+ chelator BAPTA, inhibitor of Ca2+-dependent protein kinase (PKC) G6 6976, and inhibitor of the Ca2+-calmodulin dependent protein kinase II (CaM kinase II) KN-62 were tested by adding the respective drugs to the patch pipette solution. The outcome of these treatments (Fig. 2C) provided evidence that ATPγS changes the firing properties by a mechanism dependent on increase in [Ca2+]i and PKC activity, while at the same time, this effect seems to be independent of CaM kinase II.

In another set of experiments, a cocktail of NBQX (10 μM), AP-V (50 μM), SR95531 (25 μM), and strychnine (0.3 μM) (subsequently referred to as inhibitor cocktail) was used to block AMPA, N-methyl-d-aspartate (NMDA), GABA_A, and glycine receptors, respectively. Recordings in Fig. 2D, carried out in the presence of TTX (0.3 μM), indicated no difference between responses from the two experimental conditions (control vs. inhibitor cocktail), yet they clearly show the inhibitory effect of the P2 receptor antagonist pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS). Quantification of these results excluded the possibility that the effect of ATPγS was caused by depolarization of presynaptic terminals and subsequent transmitter release (mean current amplitude evoked by 100 μA MPAM, 100 ms application: control = −271.5 ± 22.3 pA, inhibitor cocktail = −257.8 ± 21.0 pA, P = 0.6; PPADS = −19.7 ± 6.4 pA, P < 0.05;
$n = 6$; mean depolarization: control $9.7 \pm 1.5$ mV, inhibitor cocktail $10.5 \pm 1.8$ mV, $P = 0.7$; PPADS $0.75 \pm 0.2$ mV, $P < 0.05$; $n = 5$, 1-way ANOVA, Holm-Sidak post hoc analysis). However, these data do not rule out the possibility that, under physiological conditions, ATP may be coreleased at one or more synapses on SBCs.

Characterization of P2 receptor–evoked Ca$^{2+}$ responses in large SBCs

Bath perfusion of ATP reliably evoked transient calcium responses characterized by an initial peak followed by a gradual reduction to near basal levels after washout. In preliminary experiments, we verified that consistent responses could be elicited when the interstimulus interval was $\geq 15$ min. Therefore increasing concentrations of ATP and ADP were applied with 15-min breaks between consecutive applications (Fig. 3A).

Both, ATP and ADP evoked Ca$^{2+}$ responses in a concentration-dependent manner with similar time courses, yet the effects of ADP were significantly smaller than those of ATP (Fig. 3B). The EC$_{50}$ values for ATP and ADP were 112 and 192 $\mu$M, respectively.

All bath perfusion experiments were done in the presence of TTX (0.3 $\mu$M) to prevent responses evoked by spontaneous presynaptic action potentials. In addition—same as in the whole cell recordings—it was tested whether it was necessary to block synaptic transmission by applying the inhibitor cock-
Given the metabolic stability of ATP-γS (Welford et al. 1987), the mostly identical effects of ATP and ATP-γS (Fig. 4B) indicate the involvement of P2X receptors rather than activation of adenosine receptors by degraded nucleotides. Consistent with this hypothesis, the ectoATPase inhibitor ARL 67156 (50 μM) affected neither the responses to ATP nor to ADP (Fig. 4B). The involvement of P2X receptors is further suggested by the inhibitory effects of PPADS (100 μM) and suramin (100 μM), broad spectrum P2-receptor antagonists (inhibition of ATP-evoked responses by -83 ± 7%, n = 66, P < 0.01, and by -35 ± 11%, n = 57, P < 0.01, respectively; 1-way ANOVA, Holm-Sidak post hoc analysis; Fig. 4C). Strong effects of ATP and ATP-γS along with effects of ADP suggest that the Ca²⁺ transients in SBCs are mediated by both ionotropic P2X and metabotropic P2Y receptors. Therefore we next determined the effects of uridine triphosphate (UTP) (a preferential agonist at P2Y₄ and P2Y₆ receptors) and uridine diphosphate (UDP) (a preferential agonist at P2Y₄ receptors; Fig. 5A). We found that only 16 and 22% of neurons showed significant Ca²⁺ signals in response to UDP (500 μM) and UTP (500 μM), respectively. In those, the mean peak Ca²⁺ changes elicited by UDP were just 17 ± 3 and 10 ± 2% of the
Figure 3. Increases in [Ca\(^{2+}\)]\(_i\) caused by extracellular ATP and ADP are dose dependent. A: traces show time course of the Ca\(^{2+}\) responses in single cells induced by different concentrations of extracellular ATP (n = 15 cells from a single slice). To avoid changes in signal amplitudes caused by P2 receptor desensitization, 15-min breaks were introduced between applications. B: concentration-response curves for ATP and ADP. The curves were fitted with \(f = \frac{b}{a + x}\), with \(a = 112 \mu M\) and \(a = 192 \mu M\) for ATP and ADP, respectively. Data are mean ± SD (n = 82 cells for ATP and n = 27 cells for ADP). Asterisks indicate significant differences between responses evoked by ATP and ADP (\(P < 0.05\)). Percent of cells showing significant responses for ATP and ADP, respectively: 88 and 30 for 10 \(\mu M\); 98 and 81 for 100 \(\mu M\); 100 and 85 for 500 \(\mu M\). At concentrations of 1 and 5 \(mM\), all cells showed significant responses. respective ADP- and ATP-evoked responses. The maximal Ca\(^{2+}\) changes induced by UTP were larger (27 ± 4 and 18 ± 3% of the responses evoked by ADP and ATP). On the contrary, the agonists with higher potency for P2Y\(_1\) receptors, i.e., ADP (500 \(\mu M\)), ADP/BS (500 \(\mu M\)), and 2meth-S-ADP (500 \(\mu M\)) (Tokuyama et al. 1995; Vohringer et al. 2000) evoked responses with similar amplitudes. However, these Ca\(^{2+}\) transients were only ~70% of the amplitudes evoked by ATP (Fig. 5A).

To further explore the involvement of P2Y\(_1\) receptors in signal transduction in SBCs, we studied the potency of MRS 2179, a selective P2Y\(_1\) antagonist (Camaioni et al. 1998). Figure 5B shows the dose-dependent inhibitory effect of MRS 2179, which blocked the ADP-evoked responses more efficiently than the ATP-evoked responses. After a 20-min washout period, the peak ratio amplitudes significantly increased without reaching the control values before MRS 2179 administration (data not shown). In contrast to the inhibitory effect of MRS 2179, AR-C69931 MX (1 \(\mu M\)), a selective antagonist at P2Y\(_{2,13}\) receptors, did not decrease the effect of ADP (Fig. 5C), ruling out possible contributions of ADP-sensitive P2Y\(_{2,13}\) receptors. These data corroborate the notion that P2Y\(_1\) expressed by SBCs mediate the responses to ADP and, at least partially, the responses to ATP.

P2Y\(_1\) receptors couple to the phospholipase C–IP\(_3–Ca^{2+}\) pathway (King et al. 1998a; von Kugelgen and Wetter 2000). To test whether the Ca\(^{2+}\) transients evoked by ADP depend on extracellular Ca\(^{2+}\), the effect of ADP (500 \(\mu M\)) was measured in 2 mM Ca\(^{2+}\) and in low Ca\(^{2+}\) (0.1 mM) solution (Fig. 6A). Application of ADP in low Ca\(^{2+}\) ACSF reliably elicited Ca\(^{2+}\) responses, and the peak ratio amplitudes were on average 58 ± 5% of control responses (\(n = 47\), \(P < 0.01\), 1-way ANOVA, Holm-Sidak post hoc analysis; Fig. 6B). Furthermore, in the presence of the phospholipase C inhibitor U-73122 (4 \(\mu M\)) (Stam et al. 1998) Ca\(^{2+}\) responses to ATP were blocked by −47 ± 9% (\(n = 66\), \(P < 0.01\), paired t-test) and the responses to ADP by −42 ± 12% (\(n = 51\), \(P < 0.01\), paired t-test; Fig. 6C). Because of the poor washout of U-73122, viability of the cells was subsequently tested by administration of high K\(^+\) ACSF (30 mM K\(^+\)). All cells tested showed Ca\(^{2+}\) rises in response to high K\(^+\) ACSF (data not shown).

To show the contribution of internal Ca\(^{2+}\) stores, SBCs were challenged with two applications of ATP or ADP, separated by an interstimulus interval of 15 min. The inhibitor of the endoplasmic reticulum (ER) calcium ATPase cyclopiazonic acid (CPA) was perfused between the two drug applications, causing a depletion of intracellular Ca\(^{2+}\) stores. Compared with a second application under control conditions, CPA reduced the Ca\(^{2+}\) responses to ADP (500 \(\mu M\)) and, to a lesser extent, to ATP (500 \(\mu M\)) (Fig. 6D). Together, these results show that ATP and ADP may use a common signaling mechanism in SBCs, which includes activation of PLC and mobilization of Ca\(^{2+}\) from ER. However, ATP most likely evokes additional Ca\(^{2+}\) entry via P2X receptors.

P2 immunoreactivity in the rostral AVCN

To corroborate the electrophysiological and calcium imaging data suggesting purinergic action in SBCs, brain stem sections were immunostained against P2X\(_2\) and P2Y\(_{1,2,4,6}\) receptor proteins. Representative results in Fig. 7 show the P2X\(_2\) and P2Y\(_1\) immunoreactivity in the rostral AVCN.

Punctate labeling was particularly dense in perikarya of large cell bodies (∼20 \(\mu m\) diam) in the rostral AVCN. Prolongation of PLC and mobilization of Ca\(^{2+}\) from ER. However, ATP most likely evokes additional Ca\(^{2+}\) entry via P2X receptors.
obtained with antibodies against P2Y2, P2Y4, and P2Y6 receptors (Fig. 7, I–K). These findings are in good agreement with calcium imaging data, which suggested a lack of P2Y2, P2Y4, and P2Y6 receptors in SBCs.

In summary, these data provide strong evidence that P2X2 and P2Y1 receptors evoke membrane depolarization and mediate increases in cytosolic calcium concentration in large SBCs of the cochlear nucleus.

DISCUSSION

Study of P2 receptor–mediated responses in the CN of the gerbil showed that SBCs express functional P2 receptors before the onset of hearing (at P12; Woolf and Ryan 1984) and during the early phase of auditory signal processing. Extracellular ATP evoked somatic calcium signals accompanied by a moderate to strong membrane depolarization that elicited complex spikes in the latter case. Moreover, purinergic action involving P2X2 receptor activation can change the SBC discharge pattern from phasic to tonic and evoke sustained bursting activity in response to depolarizing current steps. The mechanism underlying this change in firing properties is dependent on an increase in cytosolic Ca2+ and activation of PKC and is likely to engage modulation of IK,1 conductance. The activity of SBCs is primarily driven by glutamate and shaped by inhibitory neurotransmission. In this regard, it is conceivable that ATP might be coreleased with glutamate from enbulbs of Held and/or released from astrocytes, to adjust neuronal excitability as reported elsewhere (reviewed in Abbracchio et al. 2009; Burnstock 2004). However, the actual sources of ATP in the CN remain to be elucidated in future studies.

Methodological consideration

We combined Ca2+ imaging and whole cell recordings to show that the large Fura-2–labeled cells at the rostral pole of the AVCN respond to ATPγS, a nonhydrolyzable analog of ATP.
ATP and full agonist of mammalian P2X<sub>1-6</sub> receptors (North 2002). Biocytin-filled neurons unambiguously showed electrophysiological (Cao et al. 2007; Francis and Manis 2000; Leao et al. 2005; Schwarz and Puhl 1997) and morphological properties of SBCs (Brawer et al. 1974; Osen 1969; Ostapoff et al. 1994). Further pharmacological profiling suggested that the Ca<sup>2+</sup> responses are mediated by P2 receptors and excluded possible signaling mediated by adenosine receptors. In most whole cell recordings and some calcium imaging experiments, agonists were applied at concentrations of 100 μM, consistent with estimated ATP concentration in synaptic cleft (~100 μM) upon synaptic release by brief repetitive electrical stimulation of the phrenic nerve in rat (Silinsky 1975). Most of the calcium imaging was done on an inverted microscope, thus in these experiments, agonists were typically superfused at 100 or 500 μM to provide sufficient exposure of imaged cells to the drugs.

**P2X<sub>2</sub> and P2Y<sub>1</sub> receptors mediate purinergic action in SBCs**

We consistently observed highly comparable effects of ATPyS and ATP on [Ca<sup>2+</sup>]<sub>i</sub>. Either of these P2X receptor agonists induced Ca<sup>2+</sup> responses with higher potency than ADP. Both ATP- and ADP-mediated signaling mobilize Ca<sup>2+</sup> from intracellular stores (Ralevic and Burnstock 1998), but several blockers of the P2Y<sub>1</sub>–PLC<sub>β</sub>–IP<sub>3</sub> pathway used in our study had weaker effects on the responses evoked by ATP. Moreover, the large noninactivating inward currents evoked by ATPyS (Fig. 2D) are consistent with the profile of the ionotropic P2X<sub>2</sub> receptor subtype (Werner et al. 1996). Therefore we propose that the ATP-evoked Ca<sup>2+</sup> responses in SBCs are mediated by both P2X and P2Y receptors. This assumption is in agreement with in situ hybridization data showing P2X<sub>2</sub> and P2Y<sub>1</sub> receptor mRNA in the cochlear nucleus of the mouse (Allen Brain Atlas) and rat (P2X<sub>2</sub>, Kanjhan et al. 1999). Moreover, a dense P2X<sub>2</sub> immunoreactivity was previously shown in the cochlear nucleus of the rat (Kanjhan et al. 1999; Xiang et al. 1999) and marmoset (Yao et al. 2000). Our immunohistochemical data are consistent with these reports. The strong antagonistic effect of PPADS rather than suramin (Bianchi et al. 1999; King et al. 1997) supports the notion of P2X<sub>2</sub> receptor–mediated signaling in SBCs. In addition to Ca<sup>2+</sup> influx through ionotropic P2X receptors, it is conceivable that cytosolic Ca<sup>2+</sup> accumulation is additionally promoted by activation of voltage-gated calcium channels during membrane depolarization (Lalo et al. 1998). Such voltage-activated calcium conductance was reported for rat bushy cells at similar ages (Doughty et al. 1998).

Members of the P2Y receptors comprise two classes, one activating the G<sub>q</sub>–PLC<sub>β</sub> pathway (P2Y<sub>1,2,4,6,11</sub>) and the other Gi protein (P2Y<sub>12,13</sub>) leading to inhibition of adenyl cyclase (Lazarowski et al. 2003). Postsynaptic P2Y<sub>1</sub> receptors typically activate IP<sub>3</sub> production and mobilization of intracellular Ca<sup>2+</sup> (Simon et al. 1995), which is consistent with our experimental results. The comparable effects of several P2Y<sub>1</sub> agonists (ADP, ADPβS, 2methyl-S-ADP; Waldo and Harden 2004), and the lack of effects of the respective P2Y<sub>2,4</sub> and P2Y<sub>6</sub> agonists (UTP, UDP; Communi et al. 1996a,b; Lazarowski et al. 1995), provide means to propose P2Y<sub>1</sub>-mediated Ca<sup>2+</sup> increases. This assumption is also confirmed by the inhibitory effect of the selective P2Y<sub>1</sub> antagonist MRS 2179. Moreover, P2Y<sub>1</sub> receptors show sensitivity to PPADS (Fig. 4C), whereas the P2Y<sub>11</sub> receptor is efficiently blocked by suramin, but lacks the sensitivity to PPADS (von Kugelgen 2006), ruling out the possible role of P2Y<sub>11</sub> receptors in SBC signaling. A selective antagonist at P2Y<sub>12,13</sub> receptors (AR-C69931) showed no effect on the responses evoked by ADP; hence it was concluded that only the P2Y<sub>1</sub> subtype among the ADP-sensitive P2Y receptors (P2Y<sub>1,12,13</sub>) mediates the calcium signals in SBCs.

ADP elicited larger calcium responses in the presence of 2 mM than 0.1 mM [Ca<sup>2+</sup>]<sub>0</sub> (Fig. 6, A and B), suggesting that the
calcium influx from extracellular space contributes to the Ca\textsuperscript{2+} transients. Because ADP is a very weak agonist of P2X receptors (Bianchi et al. 1999; Evans et al. 1995), it is likely that capacitive Ca\textsuperscript{2+} entry through store-operated channels contributed to the signals. The store-operated channels are functional in neuronal and glial cells and allow Ca\textsuperscript{2+} influx across the plasma membrane to refill depleted intracellular stores (Parekh and Putney 2005). In line with this, removal of extracellular Ca\textsuperscript{2+} reduced the signals evoked by P2Y\textsubscript{1} receptors, whereas reperfusion of 2 mM [Ca\textsuperscript{2+}]\textsubscript{o} resulted in a return of the [Ca\textsuperscript{2+}]i to baseline (Fig. 6A), indicating that Ca\textsuperscript{2+} entered the cell through store-operated channels. Similar observations were made previously both in neuronal and in glial cells after P2Y\textsubscript{1} receptor activation (Rubini et al. 2006; Weick et al. 2003).

Physiological relevance

In recent years, developmental aspect of purinergic signaling in the inner ear gained significant attention. Some studies postulated a developmental role of P2 receptors based on the finding of early postnatal (and partly transient) expression of P2X\textsubscript{1} and P2X\textsubscript{3} receptors in hair cells and supporting cells of the cochlea of rats and mice (Huang et al. 2006; Nikolic et al. 2001, 2003). Recently, Tritsch et al. 2007 specified that supporting cells of the Kölliker’s organ release ATP that generates synchronous activity in the inner hair cells (IHC) before the onset of sound evoked cochlear activation. Early bursting discharge activity has been also observed in spiral ganglion neurons in cats (Jones et al. 2007), in nuclei of the lower auditory brain stem, i.e., cochlear nucleus of the cat (Walsh and McGee 1988), nucleus magnocellularis and laminaris of chicks (Lippe 1994), and in the inferior colliculus of bats (Rubsam and Schafer 1990). Such spontaneous bursting is characteristic of the early postnatal development and thought to be of importance for development and consolidation of auditory pathways, refinement of tonotopic organization (Kitzes et al. 1995; Leake et al. 2006; Parks 1997), and efficacy of synaptic transmission (McKay and Oleskevich 2007). This study investigates the details of purinergic action around hearing onset, yet developmental aspects of purinergic signaling in the CN remain to be explored in the future. Our data show that activation of P2 receptors can change the firing properties of SBCs by a mechanism involving increase in [Ca\textsuperscript{2+}]i, PKC activity, and possibly modulation of I\textsubscript{KL} conductance. I\textsubscript{KL} conductance is one of the important features of neurons along the afferent auditory pathways that ensure minimal latency fluctuations and preservation of timing (Kaczmarek et al. 2005; Trussell 1999). In neurons of the AVCN and MNTB, Kv1.1 channels contrib-
ute to a low-threshold Kv current that leads to strong accommodation in vitro (firing of a single, short-latency action potential in response to prolonged depolarizing current steps) (Brew and Forsythe 1995; Cao et al. 2007; Manis and Marx 1991) and increases temporal precision of auditory signaling in vivo (Kopp-Scheinflug et al. 2003). Prominent somatic Kv1.1 subunit expression was shown for AVCN bushy cells and principal MNTB neurons (Caminos et al. 2005; Grigg et al. 2017). In bushy cells of the mouse (and in neurons of the nucleus magnocellularis, the avian homolog of the CN), inhibition of low-threshold K⁺ conductance by α-dendrotoxin enables the cells to fire tonically for the duration of a depolarizing current pulse (Cao et al. 2007; Rathouz and Trussell 1998). Such change of the firing pattern is similar to the effect induced by the application of ATPγS (Fig. 2), suggesting that P2 receptors might exert an influence on Kv1.1 channels. Consistent with this notion is the P2 receptor–mediated inhibition of the Kv1.1 channel, conveyed through an activation of PKC (Boland and Jackson 1999). In agreement with our data, bushy cells in rats and mice express cPKC (isoforms I and II) (Garcia and Harlan 1997; Garcia et al. 2000), which can be activated by Ca²⁺ and diacylglycerol, also known to be increased by P2X₁ and P2Y₁ receptor signaling (King et al. 1998a; North 2002). Although we could show that P2Y₁ receptors activate the phospholipase C–IP₃–Ca²⁺ pathway, such activation could not evoke multiple action potential firing per se. Therefore it was concluded that the activation of P2X₂ receptors seems to be necessary, probably by providing sufficient increase in cytoplasmatic Ca²⁺ caused by substantial Ca²⁺ permeability of P2X receptors (Egan and Khakh 2004). However, it remains puzzling whether P2Y₁ receptors accomplish some additional function in SBCs or perhaps exert a modulating effect on P2X₂ receptor, as has been shown in dorsal root ganglion neurons (Gerevich et al. 2005).

It was proposed that the dynamic modulation of potassium conductances may provide one mechanism for adapting the

FIG. 7. Immunohistochemical staining for P2X₂, P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptor proteins on coronal sections of the AVCN. At the rostral pole of the CN, the P2X₂ (A–C, red fluorescence, arrows in C) and P2Y₁ immunoreactivity (D–F, green fluorescence, arrows in F) is restricted to large, spherical cells and occurs in a punctate pattern. Immunoreactivity was absent in preadsorption control experiments (peptide block, insets in A and D). The confinement of P2X₂ and P2Y₁ labeling to SBCs was exemplified by staining the endbulbs of Held with anti-VGLUT1 antibody (G, green fluorescence, arrowheads) or anti-calretinin antibody (H, red, arrowheads). Double labeling showed a staining pattern apposing the P2X₂– and P2Y₁–positive surfaces of neuron somata. Distribution of the P2X₂ and P2Y₁ immunoreactivities at P27 indicates persistent expression of both receptor proteins by SBCs 2 wk after the onset of hearing (G and H, respectively). Arrow points to immunolabeled SBC and arrowheads to Purkinje neurons in the adjacent cerebellum. Immunolabeling of P2Y₂, P2Y₄, and P2Y₆ receptors showed merely background fluorescence (J, K, and L, respectively). Scale bars: A and D, 50 μm; B and E, 20 μm; C and F, 10 μm; G–K, 100 μm.
firing pattern of auditory neurons to the ambient acoustic environment (Kaczmarek et al. 2005; Song et al. 2005). In vitro, nucleus magnocellularis neurons show adaptation of spike frequency caused by the slow inactivation of low-threshold K+ conductance (Kuznetsova et al. 2008). This loss of temporal precision might contribute to adaptation to a steady auditory stimulus. In other cells, P2 receptor–mediated signaling was shown to influence neuronal excitability by modulating diverse cation conductances (Boland and Jackson 1999; Bowser and Khakh 2004; Brown and Dale 2002; Chen et al. 1994; Hu et al. 2003). It is possible that some of these mechanisms are engaged by P2 signaling in SBCs specifically to adjust neuronal responsiveness and thereby modify pattern of the sound localization.

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**GRANTS**


**REFERENCES**


