P2 receptor-mediated signaling in spherical bushy cells of the mammalian cochlear nucleus

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ABSTRACT

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 gerbil around the onset of acoustically evoked signal processing (P9-14). Brief ATP\(_{\gamma}S\) application evoked inward current, membrane depolarization, and somatic Ca\(^{2+}\) signals. Moreover, ATP\(_{\gamma}S\) 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 [Ca\(^{2+}\)]\(_i\) and PKC activity, and is presumably caused by modulation of low-threshold K\(^+\) conductance. Activation of P2Y\(_1\) receptors could not evoke these changes per se, thus it was concluded that the involvement of P2X receptors seems to be necessary. Ca\(^{2+}\) imaging data revealed that both P2X and P2Y\(_1\) receptors mediate Ca\(^{2+}\) signals in SBCs where P2Y\(_1\) receptors most likely activate the PLC-IP\(_3\) pathway and release Ca\(^{2+}\) from internal stores. Immunohistochemical staining confirmed the expression of P2X\(_2\) and P2Y\(_1\) 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.

Keywords: Mongolian gerbil (Meriones unguiculatus), large spherical bushy cells, whole-cell recordings, Ca\(^{2+}\)-imaging, auditory pathways
INTRODUCTION

Extracellular nucleotides such as ATP accomplish important functional roles in the CNS by functioning as neurotransmitters, cotransmitters, neuromodulators, 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 2004, 1972; 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 Mammano 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, affect neurotransmission of the hair cells, and modify 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 Morest 1975; Ryugo 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 the present 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.
MATERIALS AND 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 day nine to fourteen (P9-14) were decapitated and the brain quickly removed. Acute brainstem slices (200 μm) containing the most rostral region of the anteroventral cochlear nucleus were cut by means of a vibratome (Microm HM 650, Walldorf, Germany) as previously described (Milenkovic et al. 2007). The slices were cut in cold (3-4°C) low-calcium 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 myo-inositol, 0.5 ascorbic acid, continuously equilibrated with 5% CO₂ and 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 mM and 1 mM, respectively) for 30 min at 37°C and stored at room temperature (RT) until whole-cell recording. For fluorometric Ca²⁺ measurements, the slices were bulk labeled by incubation with Fura 2 acetoxymethyl ester (10 μM Fura-2 AM in ACSF, Molecular Probes) and 0.025% (w/v) pluronic acid, for 30 min at 37°C. Thereafter, the slices were washed and stored in ACSF at RT in the dark until Ca²⁺ measurement. Recordings were made at RT (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-gluconate, 5 KCl, 1 NaCl, 1 MgCl₂, 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 ATPγS. This metabolically stable ATP analogue (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 Picospritzer (General Valve Corp, 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 neighbouring cells up to a distance of about 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 the whole-cell mode and the membrane potential was recorded under current clamp. All voltages were compensated on-line for -11mV 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 paraformadehyde (PFA 4% in 0.1M phosphate-buffered saline (PBS), pH 7.3) and postfixed overnight at 4°C. The slices were then incubated for 2.5 hours at RT with Cy2-conjugated streptavidin (5 µg/ml, Jackson Immunoresearch Lab., Dianova, Hamburg, Germany), washed (3x10 min in PBS and 1x10 min in dH2O) and finally embedded in glycerol-gelatine 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 Ca\(^{2+}\) measurements

For measurements of intracellular Ca\(^{2+}\) signals, single slices were positioned in the recording chamber (volume \(\sim 0.25\) 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 6-port distribution valve connected to solution reservoirs. For low Ca\(^{2+}\) extracellular solution (low Ca\(^{2+}\)-ACSF), Ca\(^{2+}\) was reduced to 0.1 mM and Mg\(^{2+}\) increased to 2.9 mM. Measurements were done using an inverted microscope (Axiovert 200, Zeiss, Germany); the cells facing the glass coverslip were visualized with a 40x oil immersion objective (Zeiss Fluar). Pairs of images were obtained by an alternative excitation with light of 340 nm and 380 nm wavelengths generated from a monochromator (Polychrome V, TILL Photonics, Gräfelfing, Germany) and the fluorescence emission was long pass filtered (D510, Fura-2 filter set, Chroma Technology Corp., Brattleboro, VT, USA). 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 (\(R = F_{340}/F_{380}\)) or ratio changes relative to baseline (\(\Delta R\) given as percentage). Average values are given as mean ± S.E.M. unless noted otherwise. In the range of physiological Ca\(^{2+}\) concentrations the ratio values are linearly related to the changes in [Ca\(^{2+}\)]. (Grynkiewicz et al. 1985). Ca\(^{2+}\) transients were considered significant when the maximal amplitude exceeded three times the root mean square value of the baseline fluorescence (average of ten images before stimulation) of the respective ROI (\(z > 3\), \(z\)-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 P10-14 and two gerbils P27 were deeply anesthetized with sodium pentobarbitol (10mg/kg body weight, i.p.) 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 brainstem sections (30 µm) containing AVCN were cut using a vibratome (Microm HM 650, Walldorf, Germany). All immunofluorescence procedures were applied to free-floating sections at RT 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-P2X$_2$ or with rabbit anti-P2Y$_1$ antibodies in blocking solution at RT (1:200, APR-003 and APR-009, respectively, Alomone, Israel). The P2X$_2$ antibody was generated using a synthetic peptide corresponding to amino acid residues 457-472 in the C terminus of the rat P2X$_2$. This antibody has previously been used to study P2X$_2$ receptor expression in the rat brain and spinal cord (Atkinson et al. 2000; Florenzano et al. 2002; Rubio and Soto 2001; Studeny et al. 2005). The P2Y$_1$ antibody was raised against a 17-amino-acid peptide, corresponding to the residues 242-258 of rat and human P2Y$_1$ receptor, i.e. the epitope location is in the presumed third intracellular loop (i3) between the TM5 and TM6 domains. Alternatively, the slices were stained with rabbit anti-P2Y$_1$ directed to the intracellular C-terminus of the human receptor (1:500, AB9263, Chemicon). Both antibodies have been used extensively to localize P2Y$_1$ 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- P2Y$_2$, -P2Y$_4$, and -P2Y$_6$ antibodies (1:200, APR-10, APR-006, APR-011, respectively, Alomone, Israel). 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 (1:1000, SWant, Switzerland) (Bazwinsky et al. 2008; Hartig et al. 2001). In some specimens, double labeling was done with antibody against the vesicular glutamate transporter 1 (1:1500, 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 (20µg/ml in blocking solution; Jackson Immunoresearch Lab.) were incubated where appropriate for 2.5 hrs at RT. After rinsing with PBS and dH₂O, the sections were dehydrated in xylol, dried and coverslipped with entellan. The specificity of antisera for P2X₂ and P2Y₁ receptors (Alomone) was analysed 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 brainstem (not shown). Furthermore, the fluorophores related to the relevant markers were switched; for example, P2Y₁ was also revealed 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, Germany).
RESULTS

**ATPγS changes the firing properties of SBCs**

To confirm that Fura-2 AM labels SBCs in P9-14 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 Ca^{2+} transients was subsequently recorded with biocytin-filled pipettes, electrophysiologically characterized (Cao et al. 2007; Schwarz and Puil 1997; Wu and Oertel 1984) (Fig. 1Ac, 1Bc), and the effect of ATPγS on the membrane potential was recorded (Fig. 1Ab, 1Bb).

ATPγS was applied by pressure ejection in 14 slices where it evoked transient increases in [Ca^{2+}], 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 ± 2.6mV from V_m = -60mV, n=9). In 5 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 ± 4.7mV, 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. 1Ac, Bc), consistent with type II discharge pattern of AVCN bushy cells (Francis and Manis 2000; Oertel 1983; Wu and Oertel 1984). Like 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 due to hyperpolarization-activated conductances, and anode-break action potentials occurred after the pulse offset. Biocytin labeling of recorded cells revealed the morphological features of large spherical bushy cells which occupy the anterior pole of the AVCN in low frequency hearing animals (Bazwinsky et al.)
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 ADPβS was set to begin 100 ms prior to the onset of depolarizing current injection (200 ms). The number of action potentials generated during depolarizing steps was significantly increased following application of ATPγS (Fig. 2A, B) but not ADPβS (Fig. 2A) (control= 1.0 ± 0.1, n=14; ATPγS= 9.3± 1.0, n=10, p<0.05; ADPβS (100µM)= 1.2± 0.2, n=7; ADPβS (250µM)= 1.2± 0.2, n=7, p=0.54 one 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 (I_{KL}), persisted even after the end of depolarizing current (Fig. 2C post pulse). Although the application of ADPβS induced membrane depolarization and Ca^{2+} signals, these responses did not change the firing pattern even at higher agonist concentrations (250µM). To determine whether the increase in spike number induced by ATPγS was solely due to 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 Ca\(^{2+}\) chelator BAPTA, inhibitor of Ca\(^{2+}\)-dependent protein kinase (PKC) Gö 6976, and inhibitor of the Ca\(^{2+}\)/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 ATP\(_{\gamma}S\) changes the firing properties by a mechanism dependent on increase in [Ca\(^{2+}\)], and PKC activity, while at the same time this effect appears 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, 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 PPADS. Quantification of these results excluded the possibility that the effect of ATP\(_{\gamma}S\) were due to depolarization of presynaptic terminals and subsequent transmitter release (mean current amplitude evoked by 100 µM ATP\(_{\gamma}S\), 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 ± 1.5 mV, inhibitor cocktail 10.5 ± 1.8 mV, p=0.7; PPADS 0.75 ± 0.2 mV, p<0.05; n=5, one 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 at least 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 µM and 192 µM, respectively.

All bath perfusion experiments were done in the presence of TTX (0.3 µM) to prevent responses evoked by spontaneous presynaptic APs. In addition – same as in the whole cell recordings – it was tested whether it was necessary to block synaptic transmission by applying the inhibitor cocktail (10 µM NBQX, 50 µM AP-V, 25 µM SR95531, and 0.3 µM strychnine). These drugs had no effect on Ca\(^{2+}\) signals evoked by ATP (500 µM) (data not shown): mean peak ratio change relative to baseline ΔR= 24.3 ± 1.9% (n=29) for control and ΔR= 25.6 ± 2.2% (n=32) for inhibitor cocktail, p=0.4, \(t\)-test; time to peak 36.1 ± 1.3 s for control and 34.6 ± 1.3 s for inhibitor cocktail, p=0.4, \(t\)-test. As these blockers did not appear necessary, it was concluded that transsynaptic effects were unlikely and the inhibitor cocktail was not used in further experiments.

To test whether the observed effects might be in part due to activation of P1 receptors by adenosine (Ralevic and Burnstock 1998), which may be generated from ATP by the activity of ecto-nucleotidases (Zimmermann 1996), we compared the effects of ATP...
(100 µM), ATPγS (100 µM), AMP (1 mM), and adenosine (1 mM). Figure 4A plots the amplitudes of the Ca\(^{2+}\) responses as cumulative percentages for 25 investigated cells. The amplitudes clearly comprise two classes: Both ATP and ATPγS evoked comparable strong responses, whereas the application of adenosine and AMP had virtually no effect on cytosolic calcium concentration (cells responding: 2/25 for adenosine and 1/25 for AMP).

Given the metabolic stability of ATPγS (Welford et al. 1987), the mostly identical effects of ATP and ATPγS (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; one way ANOVA, Holm-Sidak post-hoc analysis) (Fig. 4C). Strong effects of ATP and ATPγS along with effects of ADP suggest that the Ca\(^{2+}\) transients in SBCs are mediated by both ionotropic P2X and metabotropic P2Y receptors. Therefore, we next determined the effects of UTP (a preferential agonist at P2Y\(_{2-}\) and P2Y\(_{4}\)-receptors) and UDP (a preferential agonist at P2Y\(_{6}\)-receptors) (Fig. 5A). We found that only 16% and 22% of neurons showed significant Ca\(^{2+}\) signals in response to UDP (500 µM) and UTP (500 µM), respectively. In those, the mean peak Ca\(^{2+}\) changes elicited by UDP were just 17 ± 3% and 10 ± 2% of respective ADP- and ATP-evoked responses. The maximal Ca\(^{2+}\) changes induced by UTP were slightly 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 µM), ADPβS (500 µM), and 2meth-S-ADP (500 µM) (Tokuyama et al. 1995;
Vohringer et al. (2000) evoked responses with similar amplitudes. Still, these Ca\textsuperscript{2+} transients were only \(~\)70\% of the amplitudes evoked by ATP (Fig. 5A).

To further explore the involvement of P2Y\textsubscript{1} receptors in signal transduction in SBCs, we investigated the potency of MRS 2179, a selective P2Y\textsubscript{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. Following 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 µM), a selective antagonist at P2Y\textsubscript{12,13} receptors did not decrease the effect of ADP (Fig. 5C), ruling out possible contributions of ADP-sensitive P2Y\textsubscript{12,13} receptors. These data corroborate the notion that P2Y\textsubscript{1} expressed by SBCs mediate the responses to ADP and, at least partially, the responses to ATP.

P2Y\textsubscript{1} receptors couple to the phospholipase C - IP\textsubscript{3} - Ca\textsuperscript{2+} pathway (King et al. 1998a; von Kugelgen and Wetter 2000). To test whether the Ca\textsuperscript{2+} transients evoked by ADP depend on extracellular Ca\textsuperscript{2+}, the effect of ADP (500 µM) was measured in 2 mM Ca\textsuperscript{2+} and in low Ca\textsuperscript{2+} (0.1 mM) solution (Fig. 6A). Application of ADP in low Ca\textsuperscript{2+} ACSF reliably elicited Ca\textsuperscript{2+} responses, and the peak ratio amplitudes were on average 58 ± 5\% of control responses (n=47, p<0.01, one way ANOVA, Holm-Sidak post-hoc analysis) (Fig. 6B). Furthermore, in the presence of the phospholipase C inhibitor U-73122 (4 µM) (Stam et al. 1998) Ca\textsuperscript{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\textsuperscript{+} ACSF (30 mM K\textsuperscript{+}). All cells tested showed Ca\textsuperscript{2+} rises in response to high K\textsuperscript{+} ACSF (data not shown).
To reveal the contribution of internal Ca$^{2+}$ stores, SBCs were challenged with two applications of ATP or ADP, separated by an interstimulus interval of 15 minutes. The inhibitor of the ER calcium ATPase cyclopiazonic acid (CPA) was perfused between the two drug applications causing a depletion of intracellular Ca$^{2+}$ stores. Compared to a second application under control condition, CPA reduced the Ca$^{2+}$ responses to ADP (500 µM) and, to a lesser extent, to ATP (500 µM) (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, brainstem sections were immunostained against P2X$_2$ and P2Y$_1$, 2, 4, 6 receptor proteins. Representative results shown in Figure 7 demonstrate the P2X$_2$ and P2Y$_1$ immunoreactivity in the rostral AVCN. Punctate labeling was particularly dense in perikarya of large cell bodies (~20 µm diameter) in the rostral AVCN. Preadsorption control experiments (peptide block; insets in Fig. 7A and D) or omission of primary antibody (data not shown) yielded a lack of immunoreaction, indicating specificity of the labeling. To classify the P2X$_2$- and P2Y$_1$-stained cells we colabeled presynaptic endings on SBCs (endbulbs of Held) with VGLUT1 (Zhou et al. 2007) or calretinin antibodies (Bazwinsky et al. 2008; Milenkovic et al. 2007). Both approaches revealed distinct immunoreactivity outlining the P2X$_2$- and P2Y$_1$-positive cells by forming discontinuous rings around the cell somata (Fig. 7C, F). This provides additional confirmation of P2X$_2$- and P2Y$_1$- labeling.
of SBCs. Figures 7G and H show the P2X$_2$ and P2Y$_1$-immunoreactivity in SBCs of P27 gerbils, suggesting that SBCs express the respective receptors in the early postnatal and subadult AVCN. Consistent with previous studies in the rat cerebellum (Amadio et al. 2007; Kanjhan et al. 1996; Moran-Jimenez and Matute 2000; Rubio and Soto 2001), we also found strong P2X$_2$ and P2Y$_1$ immunoreactivity in Purkinje neurons (Fig. 7G, H). No immunolabeling was obtained with antibodies against P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors (Fig. 7I-K). These findings are in good agreement with calcium imaging data which suggested a lack of P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors in SBCs.

In summary, the present data provide strong evidence that P2X$_2$ and P2Y$_1$ receptors evoke membrane depolarization and mediate increases in cytosolic calcium concentration in large SBCs of the cochlear nucleus.
DISCUSSION

Investigation of P2 receptor-mediated responses in the CN of gerbil revealed 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 which elicited complex spikes in the latter case. Moreover, purinergic action involving P2X<sub>2</sub> receptor activation can change the SBCs’ 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 Ca<sup>2+</sup> and activation of PKC and is likely to engage modulation of I<sub>KL</sub> 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, in order to adjust neuronal excitability as reported elsewhere (reviewed in Abbracchio et al. 2009; Burnstock 2004). Still, the actual sources of ATP in the CN remain to be elucidated in future studies.

Methodological consideration

We combined Ca<sup>2+</sup> 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 non-hydrolyzable analogue of ATP and full agonist of mammalian P2X<sub>1-6</sub> receptors (North 2002). Biocytin-filled neurons unambiguously revealed electrophysiological (Cao et al. 2007; Francis and Manis 2000; Leao et al. 2005; Schwarz and Puil 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 µM or 500 µM to provide sufficient exposure of imaged cells to the drugs.

*P2X*₂ and *P2Y*₁ receptors mediate purinergic action in SBCs

We consistently observed highly comparable effects of ATPγS and ATP on \( [\text{Ca}^{2+}]_i \). Either of these P2X receptor agonists induced \( \text{Ca}^{2+} \) responses with higher potency than ADP. Both ATP- and ADP-mediated signaling mobilize \( \text{Ca}^{2+} \) from intracellular stores (Ralevic and Burnstock 1998), but several blockers of the P2Y₁ - PLCβ - IP₃ pathway used in our study had weaker effects on the responses evoked by ATP. Moreover, the large non-inactivating inward currents evoked by ATPγS (Fig. 2D) are consistent with the profile of the ionotropic P2X₂ receptor subtype (Werner et al. 1996). Therefore, we propose that the ATP-evoked \( \text{Ca}^{2+} \) responses in SBCs are mediated by both P2X and P2Y receptors. This assumption is in agreement with in situ hybridization data showing P2X₂ and P2Y₁ receptor mRNA in the cochlear nucleus of the mouse (Allen Brain Atlas) and rat (P2X₂, Kanjhan et al. 1999). Moreover, a dense P2X₂ 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 is 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 P2X₂ receptor-mediated signaling in SBCs. In addition to \( \text{Ca}^{2+} \) influx through ionotropic P2X receptors, it is conceivable that cytosolic \( \text{Ca}^{2+} \) accumulation is additionally promoted by activation of voltage gated calcium channels during membrane depolarization of SBCs (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 Gq - PLCβ pathway (P2Y1,2,4,6,11) and the other Gi protein (P2Y12,13,14) leading to inhibition of adenyl cyclase (Lazarowski et al. 2003). Postsynaptic P2Y1 receptors typically activate IP3 production and mobilization of intracellular Ca2+ (Simon et al. 1995), which is consistent with our experimental results. The comparable effects of several P2Y1 agonists (ADP, ADPβS, 2meth-S-ADP; Waldo and Harden 2004), and the lack of effects of the respective P2Y2,4 and P2Y6 agonists (UTP, UDP; Communi et al. 1996a; Communi et al. 1996b; Lazarowski et al. 1995), provide means to propose P2Y1-mediated Ca2+ increases. This assumption is also confirmed by the inhibitory effect of the selective P2Y1 antagonist MRS 2179. Moreover, P2Y1 receptors show sensitivity to PPADS (Fig. 4C), whereas the P2Y11 receptor is efficiently blocked by suramin, but lacks the sensitivity to PPADS (von Kugelgen 2006), ruling out the possible role of P2Y11 receptors in SBC signaling. A selective antagonist at P2Y12,13 receptors (AR-C69931) showed no effect on the responses evoked by ADP, hence it was concluded that only the P2Y1 subtype among the ADP-sensitive P2Y receptors (P2Y1,12,13) mediates the calcium signals in SBCs.

ADP elicited larger calcium responses in the presence of 2 mM than 0.1 mM [Ca2+]o (Fig 6A, B), suggesting that the calcium influx from extracellular space contributes to the Ca2+ transients. Since ADP is a very weak agonist of P2X receptors (Bianchi et al. 1999; Evans et al. 1995), it is likely that capacitive Ca2+ entry through store-operated channels contributed to the signals. The store-operated channels are functional in neuronal and glial cells and allow Ca2+ influx across the plasma membrane to refill depleted intracellular stores (Parekh and Putney 2005). In line with this, removal of extracellular Ca2+ reduced the signals evoked by P2Y1 receptors, whereas reperfusion of 2 mM [Ca2+]o resulted in a return of the [Ca2+]i to the baseline (Fig. 6A), indicating that Ca2+ entered the cell through store-operated channels. Similar observations were made...
previously both in neuronal and in glial cells following P2Y1 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 P2X1 and P2X3 receptors in hair cells and supporting cells of the cochlea of rats and mice (Huang et al. 2006; Nikolic et al. 2001; Nikolic et al. 2003). Recently, Tritsch et al. 2007 specified that supporting cells of the Kölliker’s organ release ATP which generates synchronous activity in 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 brainstem, i.e. cochlear nucleus of the cat (Walsh and McGee 1988), nucleus magnocellularis and laminaris of chicken (Lippe 1994), and in the inferior colliculus of bats (Rubasamen 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). The present 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 $[Ca^{2+}]_i$, PKC activity, and possibly modulation of $I_{KL}$ conductance. $I_{KL}$ conductance is one of the important features of neurons along the afferent auditory pathways which ensure minimal latency fluctuations and preservation of timing (Kaczmarek et al. 2005; Trussell 1999). In neurons of the AVCN and MNTB, $Kv1.1$ channels contribute to a low-threshold $K$ current that leads to strong
accommodation in vitro (firing of a single, short latency AP in response to prolonged depolarizing current steps) (Cao et al. 2007; Brew and Forsythe 1995; Manis and Marx 1991) and increases temporal precision of auditory signaling in vivo (Kopp-Scheinpflug et al. 2003). Prominent somatic Kv1.1 subunit expression was revealed for AVCN bushy cells and principal MNTB neurons (Caminos et al. 2005; Grigg et al. 2000). In bushy cells of the mouse (and in neurons of the nucleus magnocellularis, the avian homologue 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 protein kinase C (PKC) (Boland and Jackson 1999). In agreement with our data, bushy cells in rats and mice express cPKC (isoforms βI and βII) (Garcia et al. 2000; Garcia and Harlan 1997) which can be activated by Ca²⁺ and diacylglycerol, also known to be increased by P2X₂ 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 AP 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⁺ due to substantial Ca²⁺ permeability of P2X receptors (Egan and Khakh 2004). Still it remains puzzling whether P2Y₁ receptors accomplish some additional function in SBCs or perhaps exert a modulating effect on P2X₂ receptor, as it 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 firing pattern of auditory neurons to the ambient acoustic environment (Kaczmarek et al. 2005; Song et al. 2005).
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 precission 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 precision of the sound localization.

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FIGURE LEGENDS:

Figure 1:

SBCs but not stellate cells show responses to ATPγS. (Aa) Fluorescence image of Fura-2 labeled cells (excitation wavelength 380 nm). Regions of interest (ROI) mark the somata of 4 cells. ATPγS (500 µM) was applied through the puff pipette (2 psi, 500 ms). Calcium responses were recorded in all 4 cells with amplitudes depending on the cells’ distance from the puff pipette. Numbers next to traces correspond to the cells outlined in the upper image. (Ab) Cell # 2 was subsequently selected for whole-cell recording and the effect of ATPγS application was measured under current clamp from the V_m = -61 mV, close to the V_rest in SBCs of the respective age (Milenkovic et al. 2007). Fast action potentials riding on the depolarization, as shown here, were recorded in 5 out of 14 neurons. Inset: Voltage trace detail from the peak response marked by asterisk. (Ac) Electrophysiological characterization revealed the characteristic properties of SBCs: depolarizing current pulse evoked single action potential and hyperpolarizing current evoked responses which sagged toward rest. Biocytin labeling of this cell shows the typical morphology of large SBCs. Data in Ab-c are from the same SBC. (Ba) Traces of 9 individual neurons recorded from a different slice. (Bb) Whole-cell recording on a neuron depicted by the black line in Ba. In most recorded neurons (9/14), ATPγS caused a moderate depolarization of the membrane potential as shown in Bb (without evoking APs as in Ab). (Bc) Electrophysiological and morphological characteristics of recorded cell point to large SBC from the rostral AVCN. Data in Bb-c are from the same SBC. (C) Stellate cell did not respond to ATPγS application.
(A) Activation of P2X receptors increases action potential firing in SBCs. Brief pressure ejection (150 ms) of ATPγS (100 µM) (gray traces) preceding the onset of depolarizing current pulse by 100 ms, elicits bursts of action potentials (top) temporally correlated to the Ca^{2+} signal (bottom). Activation of P2Y_1 receptors by ADPβS (100 µM) (black traces) evoked smaller Ca^{2+} transients and did not increase the number of APs compared to control. Ca^{2+} traces are average of 3 trials. (B) The effect of ATPγS is not solely mediated by depolarization. Depolarization by a current pulse to the same level as by ATPγS-puff followed by a standard current step did not increase the number of APs. (C) Bars show summarized data for the number of action potentials generated during depolarizing step (current pulse) and for 5 s period after the pulse (post pulse) (mean ± S.E.M.) (● p<0.05 compared to control; ○ p<0.05 compared to ATPγS, one way ANOVA on ranks, Dunn’s test post-hoc analysis). (D) Responses to ATPγS are solely mediated by the postsynaptic P2 receptors. Cocktail of inhibitors which blocks AMPA, NMDA, GABA_A, and glycine receptors (10 µM NBQX, 50 µM AP-V, 25 µM SR95531, and 0.3 µM strychnine) had no influence on the whole-cell currents (top) or on membrane depolarization (bottom) induced by a brief (100 ms) ATPγS application. Responses were inhibited by PPADS, a broad spectrum P2-receptor antagonist. Data quantification is given in the text.

Figure 3:

Increases in [Ca^{2+}], 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 due to P2 receptor desensitization, 15 min-breaks were introduced between applications (B) Concentration-response curves for ATP and ADP. The curves
were fitted with \( f = \frac{b \cdot x}{x + a} \) with \( a=112 \, \mu\text{M} \) and \( a=192 \, \mu\text{M} \), 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). % of cells showing significant responses for ATP and ADP, respectively: 88, 30 for 10 µM; 98, 81, for 100 µM; 100, 85, for 500 µM. At concentrations of 1 and 5 mM all cells showed significant responses.

Figure 4:

**P2X and P2Y receptors mediate Ca\(^{2+}\) responses in SBCs.** (A) Cumulative distribution plot of the peak ratio values obtained in 25 cells. The values are sorted by size (abscissa) between 0 and 100% (ordinate) and show clear grouping with respect to agonists indicated in the graph. (B, top) Examples of the calcium responses elicited in individual neurons. (B, bottom) Mean values (± S.E.M.) of the ratio changes. No significant difference was measured between ATP (100 µM) and ATP\(_{\gamma}\)S (100 µM) responses (n=72 and n=81 cells, respectively, p=0.87). The ectoATPase inhibitor ARL 67156 (50 µM) had no effect on the responses evoked by ATP (100 µM, n=46, p=0.62) or by ADP (100 µM; n=44, p=0.44). (C) Amplitudes of ATP-elicited [Ca\(^{2+}\)]\(_i\) increases were decreased by the P2 antagonists PPADS (100 µM) and suramin (100 µM). Data are mean ± S.E.M. Asterisks indicate values significantly different from controls (black bar) (p<0.01, one way ANOVA, Holm-Sidak post-hoc analysis).

Figure 5:

**Activation of P2Y\(_1\) receptors increases [Ca\(^{2+}\)]\(_i\) in SBCs.** (A) Mean amplitudes of the Ca\(^{2+}\) signals evoked by 500 µM ATP (n=129), ADP (n=93), ADP\(_{\beta}\)S (n=78), 2meth-S-ADP (n=55), UDP (n=50), and UTP (n=50) (mean ± S.E.M). UDP and UTP elicited significant responses in 16% and 22% of the neurons, respectively. Asterisks indicate
significant differences (p<0.01, one way ANOVA, Holm-Sidak post-hoc analysis). (B) The selective P2Y$_1$ receptor antagonist MRS 2179 inhibited the responses evoked by ATP and ADP in a concentration-dependent manner. Asterisks indicate values significantly different from controls (black bars) (p<0.01, one way ANOVA, Holm-Sidak post-hoc analysis). Data are mean ± S.E.M. (C) AR-C69931MX (1 µM), a specific P2Y$_{12,13}$-receptor antagonist had no effect on the Ca$^{2+}$ response evoked by ADP (100 µM). Left panel shows the traces of a single neuron and the right panel summary data for 29 cells (mean ± S.E.M.; p>0.05).

Figure 6:

Calcium responses to ADP and partially to ATP are mediated by the PLC - IP$_3$ pathway. (A) ADP-elicited responses are persistent in low extracellular calcium solution (0.1 mM Ca$^{2+}$; values show mean ± S.E.M. for 10 cells in one slice). When [Ca$^{2+}$]$_o$ was restored, there was a transient increase in [Ca$^{2+}$]$_i$ until it reached the control level. Repeated stimulation with ADP under control conditions evoked larger response. (B) Mean peak responses evoked by ADP under two experimental conditions: 2 mM [Ca$^{2+}$]$_o$ and 0.1 mM [Ca$^{2+}$]$_o$. Asterisks indicate significant differences (n=47 cells, mean ± S.E.M.; p<0.01, one way ANOVA, Holm-Sidak post-hoc analysis). (C) ATP- and ADP-induced calcium signals are decreased in the presence of the phospholipase C inhibitor U73122. Asterisks indicate significant difference relative to control (black bars) (p<0.01). (D) Depletion of intracellular Ca$^{2+}$ stores by cyclopiazonic acid (CPA) blocked ATP- and ADP- elicited responses in a concentration-dependent manner (mean ± S.E.M.; significant differences relative to control (black bars) are indicated by asterisks (p<0.001, one way ANOVA, Holm-Sidak post-hoc analysis)).
Figure 7:

Immunohistochemical staining for P2X$_2$, P2Y$_1$, P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptor proteins on coronal sections of the AVCN. At the rostral pole of the cochlear nucleus, the P2X$_2$ (A-C, red fluorescence, arrows in C) and P2Y$_1$ 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 - PB) (insets in A and D). The confinement of P2X$_2$ and P2Y$_1$ labeling to SBC was exemplified by staining the endbulbs of Held with anti-VGLUT1 antibody (C, green fluorescence, arrowheads) or anti-calretinin antibody (D, red, arrowheads). Double labeling revealed a staining pattern apposing the P2X$_2$- and P2Y$_1$- positive surfaces of neuron somata. Distribution of the P2X$_2$ and P2Y$_1$ immunoreactivities at P27 indicates persistent expression of both receptor proteins by SBCs two weeks 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 P2Y$_2$, P2Y$_4$, and P2Y$_6$ receptors showed merely background fluorescence (J, K, L, respectively). Scale bars: A, D: 50 µm; B, E: 20 µm; C, F: 10 µm; G-K: 100 µm.
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