Functional expression of P2X4 receptor in capillary endothelial cells of the cochlear spiral ligament and its role in regulating the capillary diameter

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Wu T, Dai M, Shi XR, Jiang ZG, Nuttall AL. Functional expression of P2X4 receptor in capillary endothelial cells of the cochlear spiral ligament and its role in regulating the capillary diameter. Am J Physiol Heart Circ Physiol 301: H69–H78, 2011. First published April 1, 2011; doi:10.1152/ajpheart.01035.2010.—The cochlear lateral wall generates the endocochlear potential (EP), which is dynamically and precisely regulated to meet the changing metabolic needs of the cochlear lateral wall. The anterior inferior cerebellar artery is the end artery to the cochlea (48, 45, 47). Its branch arterioles supply capillaries in the SL and SV (1). SL capillaries are thought to regulate the blood flow of the more distal downstream capillaries leading to the SV. The blood flow in the arteries and arterioles of the cochlea is regulated by smooth muscle contraction or relaxation. In contrast, capillaries, which are composed of a single layer of endothelial cells (ECs) and a basilar membrane, lack smooth muscle and are known to have different mechanisms to regulate blood flow (8). Recent studies have shown that pericytes, which encircle capillaries at intervals, have a contractile function in the cochlea, retina, and brain (4, 49, 18, 42). It has also been reported that ECs by themselves can have contractility (12, 7, 2, 25, 5, 30).

ATP is an important extracellular signaling molecule in a wide variety of peripheral tissues. It activates two classes of receptors: 1) P2X(1–7) ligand-gated ion channels, and 2) P2Y receptors coupled to G protein (17, 53, 13, 9, 19, 39). In the cochlea, ATP is an important signaling element in physiological and pathophysiological auditory processes (36, 15, 16, 55, 27, 28). Both peri- and endolymph ATP levels increase following acoustic stress (33, 35). Moreover, ATP plays an important role in the regulation of cochlear blood flow (CBF). Arterial perfusion of the anterior inferior cerebellar artery with ATP caused a biphasic change of CBF with an initial reduction followed by a lasting increase, which was mediated through the nitric oxide (NO) pathway (47). Furthermore, perilymphatic perfusion of ATP caused an increase of blood flow in the cochlear lateral wall (34). However, the cellular mechanisms underlying the regional blood flow changes elicited by ATP have not been elucidated.

P2X4 is the dominant P2X channel expressed in ECs and has been hypothesized to be a part of the mechanism underlying blood flow-dependent vasodilation mediated by shear stress (11, 44, 3). The most commonly used preparation in studies of EC purinoreceptor function is cultured vascular endothelial cells [human and bovine umbilical vein endothelial cells (UVECs)]. In the cultured UVECs, ATP induces Ca2+ influx possibly through the P2X4 channel and the subsequent production of NO, a vasodilator (64, 65). In their hallmark study, Yamamoto et al. (66) demonstrated that P2X4-deficient mice presented an impaired flow-dependent control of vascular tone. However, direct evidence of a P2X4 tonic current was not reported in any of the above studies. Contrary to the above findings, Wilson et al. (60) found no evidence of the characteristic P2X4 current in UVECs, which suggests a difference between cultured and native ECs. In the current study, we set out to resolve this contradiction, as we are not aware of any
report on the P2X4 current (patch-clamp data) in native ECs of freshly isolated capillaries.

To address the questions of the cellular mechanism of ATP in control of the CBF and the role of the P2X4 receptor on ECs, we made a unique preparation of freshly isolated native capillaries from the cochlear SL. These segments of capillaries had only ECs, i.e., they lacked pericytes. Through a patch-clamp technique, we found that ATP elicited a characteristic P2X4 current and a subsequent transient intracellular Ca\(^{2+}\) increase in ECs. In accordance with the in vitro finding, via use of a unique window preparation in the bony otic capsule, ATP caused a dilation of the SL capillaries in vivo through the NO signaling pathway.

**MATERIALS AND METHODS**

*Ethical Approval*

All procedures in this study were approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University.

*In Vitro Capillary Preparation for Patch-Clamp Recordings*

Adult guinea pigs (250–300 g) with positive Preyer’s reflex were anesthetized by intramuscular injection of an anesthetic mixture (60 mg/ml ketamine, 2.4 mg/ml xylazine, and 1.2 mg/ml acepromazine in saline) at a dose of 1 ml/kg and were then killed by decapitation. The cochleas were rapidly removed from the bulla and dissected in a Petri dish filled with a standard artificial perilymph composed of the following (in mM): 144 NaCl, 1.3 CaCl\(_2\), 0.9 MgCl\(_2\), 0.7 Na\(_2\)HPO\(_4\), 10 HEPES, and 5.6 glucose. The osmolarity of the solution was adjusted to 304 mosM with glucose, and the pH was adjusted to 7.4 with NaOH. All procedures were performed at room temperature.

The cochlear lateral wall (SV and SL) at the second and third turn was dissected and then digested in collagenases (II and IV) for 2 h at 37.0°C. The SL containing capillaries were obtained by peeling off the SV and trimming off the lower half of the SL containing the more distal capillaries. With the use of a dissection microscope, the remaining SL containing more proximal capillaries was dissected with a needle (30½ G) into small pieces, which were gently triturated to separate the capillaries from the surrounding tissue (fibrocytes) in a Petri dish with a glass bottom coated with poly-L-lysine. The isolated segments of capillaries were then allowed to settle onto the glass bottom for 30 min. Through the above-described procedures, all the isolated capillary segments were composed of a single layer of ECs without any pericytes left at higher magnification (10×40) by morphological identity. The finding was also confirmed by our further immunocytochemistry study with co-labeling of vascular endothelial (VE)-cadherin, desmin, and Hoechst showing that the isolated segments of capillaries were continuously perfused with a bath solution before the patch recordings.

*Solutions and Chemical Applications for Patch-Clamp Recordings*

ATP (A-9187; Sigma, St. Louis, MO), 2'- and 3'-O-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (BzATP; B-6396; Sigma), α,β-methyleneadenosine 5'-triphosphate (α,β-MeATP; M-6517; Sigma), pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS; P-178; Sigma), 5-(3-bromophenyl)-1,3-di-hydro-2H-benzofuro[3,2-ε]-1,4-diazepin-2-one (5-BDBD; 3579; Tocris Bioscience, Ellisville, MO) (6), and lanthanum chloride hydrate (L4131; Sigma) were directly dissolved in solutions. The drugs were gravity delivered at a rate of 0.35 ml/min through an array of parallel polyethylene tubes with an inner diameter of ~280 μm at the distal end (see Ref. 61 for a more detailed description). The polyethylene tube was positioned at a distance of ~350 μm from the ECs. Rapid switching of two different solutions was performed by shifting the fluid loaded tubes. Bath solutions containing the following (in mM): 141 NaCl, 5 KCl, 1.6 CaCl\(_2\), 1.2 MgCl\(_2\), 6 HEPES, 5 HEPES-Na, and 5.6 n-glucose were adjusted to a pH of 7.4 with NaOH and osmolarity to 304 mosM with n-glucose. Regular pipette solutions containing the following (in mM): 130 K-glucurate, 10 NaCl, 2 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPES, and 5 EGTA were adjusted to a pH of 7.2 with KOH and osmolarity to 298 mosM with n-glucose.

*Whole Cell Recordings of ECs*

The procedures and settings are briefly described as follows (see Ref. 61 for a more detailed description). An Axopatch 1-D amplifier (Axon Instruments) was used to record ionic currents with its low-pass filter bandwidth set to 1 kHz (four-pole Bessel). Membrane currents were recorded for episodic current-coltage (I–V) commands with a Digidata 1322A (Axon Instruments) interface and pCLAMP 8 software (Axon Instruments) at a sampling rate of 10 kHz and for simultaneous gap-free recording with Minidigi digitizer and Axoscope 9.2 software (Axon Instruments) at a sampling rate of 50 Hz. Through a gap-free recording mode, we could continuously monitor the change of current at a holding potential (Vh) of ~70 mV when ATP was administered and decided on the time to apply step voltage commands for I–V plots. The whole cell configuration was achieved by rupturing the cell membrane with suction after achieving a high-resistance seal (>2.5 GΩ). Stability of the patch was ascertained by monitoring the gap-free recording and the cell parameters [cell capacitance (C\(_{cell}\)), membrane resistance (R\(_{m}\)), and series resistance (R\(_{s}\))] during the recordings. The patch pipettes were pulled in four steps with a puller (P80/PC Sutter Instruments) from the borosilicate capillaries (WPI, 1B150F-4) with the tip diameter of ~1 μM and the initial resistance of 6–7 MΩ in our regular Na\(^+\)-rich bath and K\(^+\)-rich pipette solutions. The uncompensated R\(_{s}\), 17.1 ± 1.0 MΩ (n = 20), was corrected off-line with the equation V\(_i\) = V\(_o\) – I \(\times\) R\(_{s}\), where V\(_o\) is corrected clamping voltage, V\(_i\) is uncorrected clamping voltage, and I is current) in Excel spreadsheets and Origin 7.5 (OriginLab Technical) files. To stabilize the liquid junction potential (LJP), a salt bridge (3 M NaCl) with a ceramic tip was used as a reference electrode. The LJP (actual measurement) was 3 mV in a regular Na\(^+\)-rich bath and K\(^+\)-rich pipette solutions. The LJP was corrected in the Excel spreadsheets and Origin 7.5 (OriginLab Technical) files. The data were analyzed by clampfit 9.0 (Axon Instruments) and origin 7.5 (OriginLab Technical).

*Fluorescent Imaging of Intracellular Ca\(^{2+}\) of SL Capillaries*

The cochlear lateral wall (2nd or 3rd turn) was incubated with the fluorescent dye fluo-4 (10 μM) for 60 min. After excess fluo-4 was thoroughly washed away by the perilymph solution, the cochlear lateral wall was secured on the glass bottom of a Petri dish and perfused with the perilymph bath. The perfusion pipette was usually from 5 to 6 mm away from the ECs when the solution was switched to one containing ATP. A confocal laser microscope (Olympus Fluoview FV1000) was used to acquire time-lapse imaging of fluorescence signals (with 488-nm excitation and 520-nm emission filters). The fluorescence intensity was normalized by the intensity in the control bath before ATP or bath control application.

*Immunohistochemistry*

Isolated capillary segments. The freshly isolated capillary segments, which stuck to the glass bottom of a Petri dish, were constantly perfused with 4% paraformaldehyde with the perfusion system used for our patch-clamp experiment for 30 min. In the perfusion system, the fluid flow through a small distal perfusion tube (~280 μm) was well controlled not to move the samples and a constant aspiration was
maintained to remove excessive solutions. All the washing and solution switching procedures were performed through this perfusion system. The capillary segments were incubated in 4% paraformaldehyde for 4 h, washed in 0.02 M PBS for 30 min, permeabilized in 0.5% Triton x-100 for 30 min, and then immunoblocked in a solution of 10% goat serum in 1% bovine albumin in 0.02 M PBS for 1 h. The tissues were incubated overnight in an antibody for VE-cadherin (ab7047, mouse monoclonal; Abcam, Cambridge, MA) and desmin (ab32362, rabbit monoclonal) diluted to 1:10 and 1:100 respectively with 1% BSA-PBS. After that, the samples were washed in 0.02 M PBS for 30 min and then incubated in Alexa fluor 488 anti-rabbit and 568 anti-mouse IgG (both diluted to 1:100; Invitrogen, Eugene, OR) for 1 h and Hoechst (2 μg/ml) for 15 min. After being washed in 0.02 M PBS for 30 min, the tissues were observed on an Olympus IX81 inverted microscope fitted with an Olympus Fluoview FV1000 confocal laser microscope system. The negative controls were tissues incubated with 1% BSA-PBS in place of the primary antibody.

Whole mount cochlear lateral wall. The freshly isolated cochlear lateral wall was incubated with an artificial perilymph solution containing 10 μM dinitrofluorescein-2 diacetate (DAF-2DA; Calbiochem Novabiochem), the NO marker, for 30 min. After being washed in 0.02 M PBS for 30 min, the tissues were incubated overnight in an antibody for P2X4 (cat. no. ab82329), P2X2 (cat. no. ab10266), or P2X5 (cat. no. ab82327) (rabbit polyclonal; Abcam) diluted to 1:1,000 with 1% BSA-PBS. After that, the specimens were fixed in 4% paraformaldehyde for 4 h, washed in 0.02 M PBS for 30 min, permeabilized in 0.5% Triton x-100 for 1 h, and then immunoblocked in a solution of 10% goat serum in 1% bovine albumin in 0.02 M PBS for 30 min. The tissues were washed in 0.02 M PBS for 30 min and then incubated in Alexa fluor 568 anti-rabbit IgG for 1 h. After being washed in 0.02 M PBS for 30 min, the tissues were mounted and observed on an Olympus IX81 inverted microscope fitted with an Olympus Fluoview FV1000 confocal laser microscope system. The negative controls were tissues incubated with 1% BSA-PBS in place of the primary antibody.

**In Vivo Observation of SL Capillaries**

**Surgical preparation.** The animals were anesthetized with an injection of ketamine (40 mg/kg; Hospira, Lake Forest, IL) and xylazine (10 mg/kg; Abbott Laboratories, Chicago, IL) and were then wrapped in a heating pad with rectal temperature maintained at ~38°C. The head was fastened into a manipulator, which was heated to prevent conductive cooling. A ventrolateral surgical dissection was carried out to expose the bulla, which was then opened over its ventral surface. To observe the blood circulation in the SL capillaries, we used a method that we have previously described (41, 50). In brief, a rectangular fenestration (0.2 × 0.3 mm) into the cochlea was made by creating a window opening over the spiral ligament after scoring a rectangle of scratch marks into the bone of the fourth turn. The vessels of the cochlear lateral wall can be visualized under intravital microscope through this window opened on the cochlear lateral wall. We named the window “vessel-window” in this study. By adjusting the optical focus, we visualized the vessels of the spiral ligament. The vessels within the vessel-window were monitored with intravital video-microscopy using a long-working-distance objective lens (×20, 0.4 NA, 20 mm WD).

**Time-lapse photography.** The procedures are briefly described as follows (see Ref. 4 for a more detailed description). The cochlear

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Fig. 1. ATP elicited an inward current with desensitization. A: representative image of a segment of isolated spiral ligament (SL) capillary with an endothelial cell (EC) in whole cell configuration. B: ATP (1 mM) elicited a representative inward current with desensitization at a holding potential (Vh) of −70 mV. The desensitization current part is well fitted with a regular one-term exponential function (y = A * e^(-t/τ) + Y₀), where y is current amplitude, t is time, A in initial current, Y₀ is offset, τ is time constant, τ = 5.64 s and regression coefficient of 0.992. C: At a Vh of 0 mV, ATP (1 mM) did not elicit any obvious current. Immunofluorescence staining (top row) shows that an isolated capillary segment was colabeled with Hoechst (D), vascular endothelial (VE)-cadherin (E), and desmin (F). G: differential interference contrast (DIC) image. H: merged image. I: Hoechst. J: negative control of VE cadherin. K: negative control of desmin. L: DIC image. M: merged image.
were as follows 10.4 ± 0.4 pF for cell capacitance (C), 2.2 ± 0.1 GΩ for membrane resistance (Rm), 33.7 ± 2.0 MΩ for series resistance (R), and −27.6 ± 1.6 mV for resting potential (RP). Figure 1B shows that ATP application for more >10 s (s) caused a typical transient inward current (~100 pA) with desensitization at a holding potential of −70 mV. The desensitization curve was well fitted with a one-term exponential function (y = A exp(−αt) + Y0) with the time constant (α) = 5.64 s and regression coefficient of 0.992. The average τ was 5.29 ± 5.27 s for 1 mM ATP (n = 5). At Vh of 0 mV (Fig. 1C), ATP (1 mM) did not elicit any obvious current (n = 3), suggesting a reversal potential (Vr) of 0 mV. This finding is consistent with a nonelective cation channel (NSCC), the characteristic of P2X receptors. Colabeling of isolated capillary segments with 1) VE-cadherin (an endothelial-specific marker), 2) desmin (a pericyte-specific marker), and 3) Hoechst (stains nuclei) shows that all the isolated segments of capillary were stained with only VE-cadherin and Hoechst without any desmin labeling; thus, the isolated capillary segments did not contain any pericytes (Fig. 1, D–M).

The ATP-induced currents were dose-dependent with EC50 of 0.16 mM (Fig. 2, A and B). The current became desensitized after the first ATP application and did not fully recover after 15 min (min) or longer of washing time (Fig. 3).

The NSC blocker LaCl3 (1 mM) blocked the normalized ATP (1 mM) induced current by 93.1% [from 8.0 (n = 7) to 0.6 pA/pF (n = 4); Fig. 4, A and B]. The P2X blocker PPADS (1 mM) blocked the normalized ATP current by 81.9% [from 8.0 (n = 7) to 1.6 pA/pF (n = 3); Fig. 4]. The P2X4 specific blocker 5-BDBD (30 μM) blocked the normalized ATP current by 55.6% [from 8.0 (n = 7) to 3.6 pA/pF (n = 6); Fig. 4]. Since the ATP-induced current does not recover after a long washing time (Fig. 3), the control ATP current and the ATP current pretreated with the other chemicals were from different individual experiments. Consistent with P2X4, the channel was less sensitive to α,β-MeATP (1 mM), which elicited only 12.2% of the normalized current of regular ATP (1 mM) [from 8.0 (n = 7) to 1.0 pA/pF (n = 6); Fig. 4, A and B]. The channel was also less sensitive to the specific P2X7 agonist BzATP (1 mM), which caused 15.7% of the normalized current of regular ATP [1 mM; from 8.0 (n = 7) to 1.3 pA/pF (n = 7); Fig. 4]. Consistent with P2X4, extracellular acidification (from pH 7.4 to 5.5) reduced the normalized ATP-induced current by 76.7% [from 8.0 (n = 7) to 1.9 pA/pF (n = 4); Fig. 5, A–C]. Interestingly, the P2X4 agonist ivermectin (IVM; 3 μM) did...
not potentiate the ATP-induced current but, instead, reduced the current by 76% \( (n = 3) \); data not shown).

**ATP Elicits a Transient Increase of Intracellular Ca\(^{2+}\) in ECs and Pericytes**

Consistent with the patch-clamp data, ATP (1 mM) caused a transient increase of intracellular Ca\(^{2+}\) fluorescent signals by 85% \( (n = 5) \) in ECs and 51.5% \( (n = 5) \) in pericytes (Fig. 6, A–D). A delayed intracellular Ca\(^{2+}\) \([\text{Ca}^{2+}]_i\) fluorescent signals recovery following the peak level was observed in pericytes with respect to the ECs. No significant change of \([\text{Ca}^{2+}]_i\) was observed in the ECs and pericytes when tissues were superfused with an ATP-free bath solution.

**P2X4 Is the Predominant P2X Channel in SL ECs**

With fluorescent immunohistochemistry, we found that P2X4, like DAF-2DA (the NO marker), was expressed in ECs and pericytes (Fig. 7, A and B), and staining of P2X4 and DAF-2DA was well overlapped (Fig. 7 D). In comparison, the other slow desensitized P2X channels, such as P2X2 and P2X5, were not significantly stained in ECs and pericytes (data not shown).

**ATP Induced an In Vivo Dilation of SL Capillaries Through the NO Pathway**

Figure 8, A and B, shows the representative images of SL capillary dilation induced by superfusion of ATP (1 mM) in artificial perilymph. Figure 8, C and D, shows the representative images of the inhibitory effect of 5-BDBD (30 \( \mu \)M), a specific P2X4 inhibitor, on the ATP-induced SL capillary dilation. Figure 8, E and F, shows the representative images of the inhibitory effect of L-NAME (10 mM), a non-isofrom-specific inhibitor of nitric oxide synthase, on the ATP-induced SL capillary dilation. In vivo, the superfusion of ATP (1 mM) in perilymph significantly dilated the SL capillaries by 11.5 ± 1.4% at 70 s \( (n = 6) \); Fig. 8G). 5-BDBD (30 \( \mu \)M) blocked the
superfusion of artificial perilymph alone did not dilate or constrict the SL capillaries (data not shown).

**DISCUSSION**

**Evidence of P2X4 Receptors in ECs of SL Capillaries**

P2X receptors are membrane nonselective cation channels gated by extracellular ATP. In the current study, at the holding voltage of $-70$ mV, ATP activated an inward current that was blocked by La$^{3+}$, a known NSCC blocker, and PPADS, a nonselective purinergic receptor blocker. The finding is consistent with P2X receptors (39, 38). However, it is known that there is no single specific agonist or antagonist that can distinguish P2X from P2Y receptors (17, 53, 13, 9, 19, 39). One approach to distinguish P2X from P2Y is by examining the time course of the current onset (43); it usually takes $<10$ ms for a P2X channel to open after ATP application vs. $>100$ ms for a P2Y receptor to have a response on cells, but in our experiment settings, the tissue perfusion rate was not fast enough to use this criterion. However, the following evidence supports the presence of a P2X receptor. 1) In the current study, continuing application of ATP for $>10$ s elicited a typical P2X inward current with desensitization ($\tau = \sim 5$ s), which is a characteristic of heterologously expressed P2X receptors (slow desensitization channels) (39). 2) Consistent with P2X channels, recovery from the desensitization in our study was extremely slow (39); after 15 min of washing, a second application of ATP did not elicit a current as large as observed in the first application (Fig. 3). 3) In addition, the ATP current has a $V_r$ of 0 mV. We are not aware of any P2Y-connected channel that presents the same characteristics.

The next question is what subtype of P2X channels it is? That 5-BDBD, the specific P2X4 blocker (6), blocked most of ATP-induced in vivo dilation of SL capillaries (by 80.1%) and in vitro ATP current (by 55%) is the direct evidence that P2X4 is the dominant P2X channel in the SL capillaries. The different conditions of ECs in in vivo and in vitro experiments may account for the differential sensitivity to 5-BDBD, i.e., the sensitivity of isolated EC was likely compromised during the relatively harsh isolation procedures.

In addition, the other features of the ATP current are consistent with P2X4. Homomeric P2X2 channels can be classified into two groups in terms of the desensitization rate (39); 1) the fast desensitization channels contain P2X1 and P2X3 with $\tau = 0.01$ to 0.3 s, and 2) the slow desensitization channels contain P2X2, P2X4, P2X5, and P2X7 ($\tau = 1$ to $>10$ s). Since the ATP current has a desensitization with $\tau = 5.23$ s, the fast desensitization channels (P2X1 and P2X3) can be excluded from the candidate list. The insensitivity of the ATP current to $\alpha,\beta$-MeATP provides further evidence to exclude P2X1 and P2X3, which are sensitive to $\alpha,\beta$-MeATP (40, 39). Contribution from the homomeric P2X6 receptor can also be excluded since P2X6 is usually silent; ATP does not elicit any current from it (21, 51). Among slow desensitization channels, P2X7 is excluded in the current study based on its greater sensitivity to BzATP than to ATP and an almost total lack of desensitization following continuing application of ATP (18, 39, 23, 24). P2X2 does not seem to be likely, since the P2X2 current has a relatively long desensitization time course with a $\tau$ of $>10$ s (39, 29) and is usually potentiated by extracellular acidification (22, 59). However, the ATP current in our study is reduced by extracellular...
cellular acidification, which actually suggests P2X4 (52, 58). The second piece of evidence supporting P2X4 is that the ATP current has a desensitization rate of 5.23 s. The desensitization rate of heterologously expressed P2X4 is intermediate between P2X1 and P2X2, usually with a rate from 5 to 10 s. The third piece of evidence is such an intense immunostaining of P2X4 in ECs that it can be used as a marker like DAF-2DA (the NO marker) to outline the SL capillaries. In comparison, the immunostaining of the other two slow desensitization receptors, P2X2 and P2X5, is too weak to be detected, which is consistent with the literature (11, 44). P2X5 has a similar desensitization rate to P2X4 (39). Different from the other P2X receptors, P2X5 (rat) has much smaller ATP-elicited current (50 to 200 pA) when expressed in HEK cells (39). However, in native tissue, the current amplitudes through the P2X receptor depend on the density of the P2X channels, which varies in different tissues. Therefore, current amplitude is not a good criterion to distinguish P2X5 in native tissue. However, in this and other studies (44), P2X5 immunostaining in ECs was not detected. Although the functional and immunohistochemistry data confirm the expression of P2X4 in ECs, it would be helpful if we could find the P2X4 transcript in ECs. However, it is technically too difficult to collect the enough pure RNA of ECs without contamination of RNA from other cell types in the cochlear lateral wall.

One of the characteristics of heterologously expressed P2X4 receptor is that IVM potentiates the ATP current (20). The mechanism is unclear, although allosterical modulation of P2X4 has been suggested. However, for an unknown reason, the action of IVM has not been seen in native tissues (neurons) that express abundant P2X4 (20). The reason is unknown. In the current study, we also did not see the potentiating action of IVM on the ATP current in the freshly isolated SL ECs; instead, we saw that IVM partially inhibited the ATP current. The finding suggests that the P2X receptor in SL EC may not be a pure homomeric P2X4 but rather heteromeric P2X receptors containing the P2X4 subunit. So far, the subunit composition of native heteromeric P2X receptors is still not clear (39, 53). However, there has been evidence of heteromultimeric P2X in biochemical and functional expression studies (39, 53). Another possibility is that the P2X4 in native SL ECs of the guinea pig is to some extent different from the heterologously expressed P2X4 receptor of the rat as described before (39).

Interestingly, in the current study, EC_{50} (160 μM) of the ATP current in the native ECs is >10 times higher than in the heteroexpression system (cultured cells; 10 μM; Refs. 40, 39). The relative higher EC_{50} (at one decade more) also happens in the P2X receptors in other native tissues, such as P2X2 in the outer sulcus cells in the inner ear and P2X7 in the pericytes of retina capillaries (26, 18). The reason for the difference between native tissue and cultured heteroexpression cells is not clear. The possible causes could be 1) more contribution of ectonucleotidase in native tissues and 2) differences among species and cell types.

**ATP Dilates SL Capillaries In Vivo**

Both peri- and endolymph ATP levels increase following acoustic stress (33, 35). It has been found that perilymphatic application of ATP increases the blood flow in the cochlear lateral wall measured with laser Doppler flowmetry (34). However, the cellular mechanism underlying the ATP-induced regional blood flow increase has not been studied. The SL capillaries are exposed to perilymph and perform an important function of controlling the blood flow in the SV, which is critical to generate EP (46, 31, 63, 62, 37, 32). In this study, we found that topical perilymphatic superfusion of ATP (1 mM) dilated a single segment of the SL capillaries by 11.5%, which is a substantial change for the SL capillary network considering the power relationship of diameter change to flow resistance for the capillaries. Based on the Hagen-Poiseuille equation, $R = (vL/r^4)/(8/\eta)$ (where $R$ is resistance, $v$ is fluid viscosity, $L$ is length

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**Fig. 7. P2X4 immunofluorescence labeling of ECs and pericytes of the SL capillaries. Top row: labeling of diaminofluorescein-2 diacetate (DAF-2DA) as a nitric oxide (NO) marker (A) and P2X4 (B) in ECs and pericytes. C: DIC image. D: merged DIC image of DAF-2DA and P2X4. Bottom row: labeling of DAF-2DA in ECs and pericytes (E) and negative control of P2X4 (with the secondary antibody only) (F). G: DIC image. H: merged DIC image of DAF-2DA and the negative control of P2X4.**
of vessel, and \( r \) is radius of tube), the capillary resistance decreased by the power of 4 to 64.7% of \( R \), when the \( r \) increased by 11.5%. Based on Darcy’s law, \( F = \Delta P/R \) (where \( F \) is blood flow and \( P = \) pressure), the blood flow would increase to 154.6% of \( F \) for a single segment of the capillary. Therefore, through the dilation of the SL capillaries, ATP would increase the blood flow in the cochlear lateral wall.

In an in vitro study of capillaries, application of ATP led to contraction of retinal capillaries through pericyte constriction that resulted from the opening of P2X7 and the subsequent increase of \( \text{Ca}^{2+} \) in the pericytes (18). However, this contraction only occurred in a minority (37%) of the observed pericytes, and the mechanism was unclear. In contrast to the 11.5% diameter increase seen in vivo, in our in vitro study we did not detect any obvious constriction or dilation in the SL capillaries in spite of intracellular increase of \( \text{Ca}^{2+} \) in the ECs and pericytes after ATP (1 mM) application. The finding of capillary in vivo dilation in the cochlea and in vitro constriction in the retina suggests a different mechanism. It has been well established that increase of \( [\text{Ca}^{2+}]_i \), directly leads to the production of NO in ECs (10, 66). In arteries, ATP causes a dilation through P2X4 and the NO pathway in ECs as a classical mechanism of shear stress related dilation, i.e., opening of P2X4 results in increase of \( [\text{Ca}^{2+}]_i \), which triggers NO production and release from ECs. NO then dilates the smooth muscle underneath the ECs (11, 44, 3, 64, 65). Likewise, the same mechanism may have occurred to the SL capillaries in the current study, i.e., extracellular ATP activated P2X4 in the ECs and caused a subsequent increase of \( [\text{Ca}^{2+}]_i \) in the ECs, which may have resulted in a release of NO from the ECs. NO and its downstream signals may act on the contractile apparatus in ECs and pericytes rather than smooth muscle and cause a relaxation. It has been reported that the EC alone can contract through its contractile apparatus (12, 7, 2, 25, 5, 30). Indeed, blocking the NO pathway with L-NAME significantly inhibited the dilation in the SL capillaries by \( \sim 80.0\% \) in all animals.

**Physiologic Significance**

Accumulating evidence indicates that ATP plays an important role in regulating inner ear transduction processes (36, 15, 16, 55, 27, 28). It is known that the basal ATP level in the cochlea fluid (both peri- and endolymph) is at a low nanomolar level (36). However, the ATP level increased substantially following acoustic stress (33). The release sites of endogenous ATP have been identified to be the organ of Corti and the marginal cells of the SV. Yet, it is likely that the other cells in the cochlea are also involved. An increase of ATP and its degradation products upon acoustic stimulation could lead to a dilation of the SL capillaries, resulting in an increase in blood flow at the cochlear lateral wall. The increased blood flow provides more oxygen, which is needed to maintain a high EP, a part of the driving force for the transduction current, under acoustic stimulation. Therefore, it is likely that ATP signaling

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**Fig. 8.** ATP dilated the SL capillaries in vivo through P2X4 and the NO pathway. Representative images of the SL capillary before (A) and 70 s after (B) ATP (1 mM) application. C: representative pictures of the SL capillary with 5-BDBD (30 \( \mu \)M) superfusion alone for 20 min. D: ATP (1 mM) application for 70 s with 5-BDBD (30 \( \mu \)M) superfusion for 20 min. E: representative pictures of the SL capillary with \( N^\omega\text{-nitro-L-arginine methyl ester hydrochloride (l-NAME; 10 mM) superfusion alone for 20 min.} \) F: ATP (1 mM) application for 70 s with l-NAME (10 mM) superfusion for 20 min. G: bar graph summary of net increase of diameters (in percentage) in the SL capillaries after ATP (1 mM) application alone (\( n = 6 \)), ATP (1 mM) with 5-BDBD (30 \( \mu \)M) superfusion for 20 min (\( n = 5 \)), and ATP (1 mM) with l-NAME (10 mM) superfusion for 20 min (\( n = 6 \)). \* \( P < 0.05 \). H: bar graph summary of diameter change (in percentage) in the SL capillaries 20 min after 5-BDBD (30 \( \mu \)M) or l-NAME (10 mM) superfusion. NS, \( P < 0.05 \); \( n = 5 \) and 3.
in the cochlea dynamically regulates the blood flow in the SV through controlling the diameters of the SL capillaries based on the amplitude of acoustic stimulation. ATP also plays an important role in the pathophysiological conditions of the inner ear, such as noise-induced trauma, upon which a very high level of intracellular ATP (5–10 mM) can be released into the cochlear fluid (54, 56).

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REFERENCES

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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