ATP-Evoked Sustained Vasoconstrictions Mediated by Heteromeric P2X1/4 Receptors in Cerebral Arteries

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Background and Purpose—Current knowledge states that vasoconstrictor responses to ATP are mediated by rapidly desensitizing ligand-gated P2X1 receptors in vascular smooth muscle cells (VSMCs). However, ATP is implicated in contributing to pathological conditions involving sustained vasoconstrictor response such as cerebral vasospasm. The purpose of this study is to test the hypothesis that the stimulation of VSMC P2XR receptors (P2XRs) contributes to ATP-evoked sustained vasoconstrictions in rat middle cerebral arteries (RMCA).

Methods—Reverse transcription-polymerase chain reaction, Western blot, and immunocytochemistry were used to analyze expression of mRNA and proteins in RMCA VSMCs. Ionic currents and calcium responses were investigated using patch-clamp and confocal imaging techniques, respectively. Functional responses were confirmed using wire myography.

Results—Expression of mRNA and protein for P2X1R and P2X4R subunits was identified in RMCA VSMCs. Confocal imaging in fluo-3-loaded VSMCs showed that ATP and a selective P2XR agonist, αβmeATP, evoked similar dose-dependent increases in [Ca^{2+}]. Patch-clamp experiments identified 2 components of P2XR-mediated currents: consisting of a fast desensitizing phase mediated by homomeric P2X1Rs and a slowly desensitizing phase involving heteromeric P2X1/4Rs. Isometric tension measurements showed that ≈80%-20% of initial ATP-evoked vasoconstriction in RMCA is mediated by homomeric P2X1Rs and heteromeric P2X1/4Rs, respectively. The sustained slowly desensitizing and rapidly recovering from desensitization responses are mediated by heteromeric P2X1/4Rs.

Conclusions—This study reveals for the first time that apart from rapidly desensitizing homomeric P2X1Rs, heteromeric P2X1/4Rs contribute to the sustained component of the purinergic-mediated vasoconstriction in RMCA. Our study, therefore, identifies possible novel targets for therapeutical intervention in cerebral circulation. (Stroke. 2014;45:2444-2450.)

Key Words: adenosine triphosphate  ■ cerebrovascular circulation

It is established that endogenous ATP released from various sources, such as nerve terminals or endothelial cells, is important in the vasculature. ATP evokes contractile responses by activation of nonselective ligand-gated cation channels, P2X receptors (P2XRs), expressed in the plasma membrane of vascular smooth muscle cells (VSMCs). Opening of these channels allows the influx of cations and thus depolarization of the cell membrane. This leads to the activation of voltage-gated Ca^{2+} channels followed by an increase in [Ca^{2+}]. The ATP-mediated increase in [Ca^{2+}] leads to calmodulin-dependent phosphorylation of the myosin light chain kinase and contraction of VSMCs.

The P2X2R family consists of 7 different receptor subunits (P2X1–7), which are encoded by different genes and able to form homomeric and heteromeric functional channels with distinct pharmacological and biophysical properties. The current opinion is that vasoconstictor responses to ATP are predominantly mediated by activation of homomeric P2X1R, and there is no experimental evidence demonstrating the involvement of other functional P2XRs. However, functional homomeric P2X1Rs rapidly desensitize within milliseconds in the continuous presence of an agonist and recover from desensitization only after several minutes. Therefore, it is unclear how homomeric P2X1Rs are involved in the maintenance of the vascular tone and also in pathologies associated with increased vascular reactivity induced by prolonged elevated levels of endogenous ATP, such as during sympathetic nerve overactivity, after ischemia, and following brain trauma. Moreover, ATP is implicated in contribution to sustained cerebral vasospasm during cerebral subarachnoid hemorrhage. This study tested the hypothesis that ATP contributes to the sustained vasoconstriction of rat middle cerebral arteries (RMCA) via activation of P2XRs of VSMCs. This work identifies for the first time the presence of functional heteromeric P2X1/4R channels in VSMCs. We also show that in rat cerebral circulation, activation of slowly
desensitizing functional heteromeric P2X1/4Rs accounts for a significant sustained vasoconstrictor drive following purinergic stimulation.

Methods

Cell Preparation

All experimental procedures were in accordance with the UK Animals Scientific Procedures Act 1986 and were approved by institutional animal care and use committee. Male Wistar Kyoto rats (180–250 g, 42 animals obtained from Charles Rivers laboratories) were humanely euthanized by cervical dislocation followed by exsanguination, and left and right RMCAs were microdissected and single cells were obtained as described previously.21 Isolated cells were collected for reverse transcription polymerase chain reaction analysis or fixed for immunocytochemical experiments within 1 hour, or they were used in patch-clamp experiments within 6 hours of isolation.

Gene and Protein Expression, Calcium Imaging, and Electrophysiological Recording

For reverse transcription polymerase chain reaction, single freshly dispersed VSMCs (pool of ≈250 cells per sample) were collected using a microscope.20 Reverse transcription polymerase chain reaction gene expression analysis and immunocytochemical detection of proteins and data analysis were performed as previously described.21 See the online-only Data Supplement for the Western blotting protocols, lists of primers, and antibodies used. Electrophysiological recordings using amphotericin B perforated patch-clamp configuration of whole cell recording and calcium imaging using an Axiovert 100 inverted microscope attached to an LSM 510 laser scanning unit (Carl Zeiss, Oberkochen, Germany) were carried out as previously described.21,22 See the online-only Data Supplement for detailed description of calcium imaging and electrophysiological recording methods.

Isometric Tension Recording

RMCAs were cleaned of adherent tissue, and the endothelium was removed by passage of air bubble through the lumen of artery.25 Segments of RMCAs ≈3-mm length were mounted on a small wire myograph (Danish Myo Technology, Aarhus, Denmark) on 40-mm tungsten wires in baths containing standard physiological saline solution bubbled with 95% O2/5% CO2 and maintained at 37°C. Changes in tension were recorded using PowerLab and Chart software (ADInstruments, Oxford, UK).

Statistical Analysis and Data Presentation

Data were analyzed and plotted using MicroCal Origin 6.1 (MicroCal Software Inc, Northampton, MA) and CorelDraw Graphic Suite X6 software (Corel Corporation, Ottawa, Canada). All statistical data are presented as means±SEM with the number of cells shown for data points obtained from ≥3 animals. Comparative analysis of the data was performed using the Student t test.

Reagents

The chemicals were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise. NF279 and NF449 were purchased from Tocris (Bristol, UK).

Results

P2X1 and P2X4 Receptor Subunits Are Expressed in RMCA VSMCs

We initially investigated the expression and cellular distribution of P2XRs in RMCA VSMCs. Figure 1A illustrates that mRNA for all 7 P2XRs subunits was expressed in preparations obtained from whole segments of RMCA, whereas mRNA only for P2X1R and P2X4R subunits was expressed in preparations from individually collected VSMCs.

Immunoblotting experiment using anti-P2X1R antibody showed the presence of 2 protein bands of ≈60 and ≈120 kDa, which are the predicted molecular weights of glycosylated monomeric and dimeric subunits (Figure 1B). Immunoblots with an anti-P2X4R antibody detected a protein band at ≈43 kDa, which is the predicted monomeric P2X4 subunit weight. The anti-P2X4 antibody also detected a protein band of ≈100 kDa, which is likely to represent a glycosylated monomeric P2X4R subunit. Our immunofluorescent experiments revealed that both P2X1R and P2X4R subunits were preferentially distributed at the plasma membrane of VSMCs, with significantly greater relative fluorescence measured in the region of the plasma membrane compared with the deeper cytoplasm (Figure 1C and 1D). No immunofluorescence was observed in control staining (Figure I in the online-only Data Supplement).

Functional P2XR-Mediated Increases in [Ca2+]i

Increase in [Ca2+]i is a major determinant in generating contractility of VSMCs, and therefore, we studied if stimulation of P2XRs induced significant changes in [Ca2+]i in fluo-3-loaded VSMCs. Three-second application pulses of 10 μmol/L ATP and the selective P2X agonist 10 μmol/L αβ-meATP
produced similar robust concentration-dependent increases in fluo-3 fluorescence (Figure 2A) with effective concentrations producing 50% of maximal responses (EC50) of 0.38±0.29 and 0.59±0.24 μmol/L, respectively (Figure 2B). These results strongly suggest that ATP-induced increases in [Ca2+]i are mediated by functional P2XRs in RMCA VSMCs.

**Electric Events in RMCA VSMCs Evoked by Purinergic Stimulation**

We next investigated membrane potential and membrane current changes induced by ATP and αβ-meATP in VSMCs using whole-cell patch-clamp recording in current-clamp and voltage-clamp configurations, respectively. Figure 3A shows that 3-second application of 10 μmol/L ATP evoked 3 distinct depolarizing responses in different VSMCs: a fast transient depolarization, a transient depolarization followed by a sustained depolarization phase, and a sustained depolarization. Moreover, Figure 3B shows that 10 μmol/L ATP also evoked inward currents with 3 distinct properties in different cells, which corresponded to the kinetic changes of the 3 types of depolarizing responses described above. These observations suggest that ATP-mediated responses are composed of a fast rising fast desensitizing phase termed I_FP2X (Figures 3A, left) and a slowly desensitizing phase defined as I_SP2X (Figures 3A, right) with biphasic responses likely to consist of both these components (Figures 3A, middle). Stimulation of VSMCs with 10 μmol/L αβ-meATP evoked inward currents with properties consistent with currents composed of both I_FP2X and I_SP2X components (Figure 3C), which further suggests that P2XRs play a predominant role in mediating ATP responses in RMCA VSMCs.

**I_FP2X Displays Properties of Homomeric P2X1R-Mediated Current**

Properties of I_FP2X were investigated in VSMCs which displayed predominantly rapidly desensitizing currents evoked by purinergic stimulation. In such cells, 3-second application of 10 μmol/L αβ-meATP resulted in rapid onset inward currents with an averaged current peak density of 22.4±2.5 pA/pF at a holding potential of −60 mV (n=27; Figure 4A), which
exhibited complete desensitization within 2 seconds in presence of the agonist. Repeating the 3-second applications of 10 μmol/L αβ-meATP following initial stimulation revealed that 5.9±1.1% (n=7, P<0.001) of the current was restored after 1 minute and complete recovery of I_sp2X was observed after 8 minutes (Figure 4A). To further characterize the properties of I_sp2X, the effect of the selective P2XR antagonist NF279 was studied in cells stimulated with 10 μmol/L αβ-meATP for 3-second followed by an 8-minute interval. Figure 4B (left) shows original traces of currents induced after pretreatment with different concentrations of NF279 for 5 minutes, and Figure 4B (right) shows that NF279 inhibited I_sp2X with an inhibitory concentration producing 50% inhibition (IC_{50}) of 16±2 mmol/L (n=5–7, for each data point). The similar sensitivity of I_sp2X to NF279 to previously described recombinant homomeric P2X1Rs (IC_{50}=19 mmol/L) indicates that ATP-mediated I_sp2X is likely to be mediated by homomeric P2X1R structures.

Properties of I_sp2X Evoked by Stimulation of P2XRs

To investigate properties of I_sp2X in VSMCs displaying predominantly I_sp2X cells were stimulated with 10 μmol/L αβ-meATP for 5 seconds at a holding potential of −80 mV. Repeating 5-second applications of 10 μmol/L αβ-meATP showed that I_sp2X recovered from desensitization within 15 seconds (Figure 5A, left top). Application 10 μmol/L αβ-meATP for 25 seconds revealed that the current amplitude was only reduced by <20%, which is in stark contrast to I_sp2X which was completely abolished after an application time of <3 seconds (Figure 5A, left bottom). In VSMCs displaying αβ-meATP-evoked biphasic responses, repeated application of agonist after a 30-second interval evoked mainly slowly desensitizing component (Figure 5A, right). The experiments studying the voltage dependence of I_sp2X (Figure II in the online-only Data Supplement) revealed that its properties are consistent with characteristics of the cationic current mediated by P2XRs.

Figure 5B (left) shows original traces of currents induced by 10 μmol/L αβ-meATP for 3 seconds before and after incubation with increasing concentrations of NF279 for 5 minutes. From these traces, the mean values of the last 500 milliseconds of 3-second agonist application were calculated, normalized to the same value of the control current, and plotted against corresponding concentrations of NF279. These experiments showed that NF279 inhibited I_sp2X with an IC_{50} of 24±7 μmol/L (n=6–7; Figure 5B, right), which is 1000 times higher than the effect of NF279 on homomeric P2X1R activity (see above) and 15 times lower than for the homomeric recombinant P2X4R activity (IC_{50}>300 μmol/L). These results suggest that it is unlikely that homomeric P2X1R or homomeric P2X4R contributes significantly to I_sp2X.

We also investigated separating I_sp2X from I_sp2X in cells displaying biphasic responses to αβ-meATP using selective P2XR antagonist NF449 which has high affinity to homomeric P2X1Rs (IC_{50}=0.3 mmol/L). Thus, 5-minute incubation with 100 mmol/L NF449 inhibited rapidly desensitizing component without substantial inhibition of I_sp2X (Figure 5C, left). The difference between control current and currents in presence of NF449 revealed currents with kinetic properties of rapidly desensitizing I_sp2X. Finally, we studied the effect of ivermectin, which potentiates homomeric P2X4R activity on I_sp2X. One-minute application of 3 μmol/L ivermectin induced no significant effect on I_sp2X further indicating that this component is not mediated by homomeric P2X4Rs (Figure 5C, right, n=6, P>0.05).
Functional Responses Evoked by P2XR Stimulation

We finally investigated the functional significance of P2XR stimulation in RMCA contractility using wire myography. Applications of 10 μmol/L ATP and 10 μmol/L αβ-meATP evoked similar biphasic vasoconstrictor responses in RMCA segments (Figure 6A). Area under the curve data revealed that 10 μmol/L αβ-meATP evoked significantly larger contractile responses than 10 μmol/L ATP, with mean values of 39.8±3.4 (n=7) compared to 31.3±2.6 (n=7, P<0.05), respectively. These results are consistent with the literature showing that αβ-meATP is more potent vasoconstrictor than ATP in various arterial preparations. As our electrophysiological experiments showed a complete inhibition of I_{SPX} by 10 μmol/L NF279 without significantly altered I_{FPX} responses, the effect of 10 μmol/L NF279 was tested on contractions induced by 10 μmol/L αβ-meATP. After 15-minute incubation with the antagonist, it was still possible to evoke a significant contraction by repetitive applications of αβ-meATP (Figure 6B). The summarized data show that component resistant to 10 μmol/L NF279 accounts for >20% of initial vasoconstrictor response with normalized mean area under the curve value during the initial 15-second application of the agonist of 0.23±0.3 compared with the control response (n=7, P<0.001).

P2X1 receptors are characterized by slow resensitization and only ≈6% of the I_{FPX} initial response was observed with repetitive applications of the agonist following 1-minute intervals in our electrophysiological experiments. Repetitive 20-second application of 10 μmol/L αβ-meATP after 10-second control stimulation evoked considerable contractions with slower onset and decline of the signal compared to that of the initial contractile response to the agonist (Figure 6C). These data suggest that although I_{FPX} can contribute to the fast large initial contractile response, slowly inactivating I_{SPX} significantly contributes to sustained component of the contraction of RMCAs following stimulation of P2XRs.

Discussion

The present findings reveal that stimulation of P2XRs induced vasoconstrictor responses of RMCA, which are likely mediated by increases in [Ca^{2+}], which are evoked by activation of homomeric P2X1Rs and heteromeric P2X1/4Rs. We propose that P2X1Rs account for the rapidly declining vasoconstrictor responses and heteromeric P2X1/4Rs are responsible for sustained responses following purinergic stimulation. The involvement of heteromeric P2X1/4Rs is a novel finding and greatly increases our understanding of purinergic transmission in VSMCs.

Stimulation of RMCA VSMCs by ATP and by the selective P2X2 agonist αβ-meATP at the same concentrations resulted in similar changes in [Ca^{2+}], and electric responses in isolated RMCA VSMCs cells, as well as in similar contractile responses in intact cerebral artery preparations. These data strongly suggest that functional P2XRs play a predominant role in stimulatory action of extracellular ATP in these vessels. Overexpression studies show that αβ-meATP acts on homomeric P2X1Rs with an EC_{50} of ≈1 μmol/L, but only activates homomeric P2X4Rs at concentrations >100 μmol/L, which indicates that αβ-meATP in our experiments is not activating homomeric P2X4Rs.
Importantly, coexpression of P2X1 and P2X4 receptor subunits in *Xenopus* oocytes induces functional channel activity, which was activated by 10 μmol/L αβ-methylene-ATP and had biphasic components composed of a fast desensitization phase mediated by homomeric P2X1Rs and a slowly desensitizing phase corresponding to heteromeric P2X1/4Rs.

There is a growing body of experimental evidence suggesting that purinergic system is involved in a number of pathophysiologic cerebral conditions, particularly during cerebral vasospasm after subarachnoid hemorrhage, because purine nucleotides that stored at millimolar range in various cells can leak during their damage into the extracellular space. In addition, ATP is also released by endothelial cells under shear stress and by degranulated platelets. Moreover, there is a significant body of the evidence suggesting the contribution of ATP released from the blood clots during trauma into vasospasm in animal models. ATP was implicated in contributing to vasospasm occurring after subarachnoid hemorrhage in monkey middle cerebral arteries in vivo. In rat model of experimental cerebral ischemia, it was shown that P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid supports recovery from experimental stroke in vivo, while the P2X antagonist suramin reduces infarct volume in a model of focal brain ischemia in rats. Also, it was shown that pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid reduced the histologically estimated infarct area after the acute phase of ischemia in rats.

Our study shows the expression of P2X1 and P2X4 receptor subunits and the presence of 2 distinct components of P2XRs-mediated ionic currents in RMCA VSMCs. The larger amplitude current mediated by homomeric P2X1Rs is characterized by rapid desensitization and contributes to the initial rapid onset phase of contractile response evoked by P2X stimulation. The smaller current mediated by heteromeric P2X1/4Rs is characterized by slow desensitization and can contribute to the sustained phase of contractile response when P2X1Rs are desensitized or blocked with specific antagonist.

**Perspectives**

Our study identified a novel mechanism of vasoconstrictor action of ATP in the vasculature. We have established that the activation of heteromeric P2X1/4Rs produces potent sustained contractions in ATP-stimulated cerebral arteries. These findings may have a wider implication for the involvement of P2XRs in some pathological conditions which could be associated with prolonged exposure of VSMCs to extracellular ATP because P2X-mediated vasoconstriction may represent a significant vasoconstrictor drive resistant to current drug therapies. These pathologies could include sympathetic overactivity leading to the development of hypertension or vasospasm of arteries during trauma. In cerebral arteries, extracellular ATP released during hemorrhage is implicated in the development of the cerebral vasospasm. Human cerebral vasospasm is a major complication of the subarachnoid hemorrhage and results in ≤3-fold increase in mortality within first 2 weeks after the event. The activation of P2XRs leading to the sustained vasoconstriction of cerebral arteries could be a contributing factor to such vasospasm. Furthermore, purinergic regulation may become particularly important during other pathologies such as middle cerebral arteries occlusion.
which is characterized by impaired cerebral vasodilatation after ischemia, with maintenance of vasoconstriction.38

Identification of expression of only P2X1 and P2X4 receptor subunits in VSMCs from human small arteries39 and in myocytes from rat aorta, kidney pulmonary, and mesenteric arteries (O.V. Povstyan and M.I. Harhun, unpublished data, 2013) further supports the importance of these receptor subunits in vascular function. Together with literature evidence allow us to hypothesize that only P2X1 and P2X4 subunits compose the functional P2XR structures in major mammalian arteries. Moreover, our preliminary results40 in rat cerebral arteries suggest the predominance of the purinergic simulation in the rat cerebral vasculature over adrenergic which is in accordance with the data obtained in dog41 and human42 cerebral arteries. Additional focused studies will be needed to establish the role of individual subtypes in pathological conditions.

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Disclosures
None.

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**SUPPLEMENTAL MATERIAL**

**Supplemental Methods**

*Primers used in RT-PCR analysis*

The following primers (Invitrogen, Paisley, UK) were used in these experiments (the following data are shown in brackets: Genebank accession number, the sense bordering nucleotide position and the anti-sense bordering nucleotide position): β-actin (NM_031144, 211-230 and 862-881), P2X1 receptor (NM_012997, 290-309 and 1119-1138), P2X2 (NM_053656, 603-622 and 1262-1281) P2X3 (198-217 and 1215-1234), P2X4 (NM_031594, 337-356 and 1061-1080), P2X5 (NM_080780, 341-360 and 1335-1354) P2X6 (NM_012721, 166-185 and 957-976) and P2X7 (NM_019256, 591-610 and 1561-1582).

*Antibodies used in immunocytochemical experiments*

The following primary antibodies were used: rabbit anti-P2X1 dilution 1:300, rabbit anti-P2X4, 1:300 (both Abcam, Cambridge, UK). The donkey anti-rabbit MFP 488 secondary antibody (dilution 1:400; Mobitec, Gottingen, Germany) was used in all experiments.

*Western blotting of P2XRs*

For Western blot analysis proteins were extracted from cleaned RMCAs (pulled from 10 animals). Arteries were homogenized in ice-cold RIPA Lysis Buffer System (Santa Cruz Biotechnology, Santa Cruz, USA), further disrupted by sonication on ice and centrifuged at 15000 rpm for 20 min at 4°C. The supernatant was collected and frozen at -80°C. Protein content was quantified using the Bio-Rad DC Protein Assay method. Samples of supernatant were eluted with Laemmli sample buffer and used in one-dimensional protein gel electrophoresis. Proteins were then transferred onto PVDF membranes using iBlot (Invitrogen, Paisley, UK). Western blotting was carried out on membranes incubated with rabbit anti-P2X1 and rabbit anti-P2X4 primary antibodies (dilution 1:1000) overnight at 4°C. Membranes were then washed and incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody.
(ThermoFisher Scientific, Loughborough, UK) treated with electrochemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, USA) for 1 min and exposed to photographic films.

Electrophysiological recordings

Electrical recordings were made in PSS using the amphotericin B (200 µg·ml⁻¹) perforated-patch tight-seal recording technique in voltage- or current-clamp modes. The patch pipettes had a free-tip resistance of 4-6 MOhm when filled with pipette solution of the following composition (mmol·l⁻¹): KCl 115; NaCl 6, HEPES 10; pH adjusted to 7.3 with KOH. When the properties of Iₚₓ were examined, to eliminate the possible contribution of the current through potassium channels to the whole-cell current records, K⁺ was equimolarly replaced with Cs⁺ in both external and pipette solutions. ATP and αβ-meATP were applied by 3s-pulses (unless stated otherwise) through a glass micropipette (located within 100 µm of the cell surface) connected to a pressure ejector PicoSpritzer III (Intracel, Shepreth, UK). The electrical signals were recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, USA). Electrical signals were generated and digitized at 1 kHz using a Digidata 1200 hosted by a PC running pClamp 6.0 software (both, Axon Instruments, USA). Voltage-clamp experiments were carried out at room temperature (21-24 °C).
**Figure I.** Control immunostaining of VSMCs from RMCA for P2X1 (A) and P2X4 (B) receptors. Left panels show the transmitted light images of cells. No fluorescence was observed during fluorescent imaging (right panels). Scale bars correspond to 10 μm.
Figure II. Voltage-dependence of $I_{SP2X}$ in VSMCs from RMCA. Left panel, original trace of the current evoked by 17-s stimulation with 10 $\mu$mol·l$^{-1}$ $\alpha$$\beta$-meATP using voltage protocol shown below. Right panel, $I_{SP2X}$ current-voltage relationship revealed inward rectification of the current ($n=6$).