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Molecular identification of P2X receptors in vascular smooth muscle cells from rat anterior, posterior, and basilar arteries

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ABSTRACT

Background: Purinergic P2X receptors in vascular smooth muscle cells (VSMCs) play an important role in physiological stimulatory responses to the extracellularly released ATP. The aim of this work was to identify molecular P2X receptor subunits in VSMCs isolated from rat anterior, posterior and basilar arteries using a number of contemporary laboratory techniques.
Methods: P2X mediated ionic currents were recorded using amphotericin B perforated patch clamp method. Gene expression analysis was performed using RT-PCR in manually collected VSMCs. The expression of proteins was confirmed by fluorescent immunocytochemistry.
Results: Under voltage clamp conditions VSMCs stimulated by application of 10 μmol/l selective P2X receptor agonist αβ-meATP, the biphasic currents consisting of rapidly rising rapidly desensitizing and slowly desensitizing components were observed in freshly isolated myocytes from all three arteries. Using RT-PCR, the expression of genes encoding only P2X1 and P2X4 receptor subunits was detected in preparations from all three arteries. The expression of corresponding P2X1 and P2X4 receptor subunit proteins was confirmed in isolated VSMCs.
Conclusions: Our work therefore identified that in major arteries of rat cerebral circulation VSMCs express only P2X1 and P2X4 receptors subunits. We can propose that these P2X receptor subunits participate in functional P2X receptor structures mediating ATP-evoked stimulatory responses in cerebral vascular myocytes *in vivo*.

Introduction

Purinergic P2X receptors to ATP are non-selective cationic channels widely expressed in various tissues. To date seven distinct receptor subunit proteins have been identified (P2X1-7), each encoded by different gene [1,2]. P2X receptor subunits are able to form functional homo- and heteromeric channels in the membrane of various cells with distinct pharmacological and biophysical properties [3,4]. It is generally accepted that in vascular smooth muscle cells (VSMCs) stimulation of P2X receptors results in influx of cations leading to depolarization of the cell membrane followed by activation of the voltage gated calcium channels [5]. This in turn leads to an increase in intracellular calcium to a micromolar concentration and contraction of the myocytes [6]. From a therapeutical point of view, P2X receptors in vasculature may mediate a substantial vasoconstrictor drive which is resistant to current drug therapies, such as using adrenoceptor antagonists and calcium channels blockers [7–9]. Therefore, molecular identification of P2X receptors expressed in VSMCs of blood vessels and analysis of their pharmacological properties could serve as a basis for the development of novel therapeutic approaches for treatment of hypertension or arterial vasospasm induced by alterations in vascular tone.

P2X receptors have been widely explored in the nervous system [10]. In contrast, in vascular smooth muscle the current knowledge regarding their molecular identification is very limited. In VSMCs P2X are stimulated by ATP released from nerve terminals as well as from endothelial or blood cells [11]. It was reported that endogenous dinucleoside polyphosphates such as uridine adenosine tetraphosphate (Up(4)A) or diadenosine polyphosphates (APnA), can also stimulate P2X receptors in blood vessels [12–14]. The general opinion in the field is that purinergic stimulation in

Abbreviations: αβ-meATP, αβ-methyleneadenosine 5′-triphosphate; PSS, physiological saline solution; RT-PCR, reverse transcription polymerase chain reaction; Up(4)A, uridine adenosine tetraphosphate; VSMC, vascular smooth muscle cell.

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muscular arteries is mediated by rapidly desensitizing homomeric P2X1 receptor structures and there is a limited amount of data about the presence of other P2X receptor subunits in the membrane of VSMCs of various blood vessels [15]. In our previous work we identified that VSMCs from rat middle cerebral artery express only P2X1 and P2X4 receptor subunits [16]. These findings stress the necessity of identification of P2X receptors in VSMCs of other major brain arteries, as since the therapeutical approach targeting P2X receptors in the middle cerebral arteries, may result in a different (undesirable) effect in other blood vessels of the cerebral circulation. Therefore in this work, using gene and protein expression analysis, we identified expression of molecular P2X receptor subunits in VSMCs isolated from rat anterior, posterior, and basilar arteries.

Materials and methods

Cell preparation

All animal care and experimental procedures were in accordance with the UK Animals Scientific Procedures. Wistar Kyoto rats (200–250 g, 18 animals, Charles Rivers laboratories, Margate, UK) were sacrificed by cervical dislocation followed by exsanguinations and anterior, posterior, and basilar arteries and their branches were dissected. Isolated VSMCs were obtained by enzymatic treatment of blood vessels as in our previous experiments [17] in physiological saline solution (PSS) in mmol/l: KCl, 6; NaCl, 120; $MgCl_2$, 1.2; $CaCl_2$, 1.0; D-glucose, 10; and HEPES, 10; pH was adjusted to 7.3 with NaOH. The fragments were then incubated in nominally Ca^{2+} free PSS supplemented with collagenase type IA (1 mg/ml) and thermolysin X (0.5 mg/ml) for 15 min at 36 °C. Single cell were obtained by gentle agitation of the arterial fragments using a wide bore pipette in nominally Ca^{2+} free PSS and allowed to settle down for 30 min in experimental chambers. During cell adhesion the Ca^{2+} concentration was gradually increased to 1 mmol/l. VSMCs were then used in patch clamp experiments within 6 h of isolation, fixed for immunocytochemistry experiments or manually collected for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Electrophysiological recordings

Electrophysiological recordings were performed in voltage clamp mode using amphotericin-B perforated patch clamp technique as described previously [18]. To investigate the properties of ionic current *via* P2X receptors, K^+ was equimolarly replaced with Cs^+ in PSS in order to eliminate possible contribution of the current through potassium channels. The pipette solution of the following composition was used, mmol/l: CsCl, 115; NaCl, 6; HEPES, 10; pH adjusted to 7.3 with CsOH. The pipette solution was supplemented with 200 μg/ml amphotericin B. αβ-meATP was applied by 2-s pulses 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 (Molecular Devices, Sunnyvale, USA) and digitized using a Digidata 1200 hosted by a PC running pClamp 6.0 software (Molecular Devices). Recordings were performed at a holding potential of −60 mV at room temperature (20–22 °C).

Gene expression analysis

For RT-PCR single VSMCs (~150 cells for each analysis) were individually collected by using a glass micropipette attached to a manipulator and frozen immediately on dry ice. Non-contractile myocytes displaying morphological features of vascular interstitial cells such as filopodia [19,20] were omitted during collection. VSMC suspension was homogenized using QIAshredder columns and total RNA was extracted using RNeasy extraction kit (both Qiagen, Crawley, UK). cDNA was then obtained using Superscript II Reverse Transcriptase (Invitrogen, Paisley, UK) and used in PCR. cDNA was used as a template in a 50 μl PCR reaction containing 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} deoxynucleoside triphosphates, 0.2 μmol L^{-1} forward and reverse primers, and 2.0 U of platinum *Taq* DNA polymerase (all reagents from Invitrogen). Amplification was performed according to the following schedule using a T3 thermocycler (Biometra, Göttingen, Germany) according to the following schedule: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 59 °C for 60 s and 72 °C for 3 min, followed by a final elongation period of 10 min at 72 °C. A no-template control PCR was performed simultaneously with every reaction. Primers were designed so that they spanned at least one intron of the genomic sequence to avoid the detection of genomic DNA contamination. The analysis was carried out using at least three pools of cells separately collected from each type of artery from three different animals.

The following primers were used in the study (the following data are shown in brackets: Genbank accession number, the sense bordering nucleotide position and the anti-sense bordering nucleotide position): β-actin (NM_031144, 306–325, and 862–881) P2X1 receptor (NM_012997, 290–309, and 1119–1138), P2X2 (NM_053656, 584–603, and 1590–1609) 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).

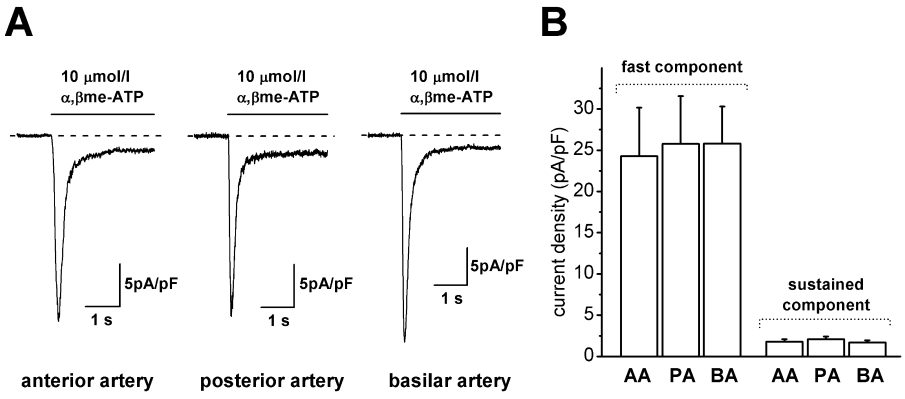

Fig. 1. P2X mediated ionic currents evoked by 10 μm/l αβ-meATP at holding potential −60 mV in VSMCs from rat anterior (AA), posterior (PA), and basilar arteries (BA). (A) Representative current traces. (B) The summarized graph for a current density for the fast desensitizing component (I_r, plotted as a mean peak current density) and for a slowly desensitizing component (I_s, plotted as a mean current density during last 1 s of the stimuli). No significant differences were found between three groups of cells for both I_r and I_s ($p > 0.05$, seven VSMCs from each cerebral artery).