NOVEL ROLE FOR P2X RECEPTOR ACTIVATION IN ENDOTHELIUM DEPENDENT VASODILATION

LOUISE S. HARRINGTON AND JANE A. MITCHELL

Unit of Critical Care Medicine, The National Heart and Lung Institute, Imperial College, Dovehouse Street, London, SW3 6LY, UK.

Corresponding Author:

Professor Jane A. Mitchell,

Unit of Critical Care Medicine,

Royal Brompton Hospital,

NHLI,

Imperial College School of Medicine,

Dovehouse Street,

London SW3 6LY

e-mail: j.a.mitchell@ic.ac.uk

Abstract

ATP is an important inflammatory and vasoactive mediator, which acts via two receptor classes; P2X and P2Y. Activation of P2X receptors has traditionally been associated with the well-characterised vasoconstrictor properties of ATP. In the current study, we have used classical bioassay to show that the P2X receptor ligand, α, β methylene ATP induces vasodilatation of rat isolated mesenteric arteries. Second order rat mesenteric arteries were mounted in myographs and vasomotor responses recorded. Both ATP and α, β methylene ATP induced a constriction followed by a vasodilation. The dilator effects of either ATP or α, β methylene ATP was slower in onset that that induced by acetylcholine. Vasodilation induced by α, β methylene ATP was endothelial cell dependent and characterised as mediated predominately by EDHF release. These results show for the first time, that P2X receptor activation results in endothelial dependent vasodilatation. These observations have important implications for our understanding of the role of purines in biological responses.

Key words: ATP; α, β methylene ATP; $P2X_1$ receptor; purinergic; nitric oxide; endothelial derived hyperpolarising factor (EDHF)

Introduction

ATP is an important mediator in the cardiovascular system. It is released by platelets, vascular and cardiac cells in response to common receptor-mediated ligands (Yang et al., 1994), physical forces and after ischemia-reperfusion (Burnstock, 1999). Once released, ATP then acts on specific receptors or is rapidly metabolised by a group of ecto-enzymes (Zimmermann, 2000) to products including ADP, AMP, and adenosine. ATP and its metabolites are potent mediators of vasomotor tone in most vascular beds (Kunapuli and Daniel, 1998); however, the mechanism by which it acts is yet to be clarified.

ATP and other purines activate purinergic (P) receptors to cause their effects in blood vessels. The vasodilator actions of ATP are mediated by the activation of P2Y receptors located on the endothelium and are caused by the release of endothelial derived relaxing factors such as NO and prostacyclin (Boeynaems et al., 2000; Ralevic and Burnstock, 1991) or EDHF (Malmsjo et al., 2002; Stanford et al., 2001).

P2X receptors are ligand-gated ion channels (Ralevic and Burnstock, 1988). Arterial P2X receptors are located on vascular smooth muscle cells (Vulchanova et al., 1996) and on the endothelium of some vessels (Glass et al., 2002; Hansen et al., 1999; Ray et al., 2002; Yamamoto et al., 2000). The vasoconstrictor role of P2X receptors on vascular smooth muscle is well studied, although in contrast, the potential role of P2X receptors on endothelial cells is not known. Activation of any receptor on endothelial cells that leads to elevation of intracellular calcium would almost certainly release endothelial derived relaxing factors. Indeed, it has been suggested that P2X receptor activation induced vasodilation of the intact mesenteric arterial bed of the rat (Ralevic, 2002); however, this phenomenon was described as endothelium independent.

Definitive studies of endothelial dependence of any ligand are not possible in the intact vascular bed because removal of the endothelial layer is technically difficult.

Thus, in the current study we have used the selective P2X ligand, α, β methylene ATP (Burnstock and Kennedy, 1985) to investigate its effects on vasodilator functions in isolated 2nd order mesenteric vessels from the rat, where the endothelium can be removed easily. Some of this work has been published in abstract form (Harrington and Mitchell, 2003).

Methods

Male Wistar rats (200 ± 15.4g) were killed by lethal exposure to CO_2 followed by cervical dislocation. The rats were maintained and killed in accordance with the European Community guidelines for the use of experimental animals.

The entire mesenteric bed was removed using ligatures, and placed into physiological salt solution (PSS; composition in mM) NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.027, and Glucose 5.5. The mesentery was pinned flat on a dissecting dish containing PSS, to allow 2nd order arteries to be cleaned of fat and connective tissue; these arteries were stored in fresh PSS solution at room temperature until use. In this study, the second order arteries had a mean internal diameter of 247μm.

Isometric myograph recordings

Using tungsten wire, segments of 2mm length of artery were mounted in a four channel Mulvany-Halpern myograph (Model 610M, Danish Myo Technology, Aarhus, Denmark). The vessels were equilibrated to 37^{o}C and the solution bubbled with 95% O_2 and 5% CO_2 for 30 minutes. The tension of the vessel was normalised to a tension equivalent to that generated at 90% of the diameter of the vessel at 100mmHg, using standard procedures as described previously (Mulvany and Halpern, 1977). Changes in arterial tone were recorded via a PowerLab/800 recording unit (ADI instruments Pty Ltd., Australia), and analysed using Chart 4.0 acquisition system (ADI instruments) on a Macintosh personal computer.

In order to assess the viability of the vessels, they were challenged 3 times with high potassium solution (KPSS; PSS with the NaCl replaced by 123.7mM KCl). Tissues were then pre-contracted with 10^{-5}M methoxamine, and once a plateau was

reached 10^{-5}M acetylcholine was added to the organ bath. Vessels which relaxed >80% in response to acetylcholine were considered to have an intact endothelium. Occasionally vessels failed this test and were disregarded.

Drug treatments

Vessels were contracted to approximately 80% of the response obtained with KPSS using methoxamine (10^{-5}M) and the effects of single additions of either ATP, (10^{-4}M), or α, β methylene ATP (10^{-6}M to 10^{-4}M) were determined. At the end of each experiment, the ability of the tissue to dilate maximally was determined by the addition of sodium nitroprusside (10^{-5}M). Since α, β methylene ATP induced a constrictor response prior to vasodilation, concentration response curves were constructed using single concentrations on individual tissues, and were therefore not cumulative.

In some experiments the nitric oxide synthase inhibitor, L-N^G nitro-L-arginine (L-NAME; 10^{-3}M), the cyclo-oxygenase inhibitor indomethacin (10^{-5}M), or apamin ($5x10^{-7}$M) plus charybdotoxin (10^{-7}M), which together inhibit EDHF responses were added. All inhibitors were added 30 mins prior to the addition of methoxamine.

In some experiments, the endothelial layer lining the lumen of the vessels was removed physically using a human hair. Complete endothelial removal was documented by an absence in the ability of acetylcholine to induce relaxation of vessels contracted with methoxamine.

Data and statistical analysis

Contractile or relaxant responses were calculated as a percentage of methoxamine induced tone. Data are given as the mean ± s.e.m. for n= 3-8 experiments. Data was analysed statistically using one way analysis of variance followed by a Dunnett's Multiple Comparison Test, using Prism 4.0. A p value of less than 0.05 was considered statistically significant.

Drugs

All drugs were purchased from Sigma Chemical Co. Gillingham, Dorset, UK. Drugs were prepared each day, except for α, β, methylene ATP, charybdotoxin and apamin, which were prepared in high concentration 'stock' solutions and were stored at -20^{o}C until used. All drugs were dissolved in aqueous solutions except for indomethacin, which was dissolved in dimethyl sulphoxide (DMSO).

Results

P2X receptor ligands induce vasodilation of methoxamine contracted mesenteric vessels.

Profound relaxation of methoxamine contracted vessels was induced by 10^{-5}M acetylcholine, 10^{-4}M ATP, 10^{-4}M α, β methylene ATP or 10^{-5}M sodium nitroprusside (Figure 1 and Figure 2). In contrast to acetylcholine or sodium nitroprusside, both ATP or α, β methylene ATP induced contractile responses prior to vasodilation (Figure 1). Interestingly, the vasodilator response induced by either ATP or α, β methylene ATP was slower in onset than that induced by acetylcholine.

Characterisation of P2X mediated response

The vasodilator actions of α, β methylene ATP were concentration dependent. α, β methylene ATP had an apparent EC_{50} of approximately 10^{-6}M (figure 3). By contrast, the contractile actions of α, β methylene ATP were not concentration related in the range used in this study (figure 4).

The vasodilator actions of α, β methylene ATP (10^{-4}M) were not inhibited by the combination of L-NAME plus indomethacin. However, dilator responses were inhibited by high K^+ or the combination of charybdotoxin plus apamin and abolished by the combination of L-NAME, indomethacin, charybdotoxin, and apamin (Figure 5). The dilator actions of α, β methylene ATP were abolished when mesenteric vessels were denuded of endothelium (figure 6 and figure 7). By contrast, the constrictor actions of α, β methylene ATP were unaffected by endothelial removal, see Figure 8.

Discussion

P2X receptors have been firmly linked to vasoconstrictor function. Where a dilator action of P2X receptors have been investigated, this phenomenon was attributed to a functional rebound effect of contraction and suggested to be endothelium independent. However, we show here for the first time, that the P2X receptor ligand α, β methylene ATP induces vasodilation, which is endothelium dependent. Furthermore we show that the vasodilator actions of α, β methylene ATP are not directly linked to the constrictor function as suggested by others (Ralevic, 2002).

We have previously shown that ATP induces three phases of vascular response in the perfused intact mesenteric bed of the rat (Stanford and Mitchell, 1998). ATP initially induces a transient dilation mediated by the co-release of NO and prostacyclin, which is followed by a constrictor response and finally a sustained dilation, which is slow in onset and mediated by EDHF (Stanford et al., 2001). Interestingly, there is a clear demarcation of vascular response associated with concentration range of ATP used. Thus, at lower concentrations, there is no constrictor response. At doses of 10^{-7} moles or above ATP induced vasoconstriction followed by the two phases on dilation (Mitchell and Stanford, 1998). Thus, the constrictor and second phase dilation induced by ATP coincide in the same dose range. This observation clearly demonstrates that a common receptor may be involved in both the constrictor and the second phase dilator response induced by ATP.

From our previous work with ATP in the perfused mesenteric bed of the rat, it was suggested that P2X receptor activation could result in vasodilation. In the current study we have indeed established this to be the case. In fact, α, β methylene ATP induces vasoconstriction followed by a profound and sustained dilation, similar in nature to the second phase dilator response induced by ATP in the intact arterial

mesenteric bed (Stanford and Mitchell, 1998). We found this response to be inhibited by high potassium and by the combination of apamin plus charybdotoxin and was absent in tissues where the endothelium had been removed. This pharmacological profile of results is consistent with EDHF being the mediator responsible for the P2X induced vasodilation.

α, β methylene ATP is a selective ligand for both $P2X_1$ and $P2X_3$ subunits (North, 2002; Surprenant et al., 2000). However, two pieces of recently published evidence suggest that $P2X_1$ seems the most likely receptor responsible for the responses we observed. Firstly, $P2X_1$ immunoreactivity is localised on the endothelial as well as the smooth muscle cell layer of blood vessels (Hansen et al., 1999) and secondly, the vasoconstrictor actions of α, β methylene ATP in mesenteric vessels is ascribed solely to activation of $P2X_1$ receptors (Lewis and Evans, 2000).

Thus, in summary, we have shown, for the first time, that the $P2X_1$ receptor ligand α, β methylene ATP is an endothelium-dependent vasodilator of rat isolated mesenteric vessels and that EDHF is the predominate mediator involved. This work illustrates a novel bi-functional role for P2X receptors, causing constriction when activated on smooth muscle and dilation when activated on the endothelium. This would be comparable to the variable functions of P2Y receptors, which mediate relaxation when located on the endothelium and contraction when situated on the smooth muscle cells (Ralevic and Burnstock, 1998).

Figures

Figure 1. Representative recorder traces of vasodilator responses of rat mesenteric arteries induced by 10^{-4}M ATP, 10^{-4}M α, β methylene ATP, 10^{-5}M acetylcholine or 10^{-5}M sodium nitroprusside (SNP). Vessels were first contracted with 10^{-5}M methoxamine.

Figure 2. Histogram depicting vasodilation of rat mesenteric arteries induced 10^{-4}M ATP, 10^{-4}M α, β methylene ATP (α, β), 10^{-5}M acetylcholine (ACh) or 10^{-5}M sodium nitroprusside (SNP). Vessels were first contracted using methoxamine (meth.). The natural drift in induced tone which occurs over time is depicted in the column for 'time control' (T.Con). Results are the mean +/- the S.E. mean (n=3-8). Statistical differences were calculated using one-way ANOVA followed by Dunnett's Multiple Comparison. Differences were considered statistical significant where p was found to be <0.05 and where $p<0.001$ *** is shown.

Figure 3. Concentration response curve illustrating the vasodilator effects of α, β methylene ATP. Isolated mesenteric vessels were contracted with methoxamine (meth.). The data shown is the mean +/- the S.E. mean for n=3-4 experiments. In these experiments a single concentration was administered to each tissue, cumulative response curves were not possible because of desensitisation of the drug.

Figure 4. Effects of α, β methylene ATP on contractile responses of mesenteric vessels precontracted with methoxamine (meth.). The concentrations shown are selected from

those depicted in Figure 3 where concentration dependent dilation is seen. The data shown is the mean +/- the S.E. mean for n=3-4 experiments. Again, a single concentration of α, β methylene ATP was administered to each tissue, since cumulative response curves were not possible due to desensitisation effect of the drug.

Figure 5. Characterisation of the dilator function of α, β methylene ATP. The figure shows the effects of the combination of L-NAME (10^{-4}M) plus indomethacin (10^{-5}M; L+I), apamin (10^{-6}M) plus charybdotoxin (10^{-7}M; A+C), L+I plus A+C (L/I/A/C) or KCl (10^{-2}M) on the vasodilator actions of α, β methylene ATP (10^{-4}M) on methoxamine (meth.) precontracted arteries. Data is shown as the mean ± s.e.m. for n=3-8 experiments. Statistical significance was established using one-way ANOVA followed by Dunnetts Multiple Comparison Test. $p<0.001$; *$p<0.05$, ***$p<0.001$.

Figure 6. Role of the endothelium in the vasodilator actions of α, β methylene ATP. The figure shows data where responses were obtained using endothelial cell intact (+EC) versus endothelial denuded (-EC) arteries precontracted with methoxamine (meth.), compared to time controls (T.Con.) Data is shown as the mean ± s.e.m. for n=3-8 experiments. Statistical significance was established using one-way ANOVA followed by Dunnetts Multiple Comparison Test. $p<0.001$; *$p<0.05$, ***$p<0.001$.

Figure 7. Representative recording of the effect of removal of endothelial cells (+/-EC) on α, β methylene ATP vasodilation.

Figure 8. Effect of removal of endothelial cells (+/-EC) on α, β methylene ATP contraction in methoxamine (meth.) precontracted mesenteric arteries. Data is shown as

the mean ± s.e.m. for n=3-8 experiments. Significant by one-way ANOVA; $p<0.001$, ***$p<0.001$ by Dunnetts Multiple Comparison Test.

Reference List

Boeynaems JM, Communi D, Savi P, and Herbert JM (2000) P2Y receptors: in the middle of the road. *Trends Pharmacol.Sci.* **21**:1-3.

Burnstock G (1999) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J.Anat.* **194 (Pt 3)**:335-342.

Burnstock G and Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoceptor? *Gen.Pharmacol.* **16**:433-440.

Glass R, Loesch A, Bodin P, and Burnstock G (2002) P2X4 and P2X6 receptors associate with VE-cadherin in human endothelial cells. *Cell Mol.Life Sci.* **59**:870-881.

Hansen MA, Dutton JL, Balcar VJ, Barden JA, and Bennett MR (1999) P2X (purinergic) receptor distributions in rat blood vessels. *J.Auton.Nerv.Syst.* **75**:147-155.

Harrington LS and Mitchell JA (2003) Activation of P2X-like receptors in rat isolated 2nd order mesenteric arteries induces vasodilation: Role of EDHF. *Br.J.Pharmacol.* In Press.

Kunapuli SP and Daniel JL (1998) P2 receptor subtypes in the cardiovascular system. *Biochem.J.* **336 (Pt 3)**:513-523.

Lewis CJ and Evans RJ (2000) Lack of run-down of smooth muscle P2X receptor currents recorded with the amphotericin permeabilized patch technique, physiological and pharmacological characterization of the properties of mesenteric artery P2X receptor ion channels. *Br.J.Pharmacol.* **131**:1659-1666.

Malmsjo M, Chu ZM, Croft K, Erlinge D, Edvinsson L, and Beilin LJ (2002) P2Y receptor-induced EDHF vasodilatation is of primary importance for the regulation of perfusion pressure in the peripheral circulation of the rat. *Acta Physiol Scand.* **174**:301-309.

Mulvany MJ and Halpern W (1977) Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ.Res.* **41**:19-26.

North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev.* **82**:1013-1067.

Ralevic V (2002) The involvement of smooth muscle P2X receptors in the prolonged vasorelaxation response to purine nucleotides in the rat mesenteric arterial bed. *Br.J.Pharmacol.* **135**:1988-1994.

Ralevic V and Burnstock G (1988) Actions mediated by P2-purinoceptorsubtypes in the isolated perfused mesenteric bed of the rat. *Br.J.Pharmacol.* **95**:637-645.

Ralevic V and Burnstock G (1991) Effects of purines and pyrimidines on the rat mesenteric arterial bed. *Circ.Res.* **69**:1583-1590.

Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol.Rev.* **50**:413-492.

Ray FR, Huang W, Slater M, and Barden JA (2002) Purinergic receptor distribution in endothelial cells in blood vessels: a basis for selection of coronary artery grafts. *Atherosclerosis* **162**:55-61.

Stanford SJ. and Mitchell JA. (1998) ATP-induced vasodilatation in the rat mesenteric bed exhibits two apparent phases. *Br. J. Pharmacol*. 125, 83P.

Stanford SJ, Gitlin JM, and Mitchell JA (2001) Identification of two distinct vasodilator pathways activated by ATP in the mesenteric bed of the rat. *Br.J.Pharmacol.* **133**:825-832.

Surprenant A, Schneider DA, Wilson HL, Galligan JJ, and North RA (2000) Functional properties of heteromeric P2X(1/5) receptors expressed in HEK cells and excitatory junction potentials in guinea-pig submucosal arterioles. *J.Auton.Nerv.Syst.* **81**:249-263.

Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North RA, and Elde R (1996) Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. *Proc.Natl.Acad.Sci.U.S.A* **93**:8063-8067.

Yamamoto K, Korenaga R, Kamiya A, Qi Z, Sokabe M, and Ando J (2000) P2X(4) receptors mediate ATP-induced calcium influx in human vascular endothelial cells. *Am.J.Physiol Heart Circ.Physiol* **279**:H285-H292.

Yang S, Cheek DJ, Westfall DP, and Buxton IL (1994) Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. *Circ.Res.* **74**:401-407.

Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch.Pharmacol.* **362**:299-309.