Pre-contraction with the thromboxane-mimetic U46619 enhances P2X receptor-mediated contractions in isolated porcine splenic artery

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Received: 8 July 2011 / Accepted: 14 November 2011 / Published online: 24 November 2011
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Abstract We have previously demonstrated that the thromboxane-mimetic U46619 enhances α_2 -adrenoceptor-mediated contractions through increased activation of extracellular signal-regulated kinase (ERK). In this study, we determined whether U46619 also enhances P2X-mediated contractions through the same pathway. Segments of porcine splenic artery were mounted in isolated tissue baths. Tissues were pre-contracted with U46619 to 10–20% of the response to 60 mM KCl prior to addition of α,β -methylene ATP (P2X receptor agonist). The effect of inhibition of ERK activation with the mitogen-activated protein (MAP)/ERK kinase inhibitor PD98059 (50 μ M), Rho kinase inhibition with Y27632 (10 μ M), p38 MAP kinase with SB203580 (10 μ M) or L-type calcium channels with nifedipine (1 μ M) on both the direct and enhanced contractions was then determined. U46619 enhanced the contractions to α,β -methylene ATP. Although PD98059 inhibited the direct contractions to α,β -methylene ATP, it had no effect on the U46619-enhanced contractions. Similarly, Y27632 and SB203580 inhibited the direct contractions to α,β -methylene ATP, but had no effect on the enhanced contractions. Nifedipine inhibited the responses to α,β -methylene ATP in the absence and presence of U46619. This study demonstrates that pre-contraction with U46619 enhances P2X-mediated contractions in the porcine splenic artery through a mechanism independent of ERK, Rho kinase and p38 MAP kinase. Further studies are required to determine the exact mechanism involved.

Keywords ERK · P2X receptors · Thromboxane · Rho kinase · p38 MAP kinase

Abbreviations

ERK extracellular signal regulated kinase
MAP mitogen-activated protein

Introduction

We have previously demonstrated that pre-contraction with the thromboxane-mimetic U46619 enhances the vasoconstriction in response to α_2 -adrenoceptor activation [2]. α_2 -Adrenoceptor-mediated vasoconstriction is dependent upon activation of extracellular signal-regulated kinase (ERK) and Rho kinase [13], and the enhancement of the α_2 -adrenoceptor-mediated vasoconstriction by U46619 in the porcine ear artery is due to an enhancement of ERK activity [2]. A recent study has demonstrated that pre-contraction with U46619 also uncovers a purine P2X-mediated contraction in response to nerve stimulation in the rat mesenteric vascular bed [12]. The mechanisms underlying this effect are unknown. However, as U46619 can also enhance P2X-mediated contractions, it is possible that the enhanced contractions to α_2 -adrenoceptor and P2X receptor activation are through a common mechanism. The synergistic interaction between thromboxane receptors and P2X receptors is likely to be important when there is co-release of ATP and thromboxane from aggregating platelets, for example, during vasospasm.

ERK is a member of the mitogen-activated protein (MAP) kinase family of protein kinases. Activation of ERK has been shown to be involved in the mechanism of vasoconstriction in a number of blood vessels in response to different agonists [6, 13 16]. The mechanism through

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which ERK causes vasoconstriction has been suggested to be through phosphorylation and hence inhibition of caldesmon [11]. Caldesmon inhibits the myosin ATPase. Therefore, inhibition of caldesmon removes this inhibitory effect and therefore promotes contraction of the smooth muscle [14]. Similarly, Rho kinase causes vasoconstriction through inhibition of the myosin phosphatase, thereby preventing dephosphorylation of myosin light chains and so enhancing vasoconstriction. Both pathways are thought to play a role in the process of calcium sensitisation whereby large contractile responses can occur with small increases in intracellular calcium [14]. Therefore, the enhancement of the P2X-mediated vasoconstriction may be through activation of these signalling pathways such that the increase in intracellular calcium through activation of the P2X receptor causes a larger contraction.

Therefore, the aim of this study was to determine whether pre-contraction with U46619 enhances the vasoconstriction in response to P2X receptor activation in porcine blood isolated splenic artery, which has a large, reproducible P2X-mediated contraction. A subsequent aim was to determine whether this enhanced contraction involves ERK and/or Rho kinase.

Methods

Isometric tension recordings

Porcine spleens were obtained from a local abattoir and transported to the laboratory in Krebs–Henseleit buffer on ice. Splenic arteries were dissected out and placed in Krebs–Henseleit buffer pre-gassed with 95% O₂/5% CO₂ and stored overnight at 4°C. The following day, endothelium-intact splenic arteries were dissected into 5-mm ring segments and suspended in an isolated tissue bath containing Krebs–Henseleit buffer maintained at 37°C and constantly gassed with 95% O₂/5% CO₂. The lower support was fixed and the upper support was connected to a force transducer (Lectromed, Letchworth, UK) linked to a PowerLab data acquisition unit (AD Instruments Ltd., Hastings, UK) via an amplifier. After a 20-min equilibration period, tension was applied to the tissue, which was allowed to relax to a final resting tension of between 2 and 3 g wt. Before each experiment, the tissues were contracted at least three times with 60 mM KCl, until the final two responses to KCl differed by less than 10%. Contractile responses were expressed as a percentage of the final KCl response.

Effect of pre-contraction on agonist-induced contractions

Tissues were pre-contracted with the thromboxane-mimetic U46619 (1–10 nM). The degree of pre-contraction obtained

under these conditions was between 10% and 20% of the response to 60 mM KCl. We have previously demonstrated that this level of tone enhances α_2 -adrenoceptor-mediated contractions in the porcine ear artery [2]. Tissues were then exposed to different concentrations of the ATP stable analogue α,β -methylene ATP (0.1 to 3 μ M). P2X receptors desensitise rapidly, and therefore, non-cumulative concentration–response curves were performed. As a comparison, cumulative concentration–response curves to the α_1 -adrenoceptor agonist phenylephrine [1] were carried out in the presence or absence of U46619. Control vessels were not pre-contracted with U46619. Concentration–response curves to phenylephrine were performed in the presence of 1 μ M rauwolscine in order to prevent activation of α_2 -adrenoceptors. Contractile responses to the agonists in the presence of U46619 were measured as a change from the plateau phase of the U46619-induced contraction, not the baseline.

Role of ERK, Rho kinase, p38 MAP kinase or protein kinase C

In order to determine whether ERK is involved in either the direct vasoconstriction in response to α,β -methylene ATP or the enhanced responses to these agonists in the presence of U46619, tissues were pre-incubated for 1 h with the selective MAP/ERK kinase inhibitor PD98059 (50 μ M; [5]). Control tissues received vehicle only (0.26% DMSO). Likewise, in order to determine whether Rho kinase, p38 MAP kinase or protein kinase C are involved in any of the contractile responses, tissues were pre-incubated for 1 h with the selective Rho kinase inhibitor Y27632 (10 μ M; [15]), or the selective p38 MAP kinase inhibitor SB203580 (10 μ M; [4]), or the selective protein kinase C inhibitor calphostin C (1 μ M; [9]).

Effect of removal of extracellular calcium

In order to determine whether extracellular calcium is involved in the vasoconstriction to α,β -methylene ATP, the Krebs–Henseleit buffer was replaced with calcium-free Krebs–Henseleit buffer in which the calcium chloride had been replaced with 2 mM EGTA, 5 min before the addition of the agonist. In order to determine the role of L-type calcium channels, tissues were exposed to 1 μ M nifedipine 1 h before addition of agonist. Control tissues received vehicle only (0.1% ethanol).

Drugs

(5Z, 9 α 11 α , 13E, 15 (S))-15-hydroxy-9 (11) methanoepoxyprosta-5,13-dien-1 oic acid (U46619) and nifedipine were obtained from Axxora, Bingham, Nottinghamshire, UK; 2-amino-3-methoxyflavone (PD98059), 4-(4-fluoro-

phenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) and calphostin C from Calbiochem, Beeston, Nottingham, UK; rauwolsine and trans-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y27632) from Tocris Bioscience, Bristol, UK and phenylephrine and α,β -methylene ATP from Sigma, Poole, Dorset, UK. The remaining chemicals were obtained from Sigma (Poole, Dorset, UK).

Data analysis

Contractile responses were expressed as a percentage of the response to 60 mM KCl and are means $\pm$ SE mean. Data were analysed using Prism (GraphPad Software Inc., San Diego, CA, USA). Concentration–response curve data were analysed using two-way ANOVA followed by a Bonferroni post hoc test. Single comparisons between groups were made using a Student's two-tailed, unpaired *t* test for normally distributed data or a Mann–Whitney *U* test for non-parametric data. A *p* value <0.05 was considered statistically significant.

Results

Effect of pre-contraction with U46619 on the responses to α,β -methylene ATP or phenylephrine

The response to the P2X receptor agonist α,β -methylene ATP in the porcine splenic artery was transient. Therefore, a non-cumulative concentration–response curve was carried out. In tissues pre-contracted with U46619, the response to α,β -methylene ATP was enhanced (Fig. 1a). As a comparison, the effect of pre-contraction with U46619 on α_1 -adrenoceptor-mediated contractions was determined. In contrast to the α,β -methylene ATP-mediated response, the response to the α_1 -adrenoceptor agonist phenylephrine was not enhanced by pre-contraction with U46619 (Fig. 1b). Indeed, there was a reduction in the maximum response to phenylephrine after pre-contraction with U46619, which is probably due to the tissues reaching maximum contraction. There was no significant difference in the pEC₅₀ values for phenylephrine (6.1 ± 0.1 in control (mean $\pm$ S E mean) compared to 5.9 ± 0.1 in the presence of U46619, $n=4$).

Effect of inhibition of ERK activation on contractile responses in the porcine splenic artery

Contractions to the P2X receptor agonist α,β -methylene ATP (0.3 to 10 μ M) in the absence of U46619 were inhibited by 50 μ M PD98059 (Fig. 2a). However, after pre-contraction with U46619, the enhanced contractions to α,β -methylene ATP were unaffected by PD98059 at 50 μ M

Fig. 1 **a** Contractile responses to α,β -methylene ATP (0.1–3 μ M) in the porcine splenic artery in the absence (control) or after pre-contraction with U46619 (+ U46619). Contractions are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of six experiments. **p* <0.05 vs. control and ***p* <0.01 vs. control, two-way ANOVA followed by Bonferroni post hoc test. **b** Concentration–response curves to phenylephrine in the porcine splenic artery in the absence (control) or after pre-contraction with U46619 (+ U46619), in the presence of 1 μ M rauwolsine. Contractions are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of six experiments. **p* <0.05 , two-way ANOVA, followed by Bonferroni post hoc test

(Fig. 2b). In tissues that were exposed to PD98059, a higher concentration of U46619 was required to produce the same level of pre-contraction obtained in control tissues (30–60 nM in the presence of 50 μ M PD98059 compared to 3–7 nM in control tissues).

Effect of Y27632 on contractile responses

Contractions to α,β -methylene ATP (0.3 to 10 μ M) in the absence of U46619 were inhibited by the Rho kinase inhibitor Y27632 (10 μ M; Fig. 3a). On the other hand, 10 μ M Y27632 had no significant effect on the responses to α,β -methylene ATP in the presence of U46619 (Fig. 3b).

In tissues that were exposed to Y27632, a higher concentration of U46619 was required to produce the same level of pre-contraction obtained in control tissues (30–80 nM in the presence of 10 μ M Y27632 compared to 1–7 nM in control tissues).

Fig. 2 a Contractile responses to α,β -methylene ATP in porcine splenic artery in the absence and presence of PD98059 (50 μ M). Contractions are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of seven experiments. * p <0.05 vs. control, Student's two-tailed, unpaired t test. # p <0.05 vs. control, two-tailed, Mann–Whitney U test. ## p <0.01 vs. control, two-tailed Mann–Whitney U test. **b** Contractile responses to α,β -methylene ATP in porcine splenic artery in the absence and presence of PD98059 (50 μ M) with pre-contraction with U46619. Contractions are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of four to nine experiments

Fig. 3 a Contractile responses to α,β -methylene ATP in porcine splenic artery in the absence and presence of Y27632 (10 μ M) without pre-contraction with the thromboxane mimetic U46619 (+ U46619). Responses are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of six experiments. * p <0.05 vs. control, Student's two-tailed, unpaired t test. ** p <0.01 vs. control, Student's two-tailed, unpaired t test. ## p <0.01 vs. control, two-tailed Mann–Whitney U test. **b** Contractile responses to α,β -methylene ATP in porcine splenic artery in the absence and presence of Y27632 (10 μ M) with pre-contraction with the thromboxane mimetic U46619 (+ U46619). Responses are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of four to six experiments

Effect of SB203580 and calphostin C on contractile responses

The p38 MAP kinase inhibitor SB203580 (10 μ M) inhibited contractions to α,β -methylene ATP in the absence of U46619, but there was no significant effect on the responses to α,β -methylene ATP in the presence of U46619 (Fig. 4a). In tissues that were exposed to SB203580, a slightly higher concentration of U46619 was required to produce the same level of pre-contraction obtained in control tissues (5–30 nM in the presence of SB203580 compared to 3–10 nM in control tissues). The selective protein kinase C inhibitor calphostin C (1 μ M) had no effect on the contractile responses to α,β -methylene ATP in the absence or presence of U46619 (Fig. 4b).

Role of extracellular calcium

Contractile responses to 1 μ M α,β -methylene ATP in the absence (contractile response $0.3 \pm 0.2\%$, $n=5$) and presence (contractile response $0.6 \pm 0.4\%$, $n=5$) of U46619 were inhibited by removal of extracellular calcium. In the presence of 1 μ M nifedipine, the contractions to 1 μ M α,β -methylene ATP in the absence or presence of U46619 were inhibited by 60% by the addition of 1 μ M nifedipine (Fig. 5). There was no difference in the concentration of U46619 required to produce the same level of contraction in tissues that were exposed to nifedipine compared to controls.

Fig. 4 **a** Contractile responses to 1 μ M α,β -methylene ATP in porcine splenic artery in the absence and presence of SB203580 (10 μ M) with and without pre-contraction with the thromboxane mimetic U46619 (+ U46619). Responses are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of six experiments. * p <0.001 vs control, Student's two-tailed, unpaired t test. **b** Contractile responses to 1 μ M α,β -methylene ATP in porcine splenic artery in the absence and presence of calphostin C (1 μ M) with and without pre-contraction with the thromboxane mimetic U46619 (+ U46619). Responses are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of five to 11 experiments

Discussion

The data presented in this study demonstrate that pre-contraction with the thromboxane mimetic U46619 leads to an enhancement of P2X receptor-mediated contractions in porcine splenic artery. This is similar to the effect of U46619 on α_2 -adrenoceptor-mediated contractions [2] and could indicate that there is a common mechanism underlying the enhanced contraction in response to P2X receptor and α_2 -adrenoceptor activation. On the other hand, the effect of U46619 is not a general phenomenon as contractile responses to the α_1 -adrenoceptor agonist phen-

Fig. 5 Contractile responses to 1 μ M α,β -methylene ATP in porcine splenic artery in the absence and presence of nifedipine (1 μ M) with and without pre-contraction with the thromboxane mimetic U46619 (+ U46619). Responses are expressed as percentage of the response to 60 mM KCl and are means $\pm$ SEM of six to ten experiments. * p <0.01 vs. control, two-tailed, Mann–Whitney U test. ** p <0.01 vs. + U46619, two-tailed, Mann–Whitney U test

ylephrine were not enhanced. This indicates that the enhancement of the P2X-mediated contraction is through a specific pathway, rather than a general increase in calcium sensitisation.

In order to determine whether the enhanced contractions to P2X receptor stimulation in the presence of U46619 are due to the same pathway identified previously for enhanced α_2 -adrenoceptor-mediated contractions, we investigated the effect of inhibition of ERK activation. We have previously demonstrated that α_2 -adrenoceptor-mediated contractions in the porcine ear artery are dependent upon activation of ERK and pre-contraction with U46619 enhances the α_2 -adrenoceptor-mediated contraction through enhancement of ERK activation [2]. In this study, we found that the direct contraction to α,β -methylene ATP in the absence of U46619 was inhibited by PD98059; however, the enhanced contraction to α,β -methylene ATP in the presence of U46619 was unaffected by PD98059, even at low concentrations of α,β -methylene ATP, indicating that ERK is not involved in the enhanced contractile response. This is in contrast to the effect of U46619 on the α_2 -adrenoceptor-mediated contraction [2]. On the other hand, we have also shown previously that pre-contraction with vasopressin enhances α_2 -adrenoceptor-mediated vasoconstriction through a mechanism that is independent of ERK activity [3], although we did not identify the mechanism involved. Therefore, the mechanism underlying the enhancement of the P2X-mediated contraction by U46619 may share more similarities with the enhancement of the α_2 -adrenoceptor-mediated vasoconstriction by vasopressin than the enhancement of the α_2 -adrenoceptor-mediated vasoconstriction by U46619. The lack of effect by PD98059 on the enhanced

P2X-mediated contraction also suggests that the presence of U46619 causes a change in the intracellular signalling cascade mediating vasoconstriction to α,β -methylene ATP away from ERK.

As the enhanced contraction to α,β -methylene ATP appears to be through a different pathway to that seen in the enhanced α_2 -adrenoceptor-mediated contraction, we investigated other potential mechanisms of contraction. Calcium sensitisation has been proposed as a mechanism by which synergy can occur between two receptors to enhance contractile responses. Protein kinase C and Rho kinase are implicated in calcium sensitising pathways through their effects on myosin phosphatase activity [7,14]. However, in this study, inhibition of protein kinase C with calphostin C had no effect on either the direct or the enhanced contractions to α,β -methylene ATP. The Rho kinase inhibitor Y27632, on the other hand, inhibited the direct contraction after stimulation of P2X receptors, indicating that Rho kinase is involved in this direct contraction. However, the enhanced contractions in the presence of U46619 were unaffected by Y27632 at concentrations up to 10 μ M, suggesting that Rho kinase is not involved in the enhanced contraction through these receptors in the presence of U46619. Again, as the direct contractions to α,β -methylene ATP were inhibited by Y27632, but not the enhanced contractions, this suggests that the presence of U46619 causes a shift in the intracellular signalling cascade mediating contraction away from Rho kinase.

p38 MAP kinase has also been implicated in calcium sensitisation in blood vessels [10] and could, therefore, play a role in the enhanced contractile responses in the presence of U46619. Again, although the direct contractions to α,β -methylene ATP were inhibited by the p38 MAP kinase selective inhibitor SB203580, the U46619-enhanced contractions were unaffected, indicating that p38 MAP kinase is also not involved in the enhanced contraction. This is further evidence that the presence of U46619 causes a shift in the intracellular signalling cascade in response to P2X receptor activation.

In the absence of extracellular calcium, both the direct and the enhanced contractions to α,β -methylene ATP were almost abolished, indicating the importance of calcium influx on both the direct and the enhanced P2X-mediated contraction. This effect is no surprise considering the nature of the P2X receptor and previous studies [8]. The contractions were also inhibited by nifedipine, indicating the calcium influx through L-type calcium channels is important in both the direct and the enhanced contractions. The effect of nifedipine is similar in both the direct and U46619-enhanced contractile responses, suggesting that the responses under both conditions are equally dependent upon calcium influx. The fact that removal of extracellular calcium almost completely abolished the contractions in the

presence of U46619 indicates that the pre-contraction with U46619 does not uncover a calcium-independent mechanism of contraction.

Taken together, the data presented in this study demonstrate that pre-contraction with the thromboxane agonist U46619 enhances the P2X response in porcine splenic artery. This is similar to the effect seen in the rat mesenteric vascular bed [12] and is also similar to the effect on α_2 -adrenoceptor-mediated responses [2]. However, unlike the effect on the α_2 -adrenoceptor-mediated contractions, there is no evidence for involvement of ERK. Furthermore, there is no evidence that the enhanced contraction is mediated through calcium sensitisation via protein kinase C, Rho kinase or p38 MAP kinase. These data demonstrate that although pre-contraction with U46619 enhances the contractile responses to P2X-receptor (this study) and α_2 -adrenoceptor-mediated contractions [2], the mechanisms of enhancement appear to be through different signalling pathways.

Acknowledgements We thank G Woods & Sons, Clipstone, Nottinghamshire for providing the pig tissue.

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