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RESEARCH PAPER

Desensitization of endothelial P2Y1 receptors by PKC-dependent mechanisms in pressurized rat small mesenteric arteries

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Background and purpose: Extracellular nucleotides play a crucial role in the regulation of vascular tone and blood flow. Stimulation of endothelial cell P2Y1 receptors evokes concentration-dependent full dilatation of resistance arteries. However, this GPCR can desensitize upon prolonged exposure to the agonist. Our aim was to determine the extent and nature of P2Y1 desensitization in isolated and pressurized rat small mesenteric arteries.

Experimental approach: The non-hydrolyzable selective P2Y1 agonist ADPβS (3 μM) was perfused through the lumen of arteries pressurized to 70 mmHg. Changes in arterial diameter and endothelial cell $[Ca^{2+}]_i$ were obtained in the presence and absence of inhibitors of protein kinase C (PKC).

Key results: ADPβS evoked rapid dilatation to the maximum arterial diameter but faded over time to a much-reduced plateau closer to 35% dilatation. This appeared to be due to desensitization of the P2Y1 receptor, as subsequent endothelium-dependent dilatation to acetylcholine (1 μM) remained unaffected. Luminal treatment with the PKC inhibitors BIS-I (1 μM) or BIS-VIII (1 μM) tended to augment concentration-dependent dilatation to ADPβS (0.1–3 μM) and prevented desensitization. Another PKC inhibitor, Gö 6976 (1 μM), was less effective in preventing desensitization. Measurements of endothelial cell $[Ca^{2+}]_i$ in pressurized arteries confirmed the P2Y1 receptor but not M_3 muscarinic receptor desensitization.

Conclusions and implications: These data demonstrate for the first time the involvement of PKC in the desensitization of endothelial P2Y1 receptors in pressurized rat mesenteric arteries, which may have important implications in the control of blood flow by circulating nucleotides.

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Keywords: P2Y1 receptor; NO; EDHF; mesenteric artery; desensitization; dilatation; endothelial cell Ca^{2+}; endothelium

Abbreviations: ADPβS, adenosine 5′-[β-thio]diphosphate; BIS-I, bisindolylmaleimide I; BIS-VIII, bisindolylmaleimide VIII; EDHF, endothelium-derived hyperpolarizing factor; Gö 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IEL, internal elastic lamina; L-NAME, N^{ω}-nitro-L-arginine methyl ester hydrochloride; MOPS, 3-[N-morpholino]propane-sulphonic acid; SK_{Ca} and IK_{Ca}, small- and intermediate-conductance Ca^{2+}-activated K^+ channel

Introduction

Extracellular nucleotides play a crucial role in the regulation of vascular function (Burnstock, 1990; Buvinic *et al.*, 2006). Physiological and pathophysiological circumstances such as platelet degranulation, hypoxia or damage in the endothelium evoke a massive release of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and uridine triphosphate (UTP) into circulation followed by their action through cell-surface P2 receptors (Abbracchio *et al.*, 2006). These purinergic receptors are divided into two groups: P2X and P2Y receptors. P2Y receptors are G protein-coupled receptors (GPCRs) located in both smooth muscle cells and endothelial cells, depending on the receptor subtype (Burnstock, 2006). All of the cloned P2Y receptors activate phospholipase C (PLC) resulting in inositol 1,4,5-trisphosphate generation and Ca^{2+} release from intracellular stores. While activation of P2Y receptors located in smooth muscle cells evokes vasoconstriction, the action of nucleotides on endothelial P2Y purinoceptors has been demonstrated to induce dilatation (Burnstock, 2006).

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P2Y1 and P2Y2 are the major nucleotide receptor subtypes expressed on rat and human vascular endothelial cells (Wang *et al.*, 2002). Pioneering investigations have demonstrated that activation of both receptor subtypes mediate dilatation in conductance (Guns *et al.*, 2005; 2006) and resistance vessels (Malmsjo *et al.*, 1999a,b; Buvinic *et al.*, 2002; Mistry *et al.*, 2003), demonstrating its relevant role in the regulation of arterial tone. In the rat arterial mesenteric bed, endothelium-dependent dilatation elicited by nucleotides acting at P2Y1 and P2Y2 receptors is mainly mediated by nitric oxide (NO) or endothelium-derived hyperpolarizing factor (EDHF) (Malmsjo *et al.*, 1999a,b; Mistry *et al.*, 2003). The complete attenuation of dilatation evoked by adenosine 5′-[β-thio]diphosphate (ADPβS), ATP and UTP by selective inhibition of nitric oxide synthase (NOS), together with small- and intermediate-conductance Ca^{2+}-activated K^+ channels (SK_{Ca} and IK_{Ca}, respectively), supports these data (Malmsjo *et al.*, 1998; 1999b; 2002; Buvinic *et al.*, 2002; Mistry *et al.*, 2003; Liu *et al.*, 2004; Winter and Dora, 2007). Studies using wire myography limit the investigator to bath application of agonists, and direct activation of smooth muscle purinoceptors can evoke vasoconstriction. This complication has been overcome by luminal application of the nucleotides in isolated pressurized arteries (Liu *et al.*, 2004; Winter and Dora, 2007), which can evoke both local and spreading dilatation through P2Y1 and P2Y2 receptors, suggesting an effective and significant ability to reduce vascular resistance and increase blood flow (Winter and Dora, 2007).

The response of the P2Y receptors is tightly regulated by mechanisms involving desensitization, a crucial physiological mechanism of adaptation observed for many GPCRs (Ferguson and Caron, 1998). Desensitization of GPCRs is a complex phenomenon that involves phosphorylation of the receptors by protein kinases such as protein kinase C (PKC), protein kinase A (PKA) or GPCR kinases (GRKs) and finally by interaction or not with the protein arrestins, attenuating the receptor signalling (Tobin, 2008). These purinergic receptors can desensitize upon prolonged stimulation with the agonist (Gainetdinov *et al.*, 2004). In platelets, P2Y1 receptor desensitization evoked by ADP has been attributed to PKC-dependent and GRK-independent mechanisms (Hardy *et al.*, 2005; Mundell *et al.*, 2006). However, the regulation of P2Y1 receptor desensitization events in endothelial cells remains poorly understood, especially in arteries under physiological conditions. In this regard, Liu *et al.* (2004) documented an attenuation of the dilatation response upon prolonged exposure to luminal perfusion of purinoceptor agonists in resistance arteries. The decay of this response, more evident after the application of non-hydrolyzable purinoceptor agonists, may be explained by receptor desensitization, but was not explored. In endothelial cells, P2Y receptor desensitization has been studied in isolated, cultured cells by measuring changes in cytosolic free Ca^{2+} (Carter *et al.*, 1990; Wilkinson *et al.*, 1994), but to date, this has not been studied in intact arteries.

In this study, we show that luminal perfusion of ADPβS stimulates dilatation and rises in endothelial cell Ca^{2+} that desensitize the P2Y1, but not the muscarinic, receptors via a PKC-dependent pathway in rat small mesenteric arteries. Therefore, desensitization of P2Y1 receptors must be considered when assessing the importance of adenine nucleotides in the control of vascular flow.

Methods

Tissue preparation for pressure myography

All animal care and experimental procedures complied with the UK Animals, Scientific Procedures, Act 1986. Male Wistar rats (200–250 g) were killed by cervical dislocation and exsanguination (Schedule 1 procedure). The mesentery was collected and placed in cold 3-[N-morpholino]propanesulphonic acid (MOPS) buffer containing (mM) NaCl 145.0, KCl 4.7, $CaCl_2{\cdot}2H_2O$ 2.0, $MgSO_4{\cdot}7H_2O$ 1.17, MOPS 2.0, $NaH_2PO_4{\cdot}H_2O$ 1.20, glucose 5.0, pyruvate 2.0, ethylenediamine-tetraacetic acid 0.02 and NaOH 2.75 adjusted to pH 7.40 ± 0.02. A third-order branch of the superior mesenteric artery was carefully dissected free of adherent tissue. A segment of the artery (internal diameter 200–300 μm) was cut and then cannulated at each end with glass pipettes (diameter 180–200 μm), which were either fixed into a static pressure myograph bath (10 mL) (120CP; Danish Myo Technology, Aarhus, Denmark) or for Ca^{2+} measurements, positioned near the base of a 2 mL temperature-regulated chamber seated in a stage insert (RC-27 chamber, PH-6 platform, Warner Instruments, Hamden, CT, USA) using micromanipulators (Winter and Dora, 2007). Following a 20 min equilibration period in MOPS solution (2 mL·min^{-1}) at 37°C, arteries in both set-ups were pressurized to 80 mmHg, longitudinally straightened and stretched a further 10% of the arterial length to optimize responses to phenylephrine. The pressure was then decreased to 70 mmHg and maintained at this level during the experiments. To avoid luminal flow, the upstream and downstream pressures were equal throughout the experiments. Endothelial viability was assessed as a >90% control relaxation to 1 μM acetylcholine (ACh) after pre-constriction with phenylephrine (1–3 μM). Most experiments were performed in the presence of the selective inhibitor of NOS, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM); control signifies the absence of L-NAME. Inhibition of cyclooxygenase was not required as indomethacin (10 μM) has been shown to have no effect on purinoceptor agonist-evoked dilatations within the rat mesenteric artery (Rodríguez-Rodríguez and Dora unpubl. obs.; Mistry *et al.*, 2003).

Measurement of diameter in pressurized arteries

Arteries mounted and pressurized in a static pressure myograph bath as described above were visualized using an inverted microscope (IX70, Olympus, Tokyo, Japan) with a 4×/0.13 NA objective, captured with a CoolSNAP *hq* CCD camera (Image Processing Solutions, Inc., North Reading, MA, USA) and recorded by the Andor iQ software (v.1.2.0, Andor, Belfast, UK). In the presence of phenylephrine (1–10 μM) added to the bath, P2Y receptor agonists were perfused through the lumen of arteries at 50 μL·min^{-1} using syringe pumps (Bee Hive, BASi, Kenilworth, UK). Consecutive, non-cumulative concentration–response curves to ADPβS (0.1–3 μM) were obtained. There was a short delay (~30–40 s) in observing the response to luminal perfusion of nucleotides due to voiding the tubing and pipette dead-space volume. For each curve, ACh (1 μM) was added to the bath after ending the purinoceptor agonist perfusion. Arteries were luminally perfused with MOPS and left to equilibrate for 15 min between each agonist and

concentration–response curve to minimize receptor desensitization (Ralevic and Burnstock, 1998; Otero *et al.*, 2000; Mundell *et al.*, 2008). To test the involvement of PKC in the relaxation and desensitization evoked by the nucleotides, the same concentration–response curves were made following PKC inhibition with bisindolylmaleimide I (BIS-I, Gö 6850, GF 109203X, 1 μM), BIS-VIII (Ro 31-7549, 1 μM) or Gö 6976 (1 μM). These inhibitors were perfused luminally (to minimize smooth muscle effects), incubated for 15 min prior to luminal perfusion of the agonist, and subsequently included in the luminal solutions containing agonist. Artery outer diameter was measured offline using motion analysis software (Metamorph Version 6.1, Universal Imaging, Downingtown, PA, USA).

Measurement of $[Ca^{2+}]_i$ changes in pressurized arteries

In separate experiments, artery segments were mounted in a small chamber seated in a heated stage as described above. Bright field images were captured with a laser scanning confocal microscope (FV300-SU, Olympus) and recorded with Fluoview Software version 5.0 (Olympus). After an equilibration period, the pressurized artery was luminally perfused with filtered (0.2 μm pore) MOPS-buffered solution containing a cell-permeable, acetoxymethyl ester form of Oregon Green 488 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetate (Oregon Green 488 BAPTA-1 AM, 10 μM) and 0.02% Pluronic F-127 for 30 min to selectively load endothelial cells, as described (Kansui *et al.*, 2008). The dye was then washed out and allowed to de-esterify and equilibrate for at least 30 min. Endothelial cell fluorescence intensity was visualized by lowering the focal plane to endothelial cells at the bottom of the artery. In a 512 × 360 pixel clip box, at least 20 cells were visible (40×/0.8 NA objective, Olympus; excitation 488 nm, emission 505 nm), and scanned images were recorded at a frequency of 2 Hz. Changes in endothelial cell $[Ca^{2+}]_i$ were investigated in response to luminal perfusion of the P2Y1 receptor agonists, as explained above, in the absence or presence of the PKC inhibitor BIS-I (1 μM). To avoid the motion due to turning on the syringe pump, vehicle (MOPS ± BIS-I) was perfused alone before rapidly switching to the pump containing the agonist (±BIS-I). This avoided any artefact due to changes in acquisition focal plane. Fluorescence intensity was measured offline using edge-detection software (Metamorph version 6.1, Universal Imaging).

Immunohistochemistry

Pressurized arteries were fixed in cold methanol for 10 min, washed with phosphate buffered saline (PBS), followed by incubation in blocking buffer solution for 1 h at 37°C (1% bovine serum albumin and 0.1% Tween-20). After that, vessels were exposed to the primary antibody overnight at 4°C. These were anti-P2Y1 receptors raised in rabbit against the residues 363–373 (CPEFKQNGDTSL) located at the extreme C-terminus of the human P2Y1 receptor (1:100) (Buvinic *et al.*, 2007), anti-P2Y1 receptor raised in rabbit against the residues 12–25 (GTDAAFLAGPGSSW) of N-terminal location of the human P2Y1 receptor (1:100) (Baurand *et al.*, 2005) and anti-M_3 muscarinic acetylcholine receptor (M_3-mAChR) (C-terminal) raised in rabbit against residues 580–589 of human M_3-mAChR (1:1000, Biodesign International, Saco, ME, USA). The tissue was subsequently washed with PBS and incubated in goat anti-rabbit Alexa Fluor 488 secondary antibody (1:100, Molecular Probes, Paisley, UK) at room temperature (1–2 h), as previously described (Dora *et al.*, 2008). The internal elastic lamina (IEL) was visualized following incubation with 0.2 μM Alexa Fluor 633 hydrazide (Molecular Probes), and the nuclei of cells were labelled with propidium iodide (7.5 μM). Following excitation at 488 and 633 nm, the fluorescence emitted at 515 and 660 nm from cells through the bottom wall of the artery was acquired through a water immersion objective (60×, NA 1.20, WD 0.28 mm, Olympus, 1024 × 1024 pixels) using same laser, pinhole and photomultiplier tube settings in all experiments. *z*-Stacks through the wall of the artery were obtained in 0.5 μm increments (FluoView Software version 5.0, Olympus) as previously described (Dora *et al.*, 2008). As a control for antibody specificity, the primary antibody was replaced with normal rabbit immunoglobulin G (IgG) (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Data analysis

Results are summarized as means ± SEM of *n* arteries, one per animal. Statistical comparisons were made using Mann–Whitney *U*-test (time courses) or one-way ANOVA with Bonferroni's post-test (histograms), where $P < 0.05$ was considered statistically significant. The dilatation evoked by each agonist was calculated as the percentage of the maximum dilatation from phenylephrine-contracted arteries. Changes in $[Ca^{2+}]_i$ are expressed as the average fluorescence from 16–20 randomly chosen individual cells (F), sampled at 15 s intervals, relative to basal intensity (F_0).

Materials

Oregon Green 488 BAPTA-1 AM (O6807) and Pluronic F-127 (P3000MP) were obtained from Molecular Probes. BIS-I and Gö 6976 were purchased from Calbiochem (EMD Chemicals, Inc., Gibbstown, NJ, USA). All the other drugs were supplied by Sigma-Aldrich (St Louis, MO, USA). All stock solutions were prepared in distilled water with the exception of BIS-I, BIS-VIII and Gö 6976, which were dissolved in dimethyl sulphoxide. Drug dilutions were made in physiological buffer.

Results

Dilatation responses to luminal perfusion of nucleotides

Luminal perfusion of vehicle alone (MOPS buffer, not shown) or 0.1 μM of ADPβS, a non-hydrolyzable, selective agonist for P2Y1 receptors, in phenylephrine-contracted (1–10 μM) segments had almost no effect on arterial diameter, both under control conditions (Figure 1) and in the presence of L-NAME (Figure 2). These data confirm the absence of flow-dependent dilatation in these arteries, as previously shown (Winter and Dora, 2007). Higher concentrations of ADPβS (0.3–3 μM) stimulated concentration-dependent dilatation that were not affected by the presence of L-NAME over the 8 min time course of luminal perfusion (Figures 1 and 2). However, in both the absence and presence of L-NAME, the responses to 1

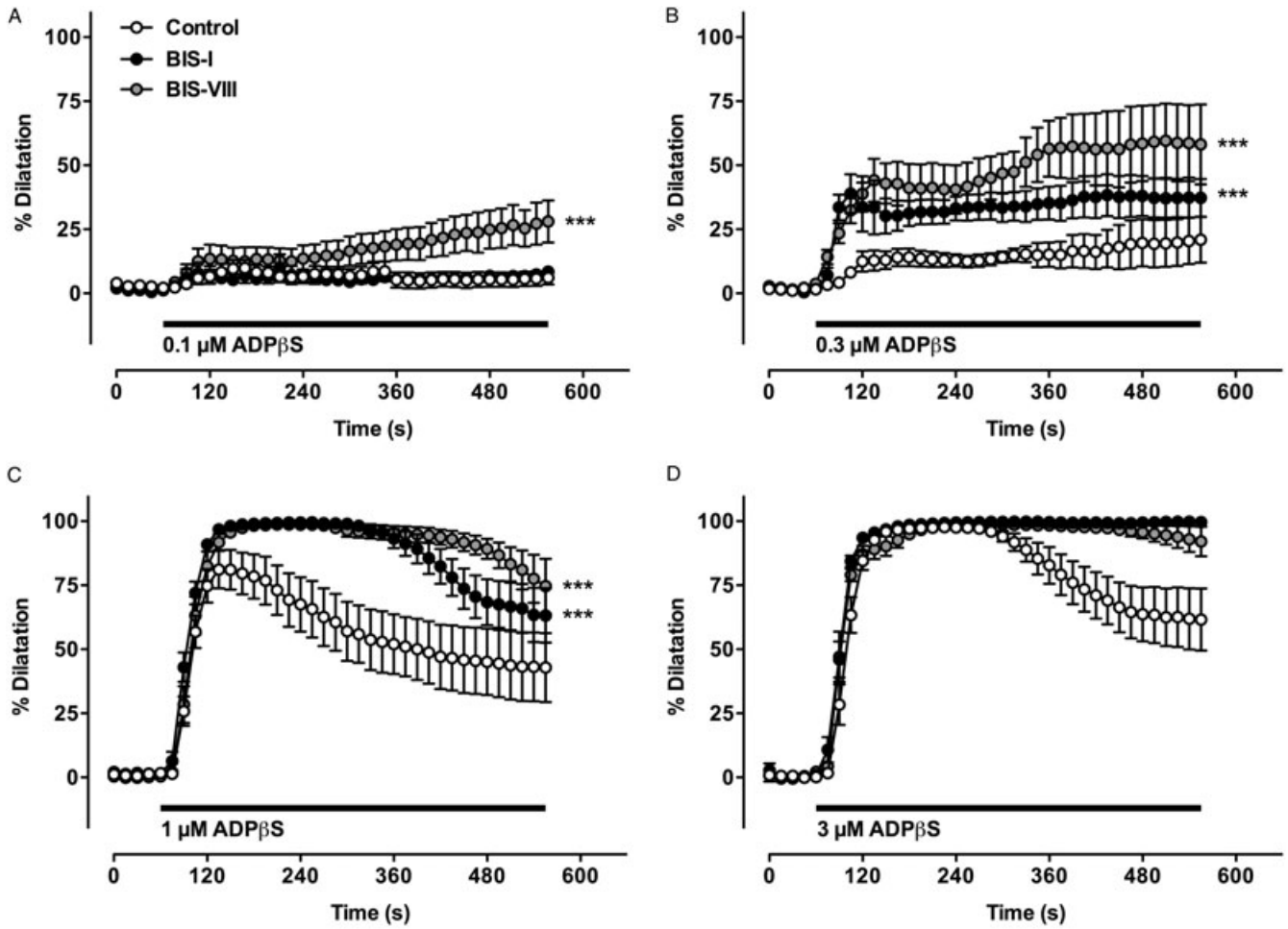

Figure 1 Dilatation responses to luminal perfusion of adenosine 5′-[β-thio]diphosphate (ADPβS) (0.1–3 μM, A–D) in rat pressurized small mesenteric artery in the absence or the presence of the selective protein kinase C inhibitor bisindolylmaleimide I (BIS-I) (1 μM) under submaximal levels of phenylephrine-evoked contraction (n = 4–7). Bars represent periods of ADPβS luminal perfusion. ***P < 0.001 versus control curve. N^{ω}-nitro-L-arginine methyl ester hydrochloride was not present. BIS-VIII, bisindolylmaleimide VIII.

and 3 μM ADPβS showed a characteristic initial peak followed by a gradual decline in dilatation over time (2–4 min), indicative of receptor desensitization. In all experiments, bath application of ACh (1 μM) was able to evoke maximum dilatation, indicating that the muscarinic (M_3) receptor was not heterologously regulated by the P2Y1 receptor (data not shown).

Because previous studies in 1321N1 cells and human platelets revealed that PKC mediates components of P2Y1 receptor desensitization, we sought to determine whether this was the case for the endothelial P2Y1 purinoceptor in pressurized arteries. In the present study, inhibition of PKC with BIS-I (1 μM), an isoform non-selective PKC inhibitor, significantly attenuated the desensitization response to the non-hydrolyzable agonist (Figures 1–3). This augmentation of dilatation was observed in both the absence and presence of L-NAME. However, it appeared that in the presence of BIS-I, the dilatation to 1 μM ADPβS was maintained to a greater extent in the presence of L-NAME (Figures 1C,2C). This was confirmed by performing paired experiments within the same arteries (Figure 2E), suggesting that a product of NO synthase was reducing the ability of ADPβS to evoke dilatation, perhaps itself causing receptor desensitization.

Another isoform non-selective PKC inhibitor, BIS-VIII also significantly attenuated the desensitization to ADPβS. In both the absence and presence of L-NAME, BIS-VIII augmented the dilatation to the lower concentrations of ADPβS (0.1–1 μM) (Figures 1–3). In contrast to BIS-I, BIS-VIII did not unmask a clear NOS-dependent desensitization (Figure 2F).

In parallel experiments, the contribution of PKC to nucleotide-evoked dilatation was investigated using Gö 6976 (1 μM), which inhibits calcium-dependent conventional PKC isoforms including PKCα, as well as the novel isoform PKCμ, but not PKCδ, ε or ζ. To more clearly study the effect against PKC, experiments were performed only in the presence of L-NAME. Inhibition of PKC with Gö 6976 did not modify the dilatation responses to 0.1 and 0.3 μM ADPβS (Figure 2). This inhibitor was, however, able to partially reverse the desensitization of the P2Y1 receptor induced by luminal perfusion to higher concentrations of ADPβS (Figures 2 and 3B). Gö 6976 was less effective at preventing desensitization to ADPβS than either BIS-I or BIS-VIII (Figures 2 and 3B).

Luminal perfusion with rottlerin (mallotoxin, 3 μM), a selective inhibitor of the Ca^{2+}-independent isoform of PKC, PKCδ, was unable to modify the agonist-induced desensitiza

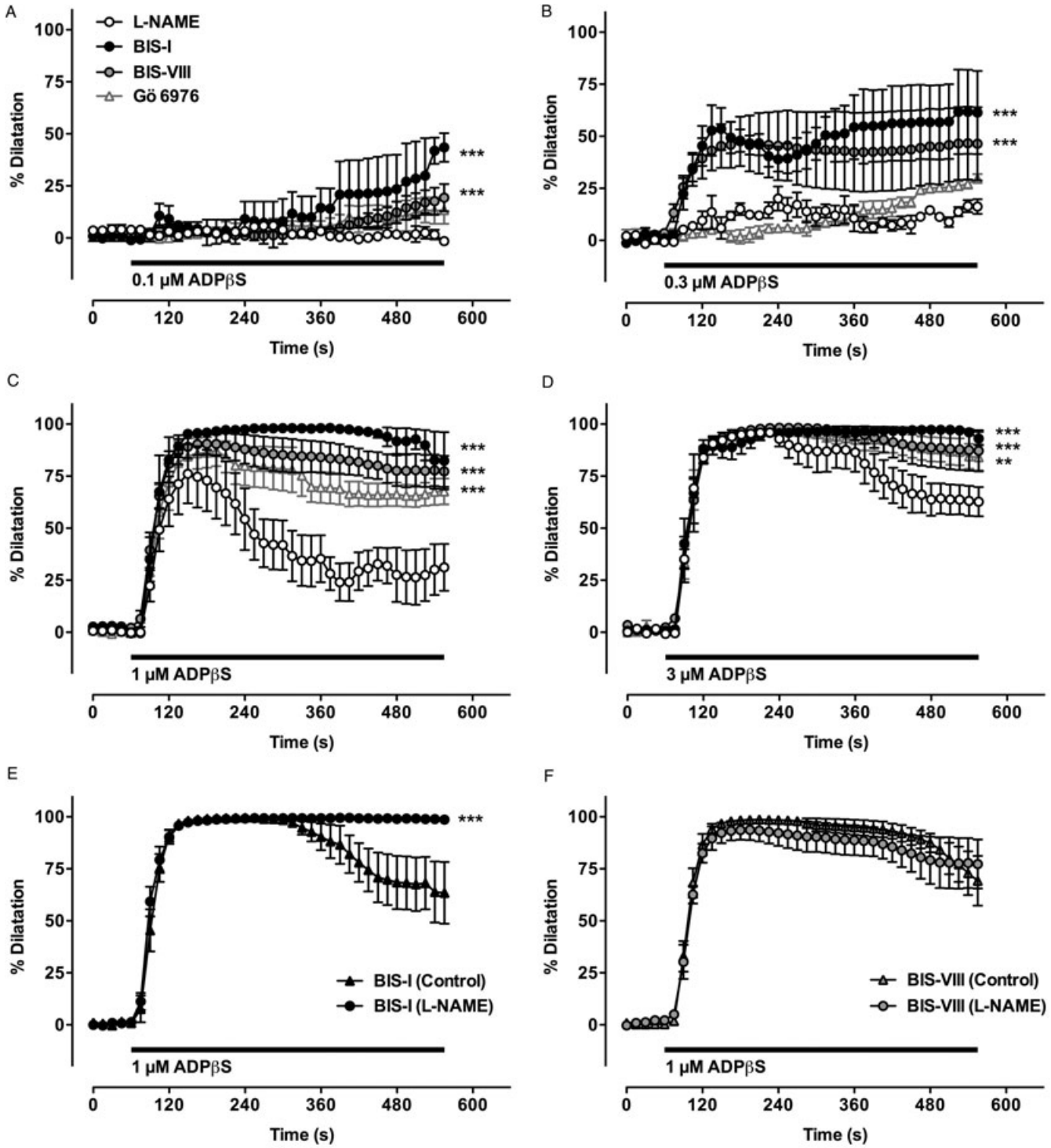

Figure 2 Dilatation responses to luminal perfusion of adenosine 5′-[β-thio]diphosphate (ADPβS) (0.1–3 μM, A–D) in rat pressurized small mesenteric artery in the absence or the presence of the selective protein kinase C inhibitors bisindolylmaleimide I (BIS-I) (1 μM), bisindolylmaleimide VIII (BIS-VIII) (1 μM) or 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö 6976) (1 μM), under submaximal levels of phenylephrine-evoked contraction (n = 3–6). Paired responses in the presence of BIS-I (1 μM) (E) or BIS-VIII (1 μM) (F) from the same arteries before (control) and after N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME). Bars represent periods of ADPβS luminal perfusion. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus L-NAME curve (A–D) or BIS-I control curve (E). L-NAME was present throughout in A–D.

tion of P2Y1 receptors (27.2 ± 0.7% dilatation to 1 μM ADPβS during plateau phase, n = 3).

To determine whether inhibition of PKC with BIS-I or BIS-VIII directly enhanced EDHF-type dilatation responses, the effects of this PKC inhibitor on concentration-dependent dilatation responses to ACh (1 nM–3 μM) were evaluated. Data revealed that the dilatation to 0.3 μM ACh was approximately the same percentage as to 1 μM ADPβS (~75% maximum

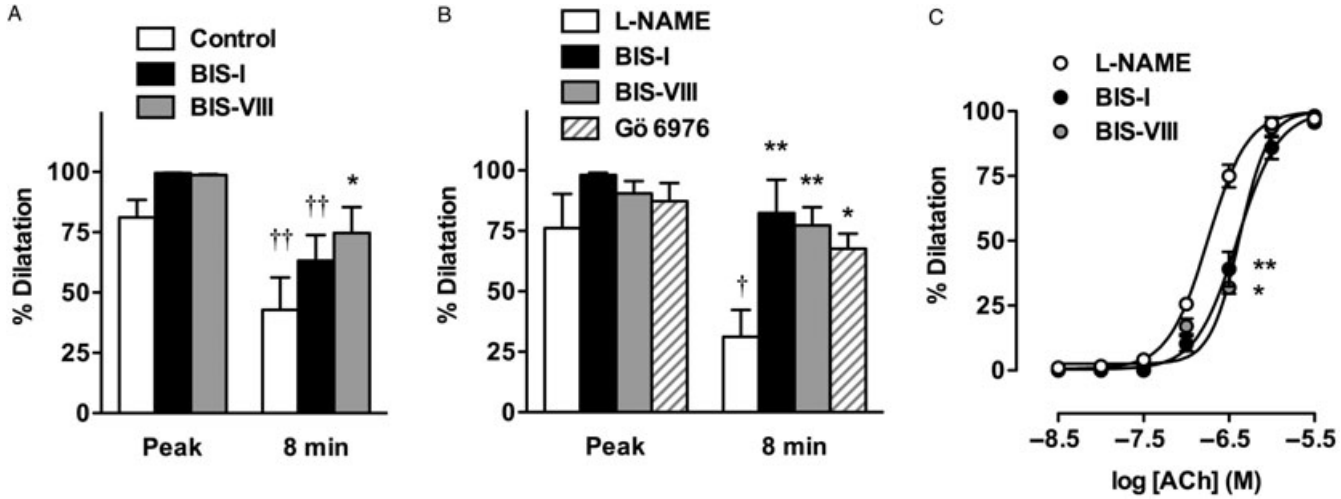

Figure 3 Effects of protein kinase C (PKC) inhibitors on adenosine 5′-[β-thio]diphosphate (ADPβS) or acetylcholine (ACh)-evoked dilatation. Effects of selective inhibition of PKC by bisindolylmaleimide I (BIS-I) (1 μM), bisindolylmaleimide VIII (BIS-VIII) (1 μM) or 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö 6976) (1 μM) in the dilatation evoked by luminal perfusion of 1 μM ADPβS in the absence (A) or presence (B) of N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME). Values are given for the maximal dilatation peak and 8 min after luminal perfusion in phenylephrine-contracted arteries (n = 4–7). (C) Responses to cumulative additions of ACh to the bath in the absence (n = 18) and presence of luminal BIS-I (n = 15) and BIS-VIII (n = 3). *P < 0.05 and **P < 0.01 versus L-NAME. †P < 0.05, ††P < 0.01 versus maximal dilatation peak.

diameter), but in contrast to the responses to ADPβS, both BIS-I and BIS-VIII significantly reduced the dilatation to ACh (Figure 2F).

Endothelial cell calcium

Determination of changes in endothelial cell intracellular Ca^{2+} ($[Ca^{2+}]_i$) was used as a further test of P2Y1 receptor desensitization evoked by ADPβS. Luminal perfusion of Oregon Green 488 BAPTA-1 AM through pressurized rat mesenteric arteries selectively loaded endothelial cells, and more than 20 endothelial cells could be simultaneously observed in each field. In general, the average basal fluorescence intensity of the Oregon Green BAPTA-1 dye stayed relatively constant within the endothelial cells for periods in excess of 2 h, suggesting that cellular loss was minimal, as previously described (Kansui *et al*., 2008). Luminal perfusion of 1 and 3 μM ADPβS induced very similar Ca^{2+} elevations in endothelial cells (Figure 4). Initial elevations in Ca^{2+} were rapidly attenuated within ~2–4 min during prolonged perfusion with the agonist, reaching values close to the baseline (Figure 4). These Ca^{2+} levels remained unchanged by constant application of ADPβS, indicating complete homologous desensitization of the P2Y1 receptors (Figure 4). Ca^{2+} elevations evoked by ADPβS were completely attenuated after incubation with the P2Y1 receptor antagonist MRS 2179 (1 μM, 5 min) (data not shown). ACh (1 μM) induced Ca^{2+} increases when added at the end of each of these experiments (F/F_0 1.34 ± 0.02, n = 7), which confirmed the ability of endothelial cells to respond to agonists of other GPCRs. In paired experiments, the participation of the PKC in the P2Y1 receptor desensitization was also evaluated by testing endothelial cell Ca^{2+} responses after luminal perfusion of the agonist in the presence of BIS-I (1 μM). Following luminal incubation with BIS-I, peak increases in endothelial Ca^{2+} in response to ADPβS were similar to control experiments (Figure 4). However, BIS-I prevented the decay in Ca^{2+} responses induced by ADPβS (Figure 4).

Cross-desensitization

In the presence of L-NAME, luminal perfusion with the P2Y2 and P2Y1 receptor agonists UTP (3 μM) and ADP (1 μM) almost fully relaxed pre-contracted arteries (Figure 5). The responses to both agonists were rapidly reversible upon cessation of luminal flow. If flow was recommenced within a short time (<3 min) the ensuing dilatation to ADP was less, and tended to fade with time, but responses to ACh remained unaffected (Figure 5A, representative of at least three experiments). These two nucleotides were then infused after ADPβS (3 μM) had caused P2Y1 receptor desensitization, to test for both homologous and heterologous desensitization (L-NAME present). At this time, co-perfusion with ADP (1 μM) did not induce further dilatation, whereas addition of the endothelium-dependent, M_3 muscarinic receptor agonist ACh evoked maximal dilatation (94.7 ± 1.5%, n = 7) (Figure 6A,B). The desensitization of P2Y1 receptors was further shown by endothelial cell $[Ca^{2+}]_i$ determinations in response to luminal perfusion of ADPβS (3 μM), which evoked an initial increase in $[Ca^{2+}]_i$ followed by a rapid decrease back to the baseline due to desensitization. The subsequent luminal application of 1 μM ADP was unable to induce an increase in endothelial cell $[Ca^{2+}]_i$, whereas addition of 1 μM ACh did (Figure 6C,D).

Under similar conditions, co-perfusion with UTP (3 μM) stimulated a small dilatation, which was much reduced compared with UTP alone, yet ACh was still able to evoke full dilatation of arteries (Figure 6E,F).

Localization of P2Y1 receptors in pressurized arteries

To determine the distribution of P2Y1 receptors in the endothelium of pressurized rat mesenteric arteries, two dif-

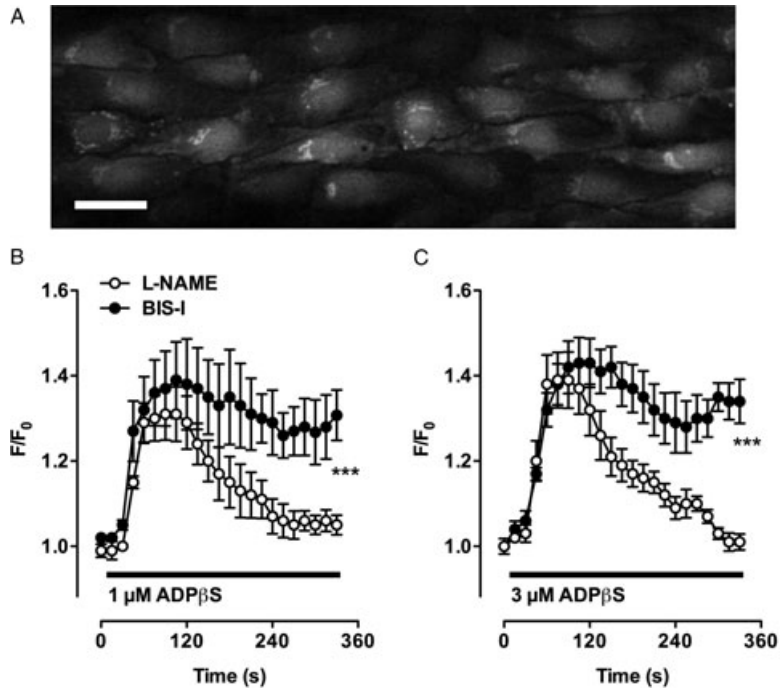

Figure 4 Endothelial cell $[Ca^{2+}]_i$ in rat pressurized small mesenteric artery following P2Y1 receptor stimulation by adenosine 5′-[β-thio]diphosphate (ADPβS). (A) Confocal micrograph of endothelial cells loaded with Oregon Green 488 BAPTA-1. Bar = 50 μm. Average data showing the time course of increases in F/F_0 in response to luminal perfusion of 1 μM (B) and 3 μM ADPβS (C) in the absence or the presence of the protein kinase C inhibitor bisindolylmaleimide I (BIS-I) (1 μM; $n = 4–7$). Bars represent periods of ADPβS luminal perfusion. ***$P < 0.001$ versus control curve. N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (100 μM) was present throughout the experiments.

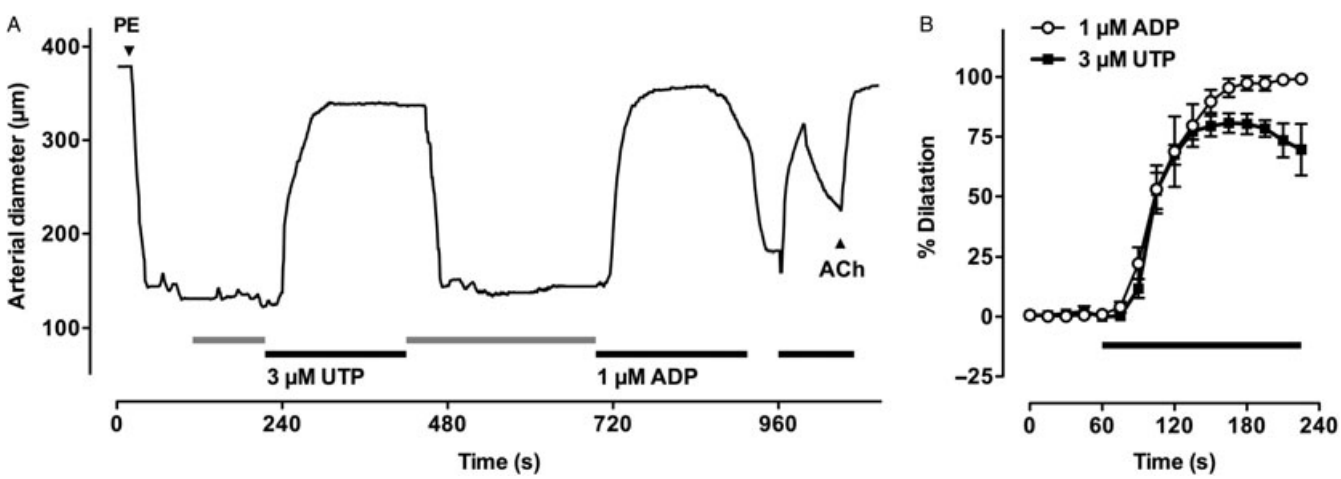

Figure 5 Dilatation responses to luminal perfusion of uridine triphosphate (UTP) (3 μM) and adenosine diphosphate (ADP) (1 μM) in pressurized rat small mesenteric artery. (A) Representative trace showing 3-[N-morpholino]propane-sulphonic acid alone (grey bar) had no effect on diameter, whereas each agonist stimulated rapid dilatation, which reversed rapidly upon washout or cessation of flow. Arterial preparations were precontracted by phenylephrine (PE). Bars represent the duration of the luminal perfusion of the agonist. (B) Summary of dilatation responses to ADP and UTP ($n = 4–5$). N^{ω}-nitro-L-arginine methyl ester hydrochloride was present throughout. ACh, acetylcholine.

ferent P2Y1 antibodies raised in rabbit targeting different epitopes (at the C- or the N-terminus) were used and limited to perfusion of the luminal surface of arteries. Immunohistochemistry revealed that P2Y1 receptors were distributed throughout the endothelial cells within pressurized rat mesenteric arteries and the labelling was similar using each of the antibodies (Figure 7). Some punctate staining for P2Y1 receptors was found at the endothelial cell borders and towards the centre of the endothelial cell, with some nuclear and strong perinuclear staining (Figure 7A,B). An overlay of the IEL demonstrated a positive correlation between strong punctate P2Y1 receptor expression and holes through the IEL. The nuclear stain propidium iodide clearly showed the orientation of endothelial cells (horizontally aligned cells) and smooth muscle cells (vertically aligned cells) (Figure 7).

In separate experiments, an antibody raised against the rat M_3-mAChR was used to show the localization of this

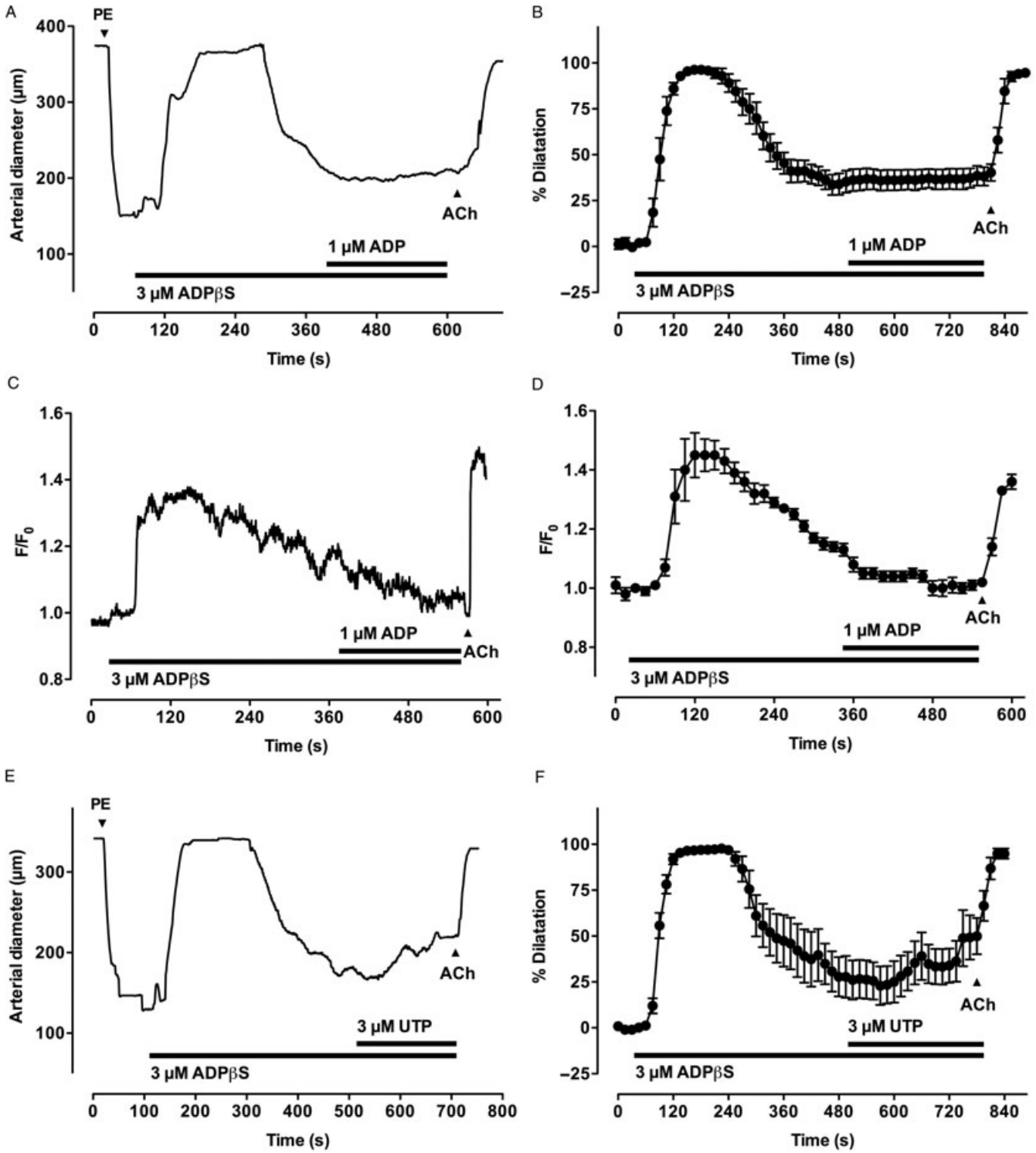

Figure 6 Dilatation responses and endothelial cell $[Ca^{2+}]_i$ to luminal perfusion of adenosine 5′-[β-thio]diphosphate (ADPβS) (3 μM) followed by ADP (1 μM, A–D) and UTP (3 μM, E–F) in pressurized rat small mesenteric arteries ($n = 5–7$). Arterial preparations were pre-contracted with phenylephrine (PE) for dilatation experiments (only). Bars represent the duration of the luminal perfusion of the agonist. Black triangles indicate addition of acetylcholine (ACh) (1 μM). N^{ω}-nitro-L-arginine methyl ester hydrochloride was present throughout.

receptor subtype. Punctate fluorescence signal was evident for M_3-mAChRs in the endothelial cell borders and some staining of endothelial cell projections in holes through the IEL (Figure 7B). Markedly less immunostaining was observed in the arteries when the primary antibody was replaced with normal rabbit IgG (Figure 7C) and not evident when the primary antibody was omitted (data not shown).

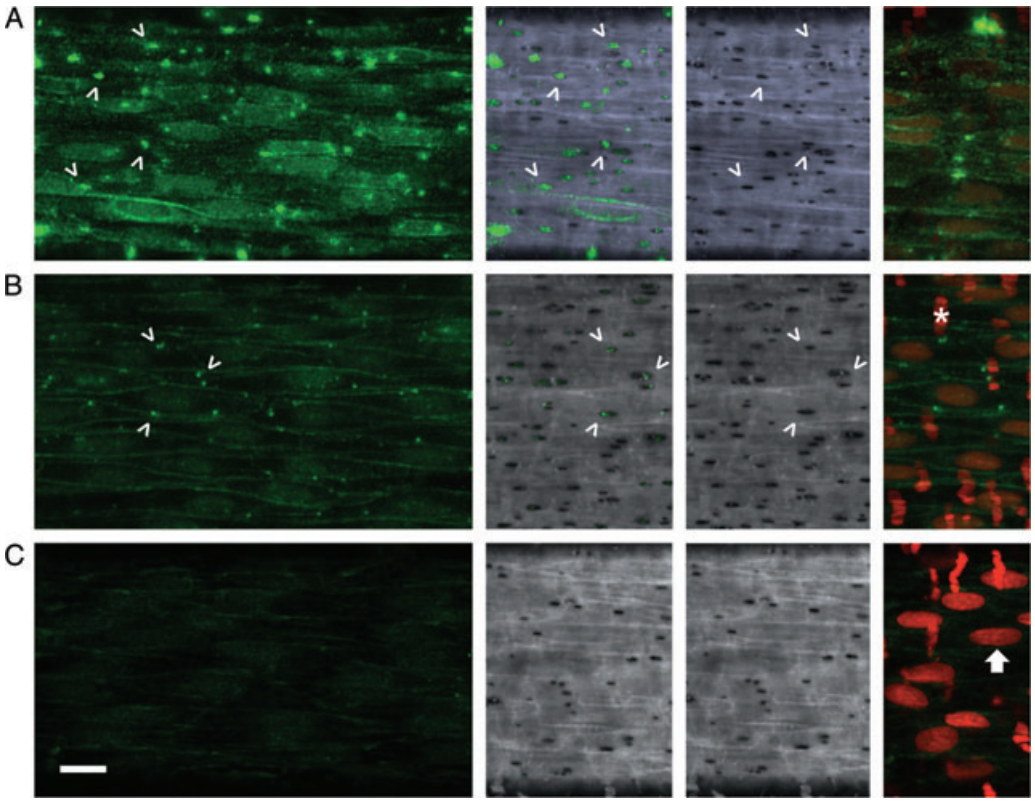

Figure 7 Immunohistochemical localization of endothelial P2Y1 and M_3 muscarinic acetylcholine receptors (M_3-mAChRs) in pressurized rat mesenteric arteries. Confocal images from the same region of a vessel stained with two P2Y1 receptor antibodies (raised in rabbit) targeting the C-terminus (A), M_3-mAChR antibody (B) or an non-specific normal rabbit immunoglobulin G (C) followed by incubation of an Alexa Fluor 488 secondary antibody (green). The internal elastic lamina (IEL) was stained with Alexa Fluor 633 hydrazide (grey) showing IEL holes. An overlay of the IEL visualization (grey) with P2Y1 receptor staining (green) demonstrated a positive correlation between strong punctate P2Y1 receptor expression and IEL holes (A). Note a weak co-localization of M_3-mAChR at IEL holes (B). Nuclei of endothelial (horizontally aligned cells, arrow) and smooth muscle cells (vertically aligned cells, asterisk) were labelled with propidium iodide (red) (A–C). White arrowheads correspond to the same region in each panel. Bar = 20 μm.

Discussion

The present study provides new insights into the dilatation evoked by P2Y1 receptor selective agonists in pressurized resistance arteries and the regulation of this response by PKC-dependent desensitization. These findings reinforce the importance of extracellular nucleotides and their purinoceptors in the control of vascular homeostasis and blood flow.

The distribution of P2Y receptors on vascular endothelial cells implies a role in the regulation of vascular tone. GPCRs present on the endothelium mediate dilatation by releasing endothelial-derived relaxing factors. In the peripheral vascular bed, both NO and EDHF contribute to relaxation to extracellular nucleotides (Malmsjo *et al.*, 1998; 1999b; 2000a,b; 2002; Buvinic *et al.*, 2002; Mistry *et al.*, 2003; Liu *et al.*, 2004; Winter and Dora, 2007). Despite their common role as dilatation mediators, P2Y1 and P2Y2 receptors differ in agonist and antagonist selectivity. It has been found that the P2Y1 receptor responds selectively to ADP compared with ATP, while ATP may act as a partial agonist, and UTP is not effective (Ralevic and Burnstock, 1998). Although much information has been generated regarding the role of the ATP- and UTP-sensitive P2Y2 receptors in the vasculature, relatively little is known about the role of ADP-preferring receptors such as P2Y1, especially in the endothelium. The observation that ADP is one of the major nucleotides released from activated platelets and endothelial cells, illustrates the importance of understanding the effects and the underlying regulation of P2Y1 receptor activation on arterial function.

In the current investigation, luminal application of the P2Y1 agonist ADPβS showed concentration-dependent dilatation, which remained unaffected in the presence of L-NAME. The dilatation was fully blocked following incubation with the P2Y1-selective antagonist MRS 2179 (1 μM) (Winter and Dora, 2007), as was that to ADP (not shown). Therefore, the dilatation can be attributed to the stimulation of P2Y1 receptors, and by inference, not P2Y2 receptors. However, full block of observed dilatation does not preclude the effects of ADP (or ADPβS) on other P2Y receptors, for example, those that affect the levels of cyclic adenosine monophosphate in endothelial cells (e.g. P2Y1/2). In themselves, these receptors may not directly lead to dilatation, but could instead regulate the channels (e.g. IK_{Ca}) and enzyme (NOS) leading to endothelium-dependent dilatation (Kou and Michel, 2007; Dora *et al.*, 2008). However, this type of G protein-dependent modulation, which is likely an important consideration, was not investigated in the present study. Given this, ADPβS should be selective for P2Y1 receptors (Alexander *et al.*, 2008).

The finding that the dilatation to ADPβS was not sensitive to L-NAME is consistent with our previous report in the same tissue. Luminal exposure to ATP, which was also sensitive to MRS 2179, indicating a P2Y1 receptor response, was not affected by the presence of L-NAME (Winter and Dora, 2007). The residual dilatation to ATP and ADPβS was attributed to EDHF due to the sensitivity to selective inhibitors of Ca^{2+}-activated potassium channels (IK_{Ca} and SK_{Ca}). These K_{Ca} channels have shown a differential contribution to the EDHF-type dilatation responses, which appears to relate to the distribution of each channel within the cell: IK_{Ca} is highly concentrated in endothelial cell projections and adjacent to myoendothelial gap junctions, whereas SK_{Ca} is also localized through the plasmalemma of endothelial cells (Sandow *et al.*, 2006; Dora *et al.*, 2008). In our study, immohistochemical data demonstrated the presence of the P2Y1 receptors in mesenteric arteries, consistent with previous data establishing their distribution in vascular endothelial cells (Volonte *et al.*, 2006). The strong punctate staining of P2Y1 receptors in the vicinity of holes through the IEL and endothelial cell borders supports a role of IK_{Ca} and SK_{Ca} in the EDHF-dependent dilatation evoked by P2Y1 receptor activation (Winter and Dora, 2007; Dora *et al.*, 2008). Interestingly, the M_3-mAChR staining was not markedly different to that of P2Y1 receptors at the level of these confocal images, so does not simply explain the lack of heterologous desensitization by the P2Y1 receptors.

In general, GPCRs can be desensitized from sustained or recurrent agonist stimulation that leads to an attenuated ability to be repetitively activated. In the present study, desensitization of the P2Y1 receptor was clearly shown by two different approaches. Firstly, during prolonged stimulation of the receptor by continuous luminal perfusion of ADPβS, a decay of the initial dilatation response was observed. Secondly, measurements of changes in endothelial cell $[Ca^{2+}]_i$ in pressurized arteries revealed an attenuation of the initial peak $[Ca^{2+}]_i$ during prolonged luminal exposure to ADPβS. The reduction in both dilatation and $[Ca^{2+}]_i$ increases remained unaltered by the subsequent luminal application of the P2Y1 receptor agonist ADP. Despite numerous studies evaluating dilatation responses evoked by extracellular nucleotides through P2Y1 receptor stimulation located in the vascular endothelium, the regulation of this activation by desensitization events remains poorly understood, especially under physiological conditions. As yet, there are no reports documenting the modulation of endogenous P2Y1 receptors by PKC in intact arteries.

Previous studies have demonstrated that ADP promotes rapid P2Y1 receptor desensitization in human platelets by PKC-dependent mechanisms, but largely independent of GRK activity (Hardy *et al.*, 2005; Mundell *et al.*, 2006). The attenuation of GPCR activity by PKC is associated with the phosphorylation of the C-terminus or third intracellular loop of the activated receptor by the serine–threonine kinase, which prevents the association of the receptor with downstream signalling proteins (Fam *et al.*, 2003). At least 12 PKC isoforms have been described and are categorized into three main groups: conventional (α, β_I, β_{II} and γ), novel (δ, ε, η and θ) and atypical PKC isoforms (ζ, λ, ι and μ) (Mochly-Rosen and Gordon, 1998). The first evidence showing the involvement of PKC in P2Y1 receptor desensitization in endothelial cells was reported in bovine pulmonary artery endothelium (Chen and Lin, 1999). In subsequent studies, PKC isoform-selective inhibitors revealed that both novel and conventional isoforms regulate P2Y1 receptor platelet activity (Hardy *et al.*, 2005; Mundell *et al.*, 2006). Some authors, however, suggest the influence of other protein kinases such as PKA on P2Y1 desensitization (Gainetdinov *et al.*, 2004).

In the present study, we have provided evidence for the involvement of PKC and also, for the first time, eNOS in P2Y1 receptor desensitization in pressurized arteries. Our data showed that both BIS-I and BIS-VIII, isoform non-selective PKC inhibitors, markedly reduced P2Y1 receptor-evoked desensitization and improved the dilatation responses induced by luminal perfusion of ADPβS. Interestingly and unexpectedly, in the presence of BIS-I, the addition of L-NAME improved the dilatation to ADPβS, or in other words more fully prevented the desensitization. Possible explanations include an inhibitory action of NO at a PKG-sensitive Ca^{2+}-permeable cation channel within endothelial cells, which would limit rises in endothelial cell Ca^{2+} (Dora *et al.*, 2001), or an effect of NO (or other eNOS-dependent molecule) to cause desensitization of the P2Y1 receptor itself. This was not investigated further, but the remaining experiments were performed in the presence of L-NAME to avoid the BIS-I-insensitive desensitization due to eNOS.

The participation of PKC in P2Y1 receptor regulation was further confirmed as the prevention of the decay in endothelial cell $[Ca^{2+}]_i$ following BIS-I incubation. Based on previous studies by others, we tried to elucidate the main PKC isozyme responsible for the P2Y1 desensitization in resistance arteries by choosing more selective PKC inhibitors, Gö 6976 for inhibiting the conventional PKC isoforms, and rottlerin, a highly selective inhibitor of PKCδ. Whereas Gö 6976 partially blocked these responses, rottlerin was unable to modify the agonist-induced desensitization of P2Y1 receptors, thus discounting the involvement of a Ca^{2+}-independent PKCδ component. These results lead us to hypothesize a key role for the Ca^{2+}-dependent and conventional PKC isoforms (PKCα, β_I, β_{II} and γ) in the desensitization of the dilatation response mediated by P2Y1 receptors in resistance arteries. This hypothesis would agree with previous evidence describing an action of PKCβ for P2Y1 receptor desensitization in endothelial cells (Chen and Lin, 1999). However, the ability of the BIS compounds to inhibit the novel isoforms of PKC cannot be ruled out. Indeed, it was found that desensitization (signalling and trafficking) of the human platelet P2Y1 receptors was strongly mediated by both conventional (PKCα) and novel (PKCδ) isoforms of PKC (Mundell *et al.*, 2006). These differences in the documented contributions of PKC isoforms to P2Y1 receptor desensitization may reflect varying degrees of participation by each protein kinase to the dilatation response, which in turn may be dependent on the level of desensitization observed, the cell type evaluated and the duration of the agonist exposure (Bailey *et al.*, 2006). This idea is further supported by the assumption that the same GPCR subtype is regulated differentially in various tissues in which it is expressed and that this is in line with the tissue-specific function of the receptor (Tobin, 2008). Accordingly, post-translational modifications of GPCRs, such as the extent of

phosphorylation, might differ among different cells and tissues, possibly changing the efficiency and specificity of the receptor activation and their capacity to be desensitized (Clark *et al.*, 1999). Differences in the attenuation of the desensitization reported between the PKC inhibitors used in our investigation could also suggest the involvement of other protein kinases in the response, perhaps via other endothelial cell P2Y receptors as mentioned above.

Finally, the homologous desensitization of the P2Y1 receptor by ADPβS was clearly demonstrated by the inability of co-perfused ADP to evoke dilatation, whereas ADP alone stimulated near maximal dilatation. Homologous desensitization of P2Y1 receptors has been previously reported in isolated endothelial cells, the bioassay being the production of inositol trisphosphate (Wilkinson *et al.*, 1994). The PKC inhibitor Ro 31-8220 (not isoform selective) augmented the response to the P2Y1 receptor agonist 2MeSATP when added alone, and in cells pre-incubated with 2MeSATP, but did not prevent the homologous desensitization. Interestingly, Ro 31-8220 was more successful at preventing homologous desensitization of $P2Y_2$ receptors (UTP response) and the heterologous desensitization of P2Y1 receptors by UTP (Wilkinson *et al.*, 1994). Homologous desensitization of P2Y1 receptors by ADPβS has been reported in platelets, where ADP was unable to evoke rises in $[Ca^{2+}]_i$ or platelet aggregation, whereas responses to 5-hydroxytryptamine remained unaffected (Baurand *et al.*, 2000). Our results also indicate partial heterologous desensitization of P2Y2 receptors by ADPβS. This type of desensitization has been shown previously in isolated endothelial cells, where the rise in $[Ca^{2+}]_i$ to ATP (acting at P2Y2 receptors) was reduced by pretreatment with ADPβS (Mateo *et al.*, 1996). This cross-desensitization of P2Y receptor subtypes does not appear to extend to muscarinic receptors in rat mesenteric arteries, as the response to ACh remained unaffected under all conditions.

In conclusion, the present study demonstrates that luminal application of the P2Y1-selective agonists ADP and ADPβS induce EDHF-type dilatation and endothelial cell Ca^{2+} increases, which rapidly desensitize by mechanisms involving activation of PKC. Given the important role of adenine nucleotides in the control of vascular tone and blood flow by potentially acting at endothelial P2Y1 receptors, the regulation of these GPCRs by desensitization reflects a key mechanism that should be considered in the maintenance of the vascular homeostasis.

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Conflict of interest

The authors state no conflict of interest.

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