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Study of the mechanisms involved in adenosine-5′-O-(2-thiodiphosphate) induced relaxation of rat thoracic aorta and pancreatic vascular bed

[1]B. Saïag, *D. Hillaire-Buys, *J. Chapal, *P. Petit, †D. Pape, B. Rault, †H. Allain & *M.M. Loubatières-Mariani

Faculté de Pharmacie, Laboratoire de Physiologie, Unité ≪Biologie de la paroi vasculaire≫, 2 Avenue du Professeur Bernard, 35043 Rennes Cedex, France; *Faculté de Médecine, Laboratoire de Pharmacologie, Boulevard Henri IV, 34060 Montpellier Cedex 01, France and †Faculté de Médecine, Laboratoire de Pharmacologie, 2 Avenue du Professeur Bernard, 35043 Rennes Cedex, France

1 The endothelium-dependent relaxation of blood vessels induced by P_{2Y}-purinoceptor activation has often been shown to involve prostacyclin and/or nitric oxide (NO) release. In this work, we have investigated the mechanisms involved in the relaxant effect of the P_{2Y} agonist, adenosine -5′-O-(2-thiodiphosphate) (ADPβS) using two complementary preparations: rat pancreatic vascular bed and aortic ring.

2 On the pancreatic vascular bed, ADPβS (1.5 and 15 μM) infused for 30 min induced a concentration-dependent vasodilatation; it was progressive during the first 10 min (first period) and sustained from 10 to 30 min (second period). Indomethacin (10 μM) delayed ADPβS-induced vasodilatation (1.5 and 15 μM) by about 6 min. N^{ω}-nitro-L-arginine methyl ester (L-NAME) (200 μM) suppressed the relaxation for about 5 min but thereafter ADPβS at the two concentrations progressively induced an increase in the flow rate. Even the co-administration of L-NAME and indomethacin did not abolish the ADPβS-induced vasorelaxation.

3 On 5-hydroxy tryptamine (5-HT) precontracted rings mounted in isometric conditions in organ baths, we observed that ADPβS induced a concentration-dependent relaxation of rings with a functional endothelium; this effect was stable for 25 min. The ADPβS relaxant effect was strongly inhibited by Reactive Blue 2 (30 μM) and was suppressed by pretreatment of rings with saponin (0.05 mg ml^{-1} for 30 min), which also abolished the acetylcholine-induced relaxation.

4 ADPβS-induced relaxation of 5-HT precontracted rings is largely inhibited by indomethacin (100 or 10 μM) or L-NAME (100 μM).

5 We conclude that: the ADPβS-induced relaxation is endothelium-dependent, mediated by P_{2Y}-purinoceptors, and at least in part linked to NO and prostacyclin release, depending on the preparation used. Furthermore, on the pancreatic vascular bed, (an)other mechanism(s) than prostacyclin and NO releases may be involved in ADPβS-induced vasodilatation.

Keywords: ADPβS; P_2-purinoceptors; L-NAME; indomethacin; Reactive Blue 2; vasodilatation; rat thoracic aorta; pancreatic vascular bed

Introduction

Purine nucleotides are potent vasoactive substances able to induce either vasodilatation via P_{2Y}-purinoceptor activation or vasoconstriction via P_{2X}- (Burnstock & Kennedy, 1986; Pearson & Gordon, 1989; Evans & Kennedy, 1994) and P_{2U}-purinoceptor activation (Saïag *et al.*, 1990; O'Connor *et al.*, 1991). P_{2Y}-purinoceptors are generally assumed to be located in vascular endothelial cells (Olsson & Pearson, 1990); however, despite this well accepted localization on the endothelium, a few papers have reported a smooth muscle P_{2Y} localization (Mathieson & Burnstock, 1985; Brizzolara & Burnstock, 1991). P_{2Y}-purinoceptor activation generally leads to an increase in inositol 1,4,5-trisphosphate (IP_3) formation and intracellular Ca^{2+} (Charest *et al.*, 1985; Okajima *et al.*, 1987; Pirotton *et al.*, 1987; Lustig *et al.*, 1992) via a guanine nucleotide-dependent phospholipase C (Cooper *et al.*, 1989). The P_{2Y} vasodilator effect is assumed to be induced by the biosynthesis and release of both prostacyclin (PGI_2) and endothelium-derived relaxing factor (EDRF) from endothelial cells (Carter *et al.*, 1988; Boeynaems & Pearson, 1990), substances known to be released by extracellular ATP and ADP (Needham *et al.*, 1987; Mitchell *et al.*, 1992). PGI_2, a metabolite derived from arachidonic acid, stimulates smooth muscle adenylate cyclase (Rubanyi, 1991) whereas EDRF, being nitric oxide (NO) or a related molecule (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Myers *et al.*, 1990), stimulates soluble smooth muscle guanylate cyclase (Murad *et al.*, 1990; Rubanyi, 1991).

In this study, we have attempted to define the role of PGI_2 and EDRF release (using cyclo-oxygenase- and NO synthase-inhibitors) in the vasodilatation induced by the stable P_{2Y}-purinoceptor agonist, adenosine-5′-O-(2-thiodiphosphate) (ADPβS) (Bertrand *et al.*, 1991). Firstly, the experiments were performed on the pancreatic vascular bed of normal rats containing a network of arteries, veins and capillaries, in which endothelial cells were intact, and previously shown to be provided with P_{2Y}-purinoceptors inducing vascular relaxation (Hillaire-Buys *et al.*, 1991). Secondly, the action of ADPβS on a large isolated artery (rat thoracic aorta) was studied. Part of this work has been reported in abstract form (Saïag *et al.*, 1994).

Methods

Rat pancreatic vascular bed

The experiments were carried out on the isolated perfused pancreas of the rat, according to the technique previously de-

[1]Author for correspondence.

scribed (Loubatières *et al.*, 1969). Male Wistar rats weighing 300–350 g received 60 mg kg^{-1} sodium pentobarbitone by i.p. injection. The pancreas was totally isolated from all neighbouring tissues and organs; it was perfused through its own arterial system with a Krebs-Ringer bicarbonate buffer (composition in mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5) containing bovine serum albumin (2 gl^{-1}) and 8.3 mM glucose. A mixture of O_2 (95%) and CO_2 (5%) was bubbled at atmospheric pressure. The pH of the solution was 7.35. The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure (in the range 40–50 cm water), selected so as to obtain a flow rate of 2.5 ml min^{-1} at the start of the experiment. Any change in pancreatic vascular bed resistance due to drug administration resulted in a change in the flow rate; the latter was measured by collecting each sample in a graduated tube during 1 min.

We carried out a kinetic study during 105 min which was divided into four periods: (1) a 45 min equilibration period (0–45 min of organ perfusion) in which basal flow rates were measured twice, at 30 and 45 min before any drug administration; (2) the next 15 min (45–60 min of organ perfusion) in which a cyclo-oxygenase inhibitor, indomethacin (10 μM) or a NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) (200 μM) or both inhibitors were added (treated) or not (control); (3) in the third period (60–90 min) ADPβS (1.5 or 15 μM) was infused in the absence of any other drug (control experiments) or in the presence of the inhibitor; (4) the final period (90–105 min) allowed us to control the reversibility of flow rate changes.

Indomethacin was used at a concentration of 10 μM since high concentrations (100 μM) can act not only by inhhibition of the cyclo-oxygenase pathway but also by inhibition of Ca^{2+}-dependent biological systems in a non-specific manner (Gorog & Kovac, 1970; Northover, 1971; Lewis & Whittle, 1977). L-NAME was used at 200 μM, a concentration that elicited a maximum fall in the flow rate in basal tone. Higher concentrations of L-NAME (500 μM and 5 mM) did not induce a larger fall in our preparation (Gross *et al.*, 1995).

The graphs represent the kinetics of flow rate. The flow rate was measured every min during the first 6 min, then 8, 10, 15, 20 and 30 min after the addition of ADPβS.

Statistical analysis

The results are expressed as means ± s.e.mean. Analysis of variance was applied, followed by the multiple comparison test (Zar, 1974).

Rat isolated thoracic aorta

The rat thoracic aorta was removed from male Wistar rats (250–350 g) which had been killed by a blow on the head followed by cerebral dislocation and exsanguination. The vessel was placed in Krebs solution (composition in mM: NaCl 94, KCl 4.7, $MgSO_4$ 0.5, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11) (Saïag *et al.*, 1990). The connective tissue was removed and 5 mm long rings were prepared. Rings were mounted horizontally under isometric conditions in 14 ml organ bath by inserting two stainless-steel wires into the lumen, according to the Bevan & Osher method (1972). Tissues were bathed in a Krebs solution at 37°C, bubbled with 95% O_2, 5% CO_2. Contractions of the aortic vascular smooth muscle were recorded with a force tranducer (Gould P50) and a Gould polygraph. Preparations were allowed to equilibrate for at least 60 min under a resting tension of 500–600 mg.

Before the beginning of each study, the functional state of the endothelium was verified by observation of the relaxation to acetylcholine (ACh) on a ring precontracted with 5-hydroxytryptamine (5-HT) (Furchgott & Zawadski, 1980).

The concentration-response relationship of ADPβS was investigated by the following methodology: after the test with ACh, the ring was washed 3 times for 20 min. After return to the resting tension, the preparation was again precontracted by 5-HT (0.5 μM) and, when the plateau of the 5-HT-induced contraction was stable (after 3–4 min), ADPβS was added in a cumulative manner with increased concentrations (from 0.01 μM to 10 μM), the highest concentration was added only when the contractile state was stable. Finally, the preparation was washed for 30 min and the functional state of the endothelium was again verified by reapplying ACh.

The chemical removal of the endothelium was performed by the addition of a saponin solution (0.05 mg ml^{-1}) (this plant glycoside causes lysis of cell membranes and disrupts their selective permeability) 3 times within 10 min. Tissues were then incubated for 20 min in the presence of saponin before washing for 30 min (Samata *et al.*, 1986; Ogawa *et al.*, 1993). The ACh test was then performed and the effect of ADPβS was tested 5 min later. In preliminary experiments we have observed that, on rings which were treated with saponin at the same concentration, the histological study (fixation by Bouin solution and ≪trichrome≫ coloration) showed that the endothelium was injured or absent.

Reactive Blue 2 (RB2) (30 μM), indomethacin (10 and 100 μM) or L-NAME (100 μM) was added for 20 min. In the presence of one of these drugs, the effects of ADPβS (1 μM) on the 5-HT-precontracted aorta was examined (for 5 min) once the contractile state was stable. Then, the preparation was washed for 30 min, and still in the presence of RB2, indomethacin or L-NAME, the ACh test was performed.

In some experiments, ADPβS (1 μM) was added for a longer period (25 min) and the effect of indomethacin (10 μM) or L-NAME (100 μM) was studied.

The results were expressed as the percentage of relaxation due to ADPβS in relation to the amplitude of the plateau of 5-HT-induced contraction just before the addition of indomethacin, L-NAME or RB2.

Drugs used

Acetylcholine, 5-HT, Reactive Blue 2 (Cibacron Blue 3GA), indomethacin and L-NAME were from Sigma (U.S.A.); saponin was from Aldrich (Germany) adenosine-5′-O-(2-thiodiphosphate)-lithium salt was from Boehringer Mannheim (Germany).

Results

Rat pancreatic vascular bed

According to previous data (Bertrand *et al.*, 1991), we have chosen two ADPβS concentrations: 1.5 μM eliciting about 60% of the maximal vasodilator effect and 15 μM inducing the maximal vasodilatation of the pancreatic vascular bed. The two concentrations of ADPβS induced a progressive vasodilatation during the first 10 min (first period) and reached a plateau which was sustained for the following 20 min (second period) (Figure 1a and b).

Indomethacin (10 μM) delayed the vasodilatation induced by the low ADPβS concentration without modifying the second period (Figure 1a). In the same way, this cyclo-oxygenase inhibitor inhibited the vasodilatation induced by the high concentration of ADPβS (15 μM) during the first 6 min, then a progressive vasodilatation appeared to reach, at 15 min, the same value as that reached by the controls in the second period (Figure 1b). This effect of indomethacin was selective for the ADPβS-induced vasodilatation since the adenosine (1.5 μM)-induced vasodilatation was not changed by indomethacin at the same concentration (data not shown).

L-NAME (200 μM) *per se* induced a large fall in the flow rate which was in the same range in all sets of experiments. When ADPβS was infused, it initially evoked a transient but not significant (1 min) reduction in the L-NAME-induced vasoconstriction (Figure 2a and b). L-NAME inhibited ADPβS-induced vasodilatation during the next 5 min but thereafter, ADPβS progressively induced an increase in the flow rate

which was dependent on the ADPβS concentration; this increase plateaued at the control levels when we used ADPβS at a concentration of 15 μM (Figure 2b).

L-NAME and indomethacin co-administration (Figure 3a and b) did not increase the fall in the flow rate induced by L-NAME alone. When ADPβS was added, we observed a rapid relaxation of vascular bed which was more pronounced when we used the high concentration. In this case the flow rate from 75 to 90 min was not significantly different from that induced by ADPβS alone.

Rat isolated thoracic aorta

In 5-HT-precontracted and endothelium-intact rings of rat thoracic aorta, ADPβS-induced relaxation was concentration-dependent with a maximal relaxation of 62%, the estimated EC_{50} being 0.09 μM (Figure 4). On these same precontracted rings, an acetylcholine-induced relaxation (1 μM) was also obtained.

We have observed that RB2 (30 μM), a P_{2Y} antagonist, strongly inhibited the ADPβS-induced relaxation by 69.6±3.1% ($n=7$).

After chemical destruction of the endothelium by saponin (0.05 mg ml^{-1}; 30 min), there was a total suppression of the acetylcholine- and ADPβS-induced relaxation on the same rings (Figure 5a and b). However, vessels could still be relaxed by papaverine (0.04%) or sodium nitroprussiate 0.01 μM (data not shown). In rings without endothelium, not only was ADPβS ineffective at inducing relaxation, but it induced a small contraction.

In 5-HT-precontracted rings with intact endothelium, indomethacin and L-NAME inhibited ADPβS induced-relaxa-

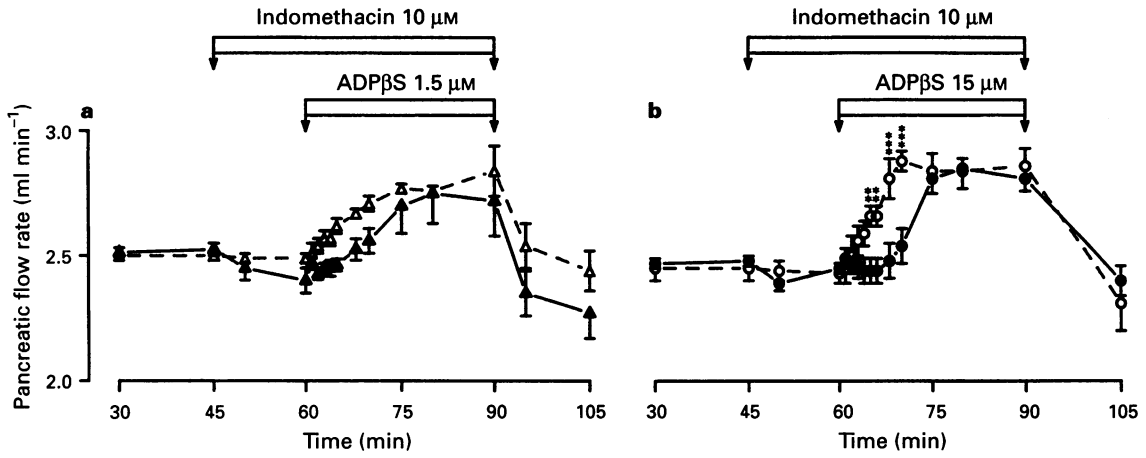

Figure 1 Effect of indomethacin on the ADPβS-induced vasodilatation of rat isolated perfused pancreas: (a) (△) ADPβS 1.5 μM alone; (▲) ADPβS 1.5 μM plus indomethacin 10 μM. (b) (○) ADPβS 15 μM alone; (●) ADPβS 15 μM plus indomethacin 10 μM. Each point represents the mean±s.e.mean of five experiments. $^{**}P<0.01$ $^{***}P<0.001$ treated versus controls.

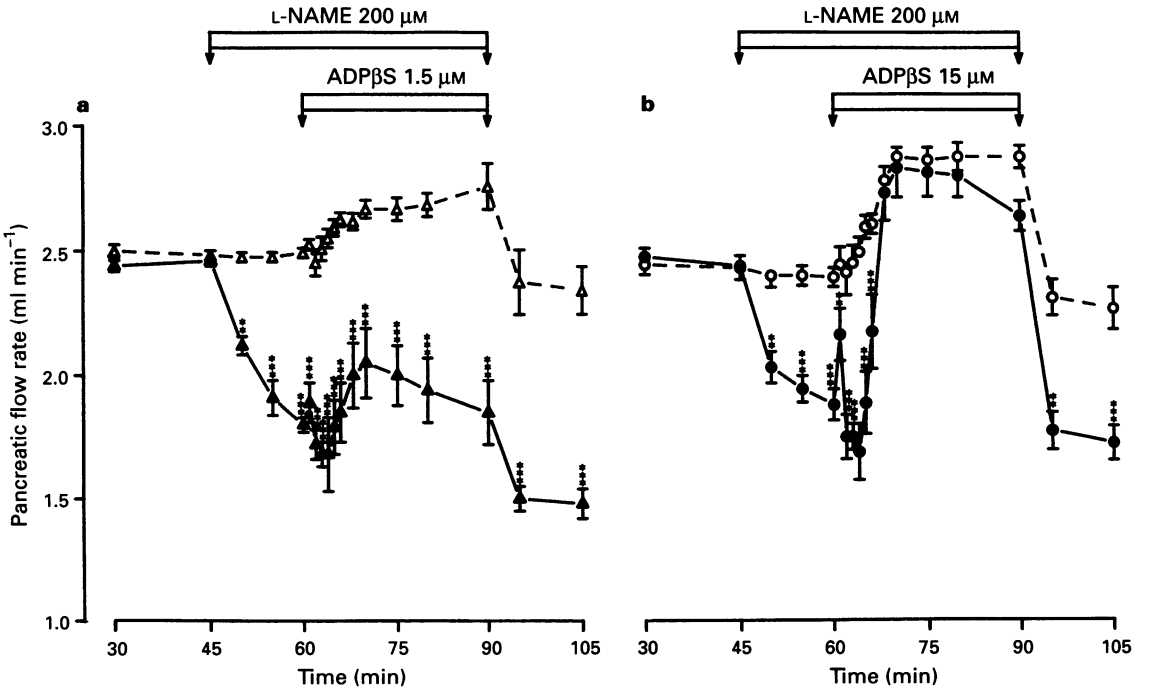

Figure 2 Effect of L-NAME on the ADPβS-induced vasodilatation of rat isolated pancreas: (a) (△) ADPβS 1.5 μM alone; (▲) ADPβS 1.5 μM plus L-NAME 200 μM. (b) (○) ADPβS 15 μM alone; (●) ADPβS 15 μM plus L-NAME 200 μM. Each point represents the mean±s.e.mean of eight experiments. $^{**}P<0.01$ $^{***}P<0.001$ treated versus controls.

tion by 72 and 60% respectively (Figure 6a and b). These results were not dependent on the duration of the administration of ADPβS since the inhibitory effect of L-NAME or indomethacin on ADPβS induced-relaxation persisted during 25 min exposure to ADPβS (Figure 7a and b).

Discussion

This study shows that the vascular relaxation induced by the purinoceptor agonist, ADPβS, can involve various mechanisms according to the preparation used. In 5-HT-precontracted rat aortic rings, the ADPβS-induced relaxation occurs via the activation of a P_{2Y}-purinoceptor located on the endothelial cells with subsequent involvement of both prostanoïd vasodilators and NO related substances; whereas, ADPβS-induced relaxation of pancreatic vascular bed, at basal tone, involves more than two mechanisms since indomethacin or L-NAME or both suppress only the first period of ADPβS-induced relaxation. The results obtained with these two different preparations underline the role of the type of vessels, their localization and/or the role of experimental conditions in the mechanisms underlying P_{2Y}-purinoceptor-induced vasodilatation.

The present study shows that within a species, the role of PGI_2 release appears to be more important in ADPβS-induced relaxation of a large isolated artery, the aorta, in comparison with that induced in the vascular pancreatic bed in which only the first period of dilatation was inhibited by indomethacin; indeed the second period of ADPβS-induced vasodilatation of the pancreatic vascular bed was insensitive to indomethacin treatment. It is known that involvement of endogenous vasodilator prostaglandins considerably varies depending on the species and the type of vessels or vascular bed studied. For example: prostaglandins are not involved in ACh-induced relaxation in rabbit aorta (Furchgott, 1984), but appear to play a role in rat aorta as observed in the present study. In the same way, prostaglandins are not involved in ATP-induced relaxation of canine femoral artery. (De Mey *et al.*, 1982); in contrast, it is well documented that ATP induces PGI_2 release from endothelial cells of human umbilical vein (Carter *et al.*, 1988), bovine aorta (Mitchell *et al.*, 1992) and pig aorta (Needham *et al.*, 1987). Despite the fact that purinoceptor-induced PGI_2 release is transient (Pearson *et al.*, 1983; Mitchell *et al.*, 1992) and subject to tachyphylaxis (Toothill *et al.*, 1988), in our study the inhibitory effect of indomethacin on ADPβS-induced relaxation of aortic artery is in favour of a real involvement of vasodilator prostanoïds; this may be related to a more potent effect of ADPβS on PGI_2 release as described by Lustig *et al.* (1992) on bovine pulmonary endothelial cells.

Concerning the involvement of NO in vascular relaxation, it is well documented that this compound plays a role in most species and types of preparations (Moncada *et al.*, 1991). The

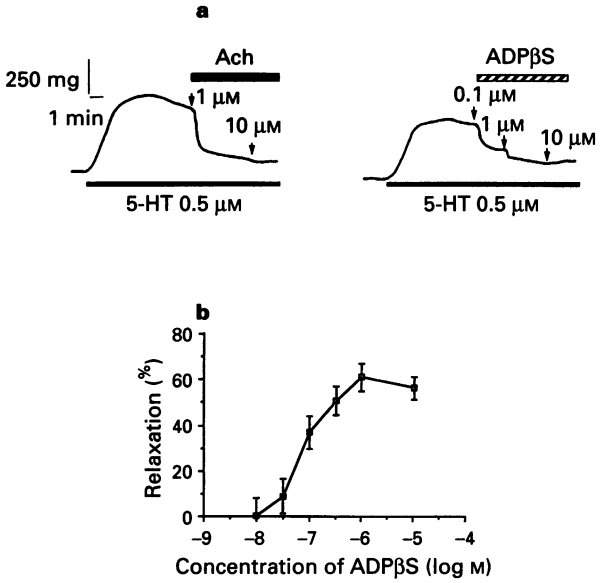

Figure 4 Concentration-response curve for ADPβS on 5-HT-precontracted rat aortic ring. (a) Right hand panel: ADPβS was added by a cumulative procedure at increasing concentrations. Left hand panel: the functional state of the endothelium was first tested with acetylcholine. (b) Results were expressed as % of relaxation of 5-HT (0.5 μM) precontracted rings. Each point represents mean ± s.e.mean ($n = 5$).

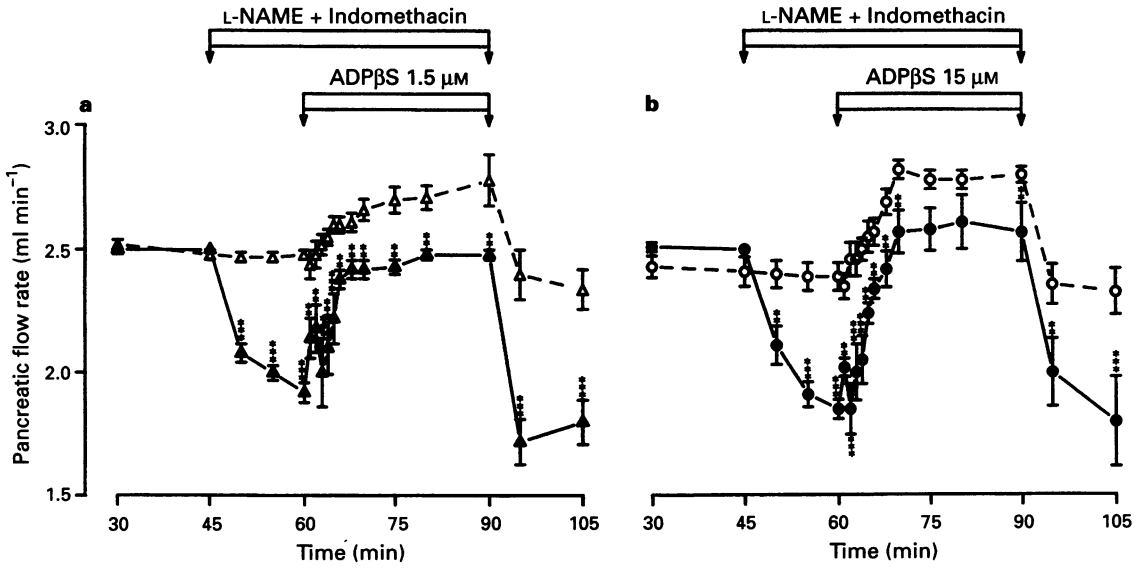

Figure 3 Effect of the co-administration of L-NAME (200 μM) and indomethacin (10 μM) on the ADPβS-induced vasodilatation of rat isolated pancreas. (a) Effect of ADPβS 1.5 μM: Controls (△); in presence of L-NAME and indomethacin (▲). (b) Effect of ADPβS 15 μM: controls (○); in presence of L-NAME and indomethacin (●). Each point represents the mean ± s.e.mean of five experiments. $^{**}P < 0.01$ $^{***}P < 0.001$ treated versus controls.

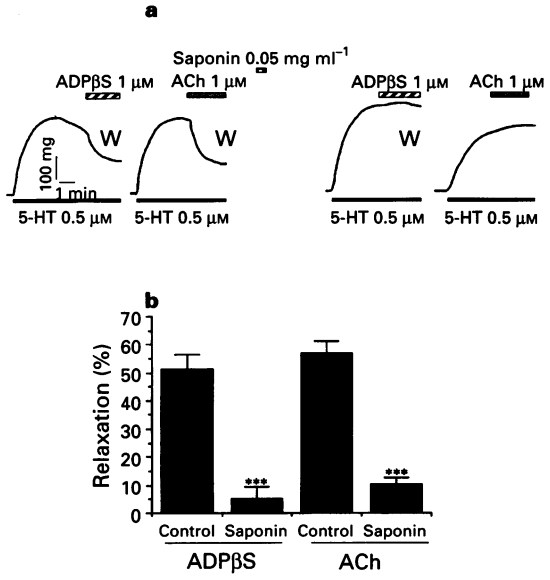

Figure 5 Effect of saponin (0.05 mg ml^{-1}) on the relaxation induced by ADPβS (1 μM) and acetylcholine (1 μM) of precontracted aortic rings. (a) Control responses were obtained to ADPβS and acetylcholine (Left hand panel). Saponin (0.05 mg ml^{-1}) was added for 30 min, then ADPβS and acetylcholine were tested and no relaxation was observed. (Right hand panel). (b) Results expressed as % of relaxation to 5-HT (0.5 μM) precontracted rings. Control = ADPβS or acetylcholine-induced relaxation before addition of saponin. Data are shown as mean ± s.e.mean ($n=9$). ***$P<0.001$.

release of this important mediator has been described as sustained and independent of the constant infusion of its precursor, L-arginine, because of the high intracellular level of the latter, about 100 μM (Mitchell *et al.*, 1990). Since L-NAME evoked a potent vasoconstriction *per se* in pancreatic vascular bed, it can be suggested that a basal release of NO may be responsible for maintaining a vasodilator tone in this preparation as described in other systems (Rees *et al.*, 1989; Gardiner *et al.*, 1990). The L-NAME-induced vasoconstriction was not observed in the ring aorta preparation, in which vascular tone was restored. A lack of effect of L-NAME alone in preparations in which vascular tone has been restored, was previously described in rat renal artery (Wu *et al.*, 1994). In experiments performed in pancreatic vascular bed, after the first inhibition of ADPβS-induced relaxation by L-NAME, ADPβS reveals its vasorelaxant effect despite the presence of NO synthase inhibitor; this L-NAME-resistant part of ADPβS-induced relaxation was dependent on the ADPβS concentration. Furthermore, the co-administration of L-NAME and indomethacin was unable to prevent the relaxant effect of ADPβS.

Considering the insensitivity of ADPβS-induced dilatation to indomethacin and L-NAME in the pancreatic vascular bed during the second period, it can be suggested that (an)other mechanism(s) is (are) involved. The existence of another relaxant factor released by endothelial cells has been proposed in the ACh-induced vasodilatation; this factor is neither a prostanoïd (Feletou & Vanhoutte, 1988) nor an activator of guanylate cyclase enzyme activity but a smooth muscle hyperpolarizing factor (Chen *et al.*, 1988; Taylor & Weston, 1988) called «endothelium-derived hyperpolarizing factor»

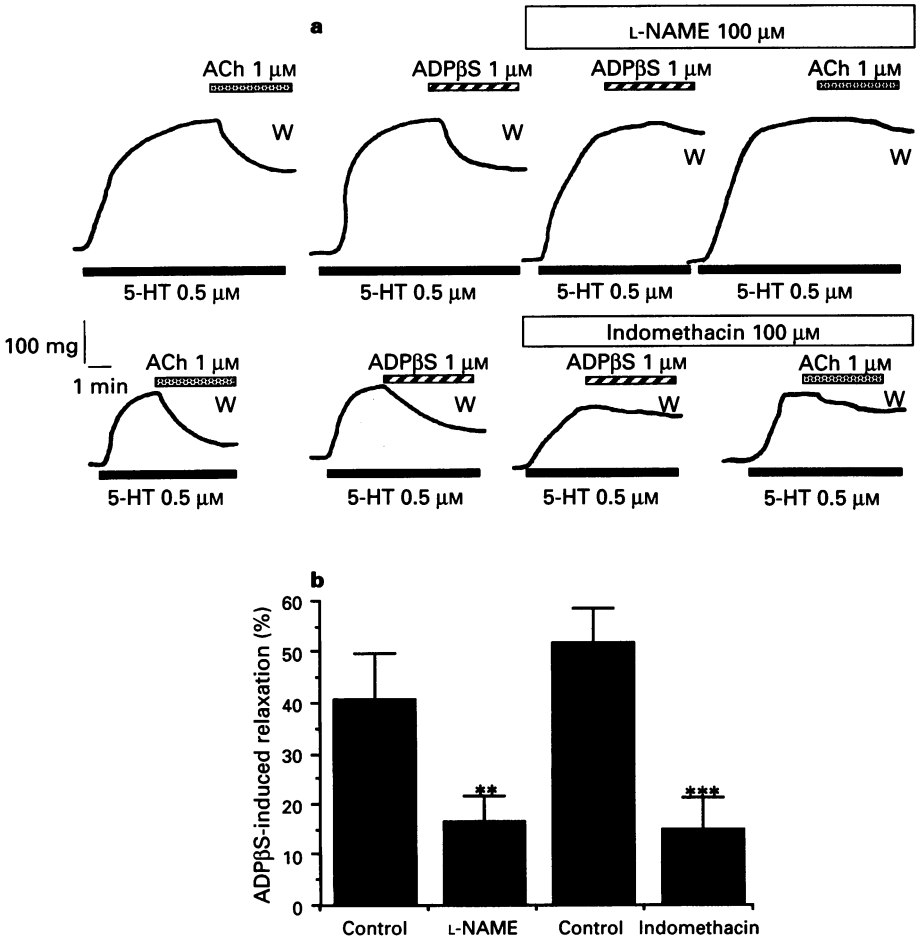

Figure 6 Effect of L-NAME (100 μM) and indomethacin (100 μM) on the ADPβS (1 μM)-induced relaxation of precontracted aortic rings. (a) On precontracted aortic rings, after inducing control relaxations to ADPβS or ACh, L-NAME or indomethacin were added for 20 min. ADPβS or ACh was then readministered. (b) Results expressed as % of relaxation to 5-HT (0.5 μM)-precontracted rings. Control = ADPβS-induced relaxation before addition of L-NAME or indomethacin, L-NAME and indomethacin were added 20 min before 5-HT induced contraction. Data are shown as means ± s.e.mean ($n=5$). **$P<0.01$, ***$P<0.001$.

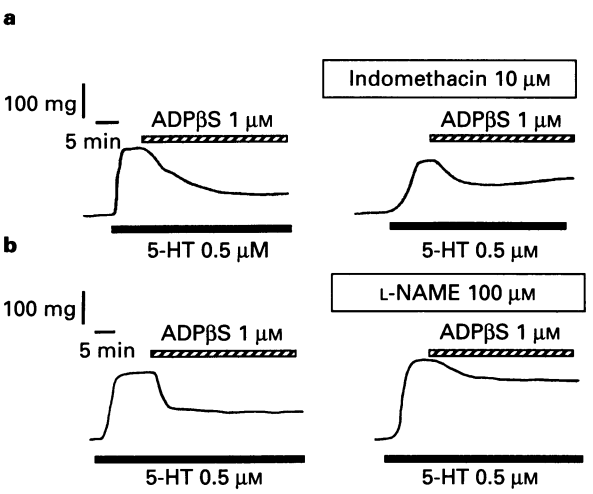

Figure 7 The long term (25 min) ADPβS (1 μM)-induced relaxation of precontracted aortic rings: (a) Effects of indomethacin (10 μM) (b) Effects of L-NAME (100 μM). Drugs were added 20 min before 5-HT-induced contraction ($n=3$).

(EDHF). Recently, it has been demonstrated that, in the perfused rat mesenteric arterial bed, in response to ACh and histamine, both EDHF and endothelium-derived nitric oxide radical (EDNO) were released by the endothelium, these factors inducing a relaxation of the vascular smooth muscle (Adeagbo & Triggle, 1993). The EDHF-induced hyperpolarization has been shown, according to the preparation, to involve an activation of the sodium-potassium pump (Feletou & Vanhoutte, 1988) and/or a direct opening of ATP-dependent or calcium-dependent potassium channels (Standen *et al.*, 1989; Adeagbo & Malik, 1990). It is important to note that L-NAME or indomethacin evokes a potent and long-lasting inhibition of the ADPβS-induced relaxation on the rat thoracic aorta. Thus, an important participation of EDHF in this large artery could be ruled out; this contrasts with the transient effect of both inhibitors in the pancreatic vascular bed containing small resistance arteries. This study may be in agreement with the concluding remarks of Garland *et al.* (1995) on the respective influence of NO and EDHF on vascular smooth muscle according to the vessels: the former being predominant in large arteries and the latter in small resistance vessels; it suggests the potential role of EDHF in the purinergic control of vascular tone.

In conclusion, this comparative study shows that the vasodilatation induced by ADPβS via P_{2Y}-purinoceptors is mediated by complex mechanisms differently involved in rat aorta and pancreatic vessels. We propose the following hypothesis for the explanation of our results on two different experimental models: (1) in the two models, the P_{2Y}-purinoceptor is present on the endothelial cell and its activation by ADPβS induces releases of PGI_2 and NO, which subsequently induce the relaxation of the smooth muscle cell; (2) in the pancreatic vascular bed, ADPβS induces relaxation not only through PGI_2 and NO release from the endothelium, but also through an (other) more delayed mechanism(s); the precise localization (endothelial or smooth muscle cells) and purinoceptor subtype involved remain to be elucidated, but implication of EDHF may be suggested.

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