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Modulation by nicotinamide adenine dinucleotide of sympathetic and sensory-motor neurotransmission via P_1-purinoceptors in the rat mesenteric arterial bed

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1 The pharmacological actions of the purine nucleotides β-nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide phosphate (β-NADP), adenosine 5'-diphosphoribose (ADP-ribose), the vitamin nicotinamide and structural analogues of NAD and NADP were tested in the isolated perfused mesenteric arterial bed of the rat. Prejunctional effects of NAD were tested against sympathetic vasoconstriction at basal tone, and against sensory-motor vasodilatation at raised tone.

2 NAD and NADP had no vasoconstrictor action but were weak vasodilators of the raised-tone mesenteric arterial bed. A rank order of vasodilator potency of ADP >> ADP-ribose >> NADP ≥ NAD = adenosine was observed. The P_1-purinoceptor antagonist, 8-*para*-sulphophenyltheophylline (8-*p*SPT; 3 μM) inhibited vasodilator responses to NAD (pK_B of 6.61 ± 0.21, $n = 7$) and adenosine (pK_B of 5.78 ± 0.14, $n = 6$), but not those elicited by NADP, ADP and ADP-ribose. Nicotinamide, and analogues of NAD and NADP, namely nicotinamide-1,N^6-ethenoadenine dinucleotide phosphate, β-nicotinamide mononucleotide, nicotinamide hypoxanthine dinucleotide phosphate, nicotinamide hypoxanthine dinucleotide, nicotinamide guanine dinucleotide, and nicotinamide-1,N^6-ethenoadenine dinucleotide had no vasoconstrictor or vasodilator actions (at doses of up to 50 nmol).

3 At basal tone, electrical field stimulation (EFS) (32 Hz, 1 ms, 90 V, 5 s) at 2 min intervals elicited reproducible vasoconstrictor responses due to activation of sympathetic nerves. NAD and adenosine (10-100 μM) inhibited these responses in a concentration-dependent manner with similar potencies. Nicotinamide had no effect on sympathetic vasoconstriction at concentrations of up to 0.1 mM. Postjunctional effects of NAD (100 μM), as tested on constrictor responses to NA (5 nmol), accounted for approximately 60% inhibition at this concentration.

4 In preparations in which tone had been raised with methoxamine (10-40 μM), EFS (8 Hz, 0.1 ms, 60 V, for 30 s) elicited vasodilatation due to activation of sensory-motor nerves. This vasodilatation was inhibited by NAD and adenosine (0.1-100 μM) in a similar concentration-dependent manner: pD_2 values were 6.2 ± 0.10 ($n = 11$) and 6.1 ± 0.15 ($n = 6$) for NAD and adenosine respectively. Nicotinamide had no effect on sensory-motor vasodilatation at concentrations of up to 0.1 mM.

5 Inhibition of sympathetic constriction by NAD and adenosine was antagonized by 8-*p*SPT (3 μM). Inhibitory effects of NAD and adenosine on sensory-motor vasodilatation were similarly antagonized by 8-*p*SPT (1 μM), pK_B values were 6.72 ± 0.21 for NAD and 6.36 ± 0.22 for adenosine, resulting in parallel rightward shifts in the concentration-inhibitory effect curves.

6 The adenosine deaminase inhibitor, pentostatin (1 μM), augmented the inhibitory effects of NAD and adenosine. Concentration-inhibitory effect curves for NAD and adenosine on sympathetic vasoconstriction and sensory-motor vasodilatation were shifted to the left without a change in the maximum.

7 It is concluded that NAD can act as a modulator of sympathetic and sensory-motor transmission in rat mesenteric arteries via P_1-purinoceptors possibly via direct actions but with a contribution of adenosine formed following breakdown of NAD or released pre- and/or postjunctionally. Structure-activity relationships of NAD, NADP, ADP and ADP-ribose showed that the P_1-purinoceptor activity of NAD is abolished after removal of nicotinamide, or ribose plus nicotinamide, to yield the structurally-related ADP-ribose and ADP respectively, or when there is phosphorylation of the 2'-hydroxyl group of NAD to yield NADP.

Keywords: Nicotinamide adenine dinucleotide; purinoceptors; rat mesenteric arterial bed; sympathetic transmission; sensory-motor transmission

Introduction

Extracellular purine nucleosides and nucleotides influence many biological process via P_1- and P_2-purinoceptors (Drury & Szent-Gyorgyi, 1929; Green & Stoner, 1950; Burnstock, 1978; Ralevic & Burnstock, 1991a). At present there are five well-established divisions of the P_2-purinoceptor: P_{2X}, P_{2Y}, P_{2Z}, P_{2T} and P_{2U} (synonymous with P_{2N}) (Burnstock & Kennedy, 1985; Gordon, 1986; Hoyle, 1992; Abbracchio & Burnstock, 1994); however, in a revision of purine receptor taxonomy 'P2X' and 'P2Y' will comprise extended families incorporating these receptors based on whether they function as receptor-operated ion channels, or are coupled to G-proteins, respectively (Abbracchio & Burnstock, 1994; Fredholm *et al.*, 1994). The term P_{2Z} purinoceptor will be retained since this mediates responses to ATP^{4-} by the opening of a nonselective ion pore. P_1-purinoceptors are activated by adenosine > adenosine 5'-monophosphate (AMP) > adenosine 5'-diphosphate (ADP) > adenosine-5'-triphosphate (ATP), and are antagonized by methylxanthines such as caffeine, theophylline and 8-phenyltheophylline; P_2-purinoceptors are selectively activated by ATP or ADP and are not antagonized by methylxanthines (Burnstock & Kennedy, 1985; Hoyle, 1992; Abbracchio & Burnstock, 1994). P_1-purinoceptors have been further subdivided into A_1, A_2 and A_3 classes, and antagonists with a degree of selectivity have been developed (see

Burnstock & Buckley, 1985; Cooper & Londos, 1988; Kennedy, 1990; Abbracchio *et al.*, 1993).

The coenzyme β-nicotinamide adenine dinucleotide (NAD), together with its phosphorylated and reduced forms, belongs to the family of naturally occurring adenine dinucleotides which are attracting considerable recent interest because of their potent and diverse actions as extracellular signal molecules (Hoyle, 1990; Ogilvie, 1992; Schluter *et al.*, 1994). In many tissues adenine dinucleotides act through nucleoside and nucleotide receptors; however, in some cases they appear to act via their own distinct dinucleotide receptors (see Hoyle, 1990). In the brain NAD-binding sites, distinct from those reported for adenosine, have been visualized autoradiographically (Candy *et al.*, 1984; Snell *et al.*, 1985). In the periphery and in the central nervous system the actions of NAD appear to be very similar to those of adenosine, for example: causing hyperpolarization and relaxation of the smooth muscle of the guinea-pig taenia coli (Romanenko, 1980; Romanenko *et al.*, 1980; Burnstock & Hoyle, 1985), causing prejunctional inhibition of sympathetic neuromuscular transmission in the vas deferens (Stone, 1981) and inhibiting synaptic transmission by decreasing glutamate release in rat hippocampal slices (Galarreta *et al.*, 1993). NAD may be partially degraded to adenosine, which subsequently acts on P_1-purinoceptors (Bruns, 1980a; Burnstock & Hoyle, 1985), or it may evoke release of adenosine from the tissue and activate P_1-purinoceptors via this mechanism (Stone, 1981). Phosphorylation of the 2'-hydroxyl group of NAD yields NADP which acts like a P_2-purinoceptor agonist (Burnstock & Hoyle, 1985).

Adenosine 5'-diphosphoribose (ADP-ribose) is an ADP moiety with a second ribose sugar attached to the β-phosphate via an esteric linkage. ADP-ribose is chemically related to NAD and NADP; NAD can be regarded as ADP-ribose covalently attached to the vitamin nicotinamide by a high energy β-N-glycosidic bond. Although its intracellular roles have been widely studied (Hayaishi & Ueda, 1977; Ueda & Hayaishi, 1985; Hussain *et al.*, 1989) information about the actions of ADP-ribose on extracellular receptors is limited. Hoyle & Edwards (1992) showed that ADP-ribose has a mixed pharmacological profile in the guinea-pig taenia coli, evoking both P_1 (A_2)- and P_{2Y}-purinoceptor-mediated responses, while being inactive at P_{2X}-purinoceptors in the guinea-pig vas deferens. To our knowledge, the extracellular actions of ADP-ribose in blood vessels have not been investigated.

The main aim of the present study was to examine the extracellular actions of NAD in the rat mesenteric arterial bed to see whether it acts at a specific class of adenine dinucleotide receptor or mimics the actions of adenosine at the P_1-purinoceptor. The study also aimed to examine the pharmacological profiles of the structurally related NADP, ADP and ADP-ribose. To this end, prejunctional effects of NAD and nicotinamide on sympathetic and sensory-motor transmission were tested. In rat mesenteric arteries noradrenaline (NA) is the principal sympathetic transmitter, and calcitonin gene-related peptide (CGRP) and principal vasodilator transmitter released from sensory nerves (Kawasaki *et al.*, 1988). Postjunctional actions of NAD, NADP, nicotinamide, ADP, ADP-ribose and analogues of NAD and NADP were also examined.

Methods

Isolated mesenteric arterial bed preparation

Male Wistar rats (300–350 g) were killed by asphyxiation with CO_2. Mesenteric beds were isolated and set up for perfusion as described previously (Ralevic *et al.*, 1994). The abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was severed, the gut dissected away and the preparation mounted on a stainless steel grid (7 × 5 cm) in a humid chamber (custom made at University College London). The preparation was perfused at a constant flow rate of 5 ml min^{-1} with a peristaltic pump (model 7554–30, Cole-Parmer Instrument Co., Chicago, Illinois, U.S.A.). The perfusate was Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8, gassed with 95% O_2:5% CO_2 and maintained at 37°C. Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (model P23XL, Viggo-Spectramed, Oxnard, CA, U.S.A.) on a side arm of the perfusion cannula, and recorded on a polygraph (model 7D, Grass Instrument Co., Quincy, Mass, U.S.A.). Preparations were allowed to equilibrate for 30 min prior to experimentation.

Assessment of direct actions at basal and raised tone

The direct effects of agents were assessed at basal and raised tone by injecting 50 μl of these agents at doses of up to 1.5 μmol into a rubber septum proximal to the preparation. Vasodilator responses were examined in mesenteric arterial bed preparations in which the tone had been raised by continuous perfusion with methoxamine (10–50 μM).

Assessment of neuromodulatory effects on perivascular sympathetic and sensory-motor nerves

At basal tone intermittent pulses of EFS (32 Hz, 90 V, 1 ms for 5 s) were given at 2 min intervals to elicit vasoconstrictor responses due to activation of perivascular sympathetic nerves. These responses could be blocked by guanethidine (5 μM) or by prazosin (1 μM). In separate preparations guanethidine (5 μM) was added to the perfusate to block sympathetic transmission and methoxamine added to raise the tone of the preparations. EFS (8 Hz, 60 V, 0.1 ms for 30 s) elicited vasodilatation due to activation of sensory-motor nerves. These responses are blocked by tetrodotoxin (1 μM) or by capsaicin (Ralevic *et al.*, 1994). The effects of each of adenosine, NAD and nicotinamide on sympathetic vasoconstriction and sensory-motor vasodilatation were examined by adding these to the perfusate cumulatively (0.03–100 μM). Postjunctional effects of NAD (10, 30 and 100 μM) were tested against constrictions to repeated doses of NA (5 nmol in 50 μl) applied at 5 min intervals. These responses were reproducible for the duration of the experiment and produced a similar magnitude of constriction as EFS. Each concentration of NAD was in contact with the tissue for 10 min separated by at least 10 min washout, which allowed two applications of NA to be tested per concentration of NAD. Postjunctional effects of NAD at raised tone were tested against relaxation to CGRP (15 pmol). Pentostatin (1 μM) and 8-*p*SPT (1-10 μM) were added to the perfusate either after an initial control stimulation of sensory-motor nerves or at the onset of the experiments for sympathetic transmission, but always allowing 20 min incubation with the drug. Preliminary studies showed that 1 μM 8-*p*SPT produced a significant reversal of inhibition of sensory-motor neurotransmission, whereas 3 μM was required to reverse NAD- and adenosine-induced inhibition of sympathetic neurotransmission.

Drugs used

Drug dilutions were made up daily from stock solutions of 10 or 100 mM (concentrates stored frozen) in distilled water. The following drugs were obtained from Sigma: adenosine 5'-diphosphate (sodium salt), adenosine 5'-diphosphoribose (sodium salt), adenosine hemisulphate, β-nicotinamide adenine dinucleotide, β-nicotinamide adenine dinucleotide phosphate (sodium salt), nicotinamide-1,N^6-ethenoadenine dinucleotide

phosphate, β-nicotinamide mononucleotide, nicotinamide hypoxanthine dinucleotide phosphate (disodium salt), niacinamide, nicotinamide hypoxanthine dinucleotide (sodium salt), nicotinamide-1,N^6-ethenoadenine dinucleotide, nicotinamide guanine dinucleotide, noradrenaline bitartrate, 8-(*para*-Sulphophenyl) theophylline was from Research Biochemicals International. Pentostatin (2'-deoxycoformycin) was a generous gift from Parke-Davis, Ann Arbor, MI, U.S.A.

Data analysis

Vasodilator responses were measured as changes in perfusion pressure (mmHg) and evaluated as a percentage of the methoxamine-induced tone. Inhibition of sympathetic vasoconstriction by NAD or adenosine was evaluated as a percentage of the preceding control stimulation in the absence of these agents. Inhibition of sensory-motor vasodilatation was evaluated as a percentage of the control vasodilatation (calculated as a percentage of the methoxamine-induced increase in tone) in the absence of drugs. Results are presented as means ± s.e.mean, followed by the number of observations in parentheses (n). pD_{20} and pD_{30} are defined as the negative log of the dose (in mol) that evokes a decrease in perfusion pressure of 20 and 30 mmHg respectively. pD_2 is defined as the negative log of the dose or concentration (as appropriate) of agonist required to produce 50% of the maximal response. pK_B was evaluated from the formula $K_B = [B]/(DR-1)$, where B = concentration of agonist and DR (dose-ratio) = the difference between pD_2 values in the absence and presence of antagonist. Differences between means were determined by Student's unpaired or paired t test and were considered significant when $P<0.05$.

Results

Direct actions of NAD, NADP, nicotinamide, adenosine, ADP, ADP-ribose and analogues of NAD and NADP

At basal tone NAD, NADP, nicotinamide, ADP-ribose and analogues of NAD and NADP had no vasoconstrictor actions. In experiments with raised tone, methoxamine (10–50 μM) increased the tone of the preparations by 60.85 ± 3.17 from a basal tone of 37.64 ± 1.21 mmHg (n = 14). Nucleotides elicited vasodilatation with a potency order of ADP >> ADP-ribose >> NADP > NAD = adenosine (Figure 1). pD_2 values were 10.33 ± 0.12 (n = 8) for ADP, 8.20 ± 0.14 for ADP-ribose (n = 7) and 7.59 ± 0.06 for NADP (n = 12). There was no significant difference between the pD_{30} values for NAD and adenosine, which were 6.73 ± 0.07 (n = 7) and 6.91 ± 0.14 (n = 8) respectively. Maximum vasodilatation to NADP, 35.49 ± 3.72% at 0.5 μmol (n = 12), was significantly smaller than that of the other vasodilators, and was less than half of the maximum vasodilatation to ADP, 84.20 ± 2.80% (n = 8). At the highest dose of NADP, vasoconstriction following the initial relaxation was sometimes observed. Nicotinamide had no vasodilator actions. None of the analogues of NAD and NADP had vasodilator actions at doses of up to 50 nmol; at the highest dose tested (500 nmol) only nicotinamide hypoxanthine dinucleotide, nicotinamide hypoxanthine dinucleotide phosphate and nicotinamide-1,N^6-ethenoadenine dinucleotide elicited weak vasodilatation in two of four preparations.

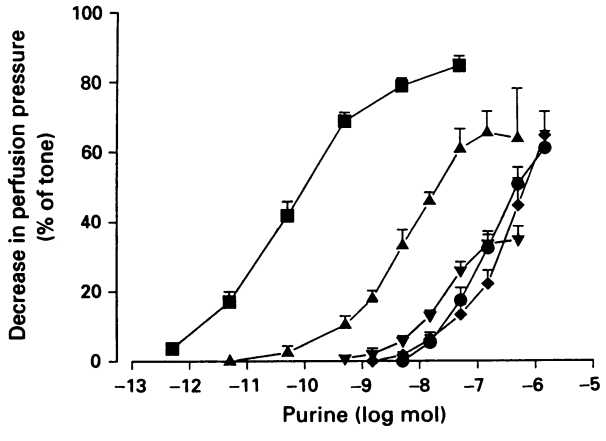

Figure 1 Dose-response curves showing vasodilator responses of the rat mesenteric arterial bed to ADP (■, n = 8), ADP-ribose (▲, n = 8), NADP (▼, n = 12), NAD (◆, n = 8) and adenosine (●, n = 8). Responses are shown as percentage decrease in methoxamine-evoked tone. Data are given as means ± s.e.mean. For abbreviation in this and subsequent figures, see text.

*Effect of 8-*para*-sulphophenyltheophylline (8-pSPT) on vasodilator responses*

Addition of the P_1-purinoceptor antagonist 8-*p*SPT (3 μM) to the perfusate caused a tendency for the tone of the preparations to increase; by reducing the methoxamine concentration the increase in tone (66.75 ± 6.60 mmHg, n = 12) was not statistically different from that in the absence of 8-*p*SPT. 8-*p*SPT (3 μM) antagonized responses to NAD and adenosine, shifting to the right the dose-response curves (Figure 2). pD_{20} values for adenosine were 7.22 ± 0.17 and 6.78 ± 0.14 (n = 6) in the absence and presence of 8-*p*SPT ($P<0.05$). pD_{20} values for NAD were 7.07 ± 0.12 and 5.93 ± 0.24 (n = 7) in the absence and presence of 8-*p*SPT ($P<0.001$). In the presence, but not in the absence, of 8-*p*SPT there was a significant difference between the pD_{20} values for adenosine and NAD ($P<0.05$). pK_B values for the effect of 8-*p*SPT on adenosine, 5.78 ± 0.14 (n = 6), and NAD, 6.61 ± 0.21 (n = 7), were also significantly different ($P<0.01$). Vasodilatations due to NADP, ADP and ADP-ribose were not antagonized by 8-*p*SPT (Figure 2).

Effect of NAD and adenosine on sympathetic vasoconstriction

At basal tone intermittent pulses of EFS (32 Hz, 1 ms, 90 V for 5 s) at 2 min intervals elicited constrictor responses due to activation of sympathetic nerves (Figure 3a). These averaged 26.1 ± 2.7 mmHg (n = 42) at the start of experimentation and were relatively stable being 27.5 ± 2.0 mmHg (n = 42) at the end of the experiment. Vasoconstrictor responses were attenuated in a concentration-dependent manner by NAD and adenosine (10–100 μM) (Figures 3a, b and 4). The concentration-inhibitory effect curves to NAD and adenosine were superimposable. There was no significant difference between inhibition of sympathetic vasoconstriction by NAD and adenosine at any concentration. Inhibition attained at the highest concentration tested (100 μM) was 63.54 ± 9.0% (n = 8) and 56.55 ± 3.69% (n = 7) for NAD and adenosine respectively. Nicotinamide had no effect on sympathetic vasoconstriction at concentrations of up to 100 μM.

The inhibitory effects of 10 μM NAD and adenosine were abolished by 8-*p*SPT (3 μM), and were significantly antagonized, by approximately 70% at 30 μM NAD and adenosine (Figure 4). At 100 μM NAD responses were significantly antagonized, by approximately 50%, by 8-*p*SPT ($P<0.01$); inhibition by NAD was 68.49 ± 8.03% (n = 4) and 32.58 ± 4.84% (n = 8) in the absence and presence of 8-*p*SPT respectively. In contrast, inhibition by 100 μM adenosine was unaffected by 8-*p*SPT; inhibition was 56.55 ± 3.69% (n = 4) and 58.99 ± 6.0% (n = 8) in the absence and presence of 8-*p*SPT respectively.

Inhibition of adenosine deaminase with pentostatin augmented the inhibitory effects of both NAD and adenosine,

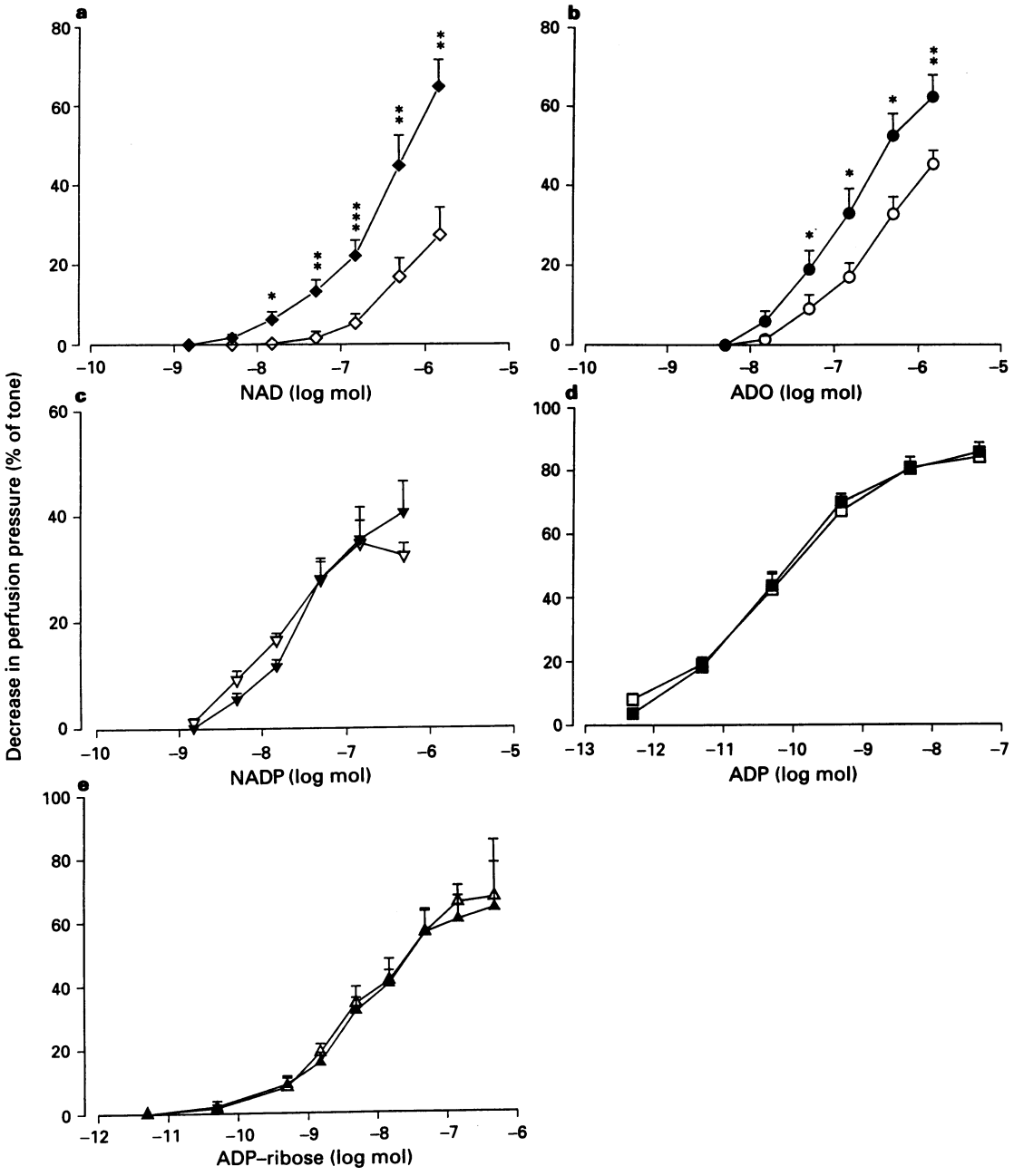

Figure 2 Dose-response curves showing vasodilator responses of purine compounds in the rat mesenteric arterial bed in the absence (closed symbols) and presence of 8-*para*-sulphophenyltheophylline (3 μM) (open symbols): (a) NAD (◆, $n = 8$); (b) adenosine (●, $n = 6$); (c) NADP (▼, $n = 6$); (d) ADP (■, $n = 7$); (e) ADP-ribose (▲, $n = 8$). Data are given as mean ± s.e.mean.

lowering the threshold for inhibition to approximately 3 μM without changing the maximum (Figure 4).

Exogenously applied doses of NA (5 nmol) elicited vasoconstriction of 25.71 ± 2.33 mmHg under control conditions in eight preparations. NAD inhibited these responses by 5.49 ± 1.92% (10 μM NAD), 24.33 ± 3.42% (30 μM NAD) and 38.11 ± 3.77% (100 μM NAD).

Effect of NAD and adenosine on sensory-motor vasodilatation

Methoxamine raised the tone of the preparations by 48.25 ± 2.0 mmHg from 33.32 ± 0.6 mmHg ($n = 47$). EFS (8 Hz, 0.1 ms, 60 V, 30 s) elicited frequency-dependent vasodilatation of the mesenteric arterial bed preparations. Mean vasodilatation in the absence of agents was 48.4 ± 2.3% ($n = 47$). In the presence of NAD or adenosine (0.1–100 μM) vasodilator responses were attenuated in a concentration-dependent manner (Figures 3b and 5). There was no significant difference in the maximum inhibition, approximately 67.73 ± 6.42% ($n = 6$) and 71.81 ± 9.32% ($n = 10$) inhibition by 30 μM adenosine and NAD respectively. Relaxation to 15 pmol CGRP in the presence of 10 μM NAD was 42.09 ± 8.99% ($n = 6$), which is similar to that obtained in the absence of NAD (Ralevic *et al.*, 1994). Above 30 μM, NAD and adenosine had some postjunctional effects causing the tone of the preparation to drop. Nicotinamide had no inhibitory effects on sensory-motor vasodilatation.

8-*p*SPT (1 and 10 μM) blocked the inhibitory effects of NAD and adenosine, shifting to the right the inhibitory-effect curves. The pD_2 of NAD was significantly decreased from 6.20 ± 0.1 ($n = 11$) in control conditions to 5.40 ± 0.18 ($n = 6$) and 5.08 ± 0.13 ($n = 4$) in the presence of 1 μM 8-*p*SPT ($P < 0.01$) and 10 μM 8-*p*SPT ($P < 0.001$). The pD_2 of adeno-

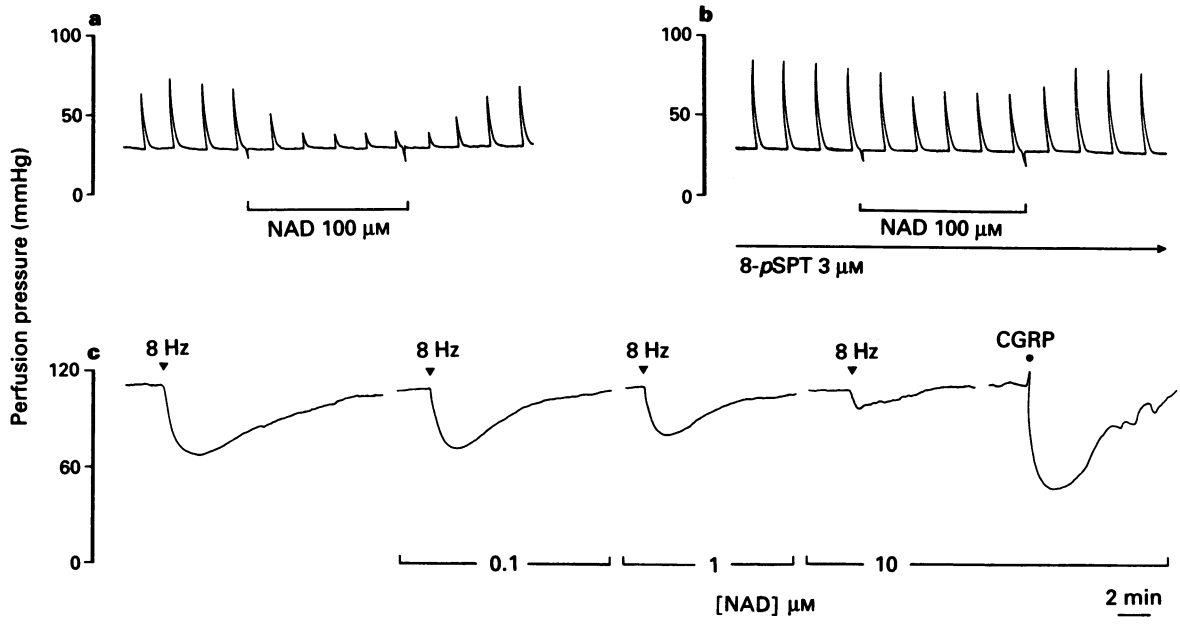

Figure 3 Representative traces showing effect of nicotinamide adenine dinucleotide (NAD) on responses of the rat isolated mesenteric arterial bed to electrical field stimulation (EFS) at basal tone (a,b) and at raised tone (tone raised with methoxamine) in the presence of guanethidine (c). (a) Inhibition of sympathetic constriction to EFS (32 Hz, 90 V, 1 m, 5 s) by 100 μM NAD, (b) reversal of sympathetic inhibition by 100 μM NAD with 8-*para*-sulphophenyltheophylline (8-*p*SPT, 3 μM), (c) concentration-dependent inhibition of sensory-motor vasodilatation to EFS (8 Hz, 60 V, 0.1 ms, 30 s) by NAD (0.1, 1 and 10 μM) and lack of effect of 10 μM NAD on relaxation to a dose of calcitonin gene-related peptide (CGRP, 15 pmol).

sine was significantly decreased from 6.10 ± 0.15 (n = 6) to 5.58 ± 0.16 (n = 6) in the presence of 1 μM 8-*p*SPT (P < 0.05) (Figure 5). There was no significant difference in pK_B values (mean ± 95% confidence limits) for 8-*p*SPT with adenosine, 6.36 ± 4.14, 8.59, and NAD, 6.72 ± 4.59, 8.85.

Pentostatin (1 μM) potentiated the effects of NAD and adenosine (Figure 5), producing a parallel shift to the right of the concentration-inhibitory effect curves without affecting the maximum. In the presence of pentostatin the pD_2 of NAD increased to 6.51 ± 0.17 (n = 7) and of adenosine to 6.31 ± 0.1 (n = 5), although these differences did not reach statistical significance.

Discussion

The results of this study show that NAD mimics the actions of adenosine in rat mesenteric arteries; it can act as a prejunctional modulator of sympathetic and sensory-motor neurotransmission and is also a relatively weak postjunctional vasodilator. Attenuation of pre- and postjunctional effects of NAD by 8-*p*SPT indicates an action at P_1-purinoceptors, while potentiation of inhibitory neuromodulation by the adenosine deaminase inhibitor, pentostatin, suggests that at least part of the effect of NAD is mediated via adenosine. The structurally related NADP and ADP-ribose were both vasodilators but were functionally dissimilar to NAD since they did not act at P_1-purinoceptors.

Several possibilities are considered for the mechanism(s) by which NAD elicits its pre- and postjunctional effects: it may act directly at nucleoside, nucleotide or dinucleotide receptors; it may act via adenosine following breakdown; finally, NAD may act via adenosine released from neural or other tissues (Stone, 1981; Stone & Perkins, 1981). Distinct receptors for the adenine dinucleotides have been described for NAD in the central nervous system (Richards *et al.*, 1983a,b; Snell *et al.*, 1985), and for certain of the α,ω-polyphosphates in some peripheral and central tissues (Hilderman *et al.*, 1991; Pintor *et al.*, 1993). To date there is no evidence for distinct adenine dinucleotide receptors in rat mesenteric arteries. Postjunctional effects of α,ω-polyphosphates in these vessels have been shown to be mediated via nucleotide receptors (Ralevic *et al.*, 1995); vasoconstriction is via P_{2X}-purinoceptors on the smooth muscle; vasodilatation is via P_{2Y}-purinoceptors on endothelial cells (Ralevic & Burnstock, 1988; 1991b).

The results of the present study show that P_1-purinoceptors are implicated in the effects of NAD because of antagonism by 8-*p*SPT. NAD was equipotent with adenosine with respect to pre- and postjunctional effects, suggesting that it acts directly at P_1-purinoceptors. However, potentiation of the effects of NAD by pentostatin indicates that at least part of its actions are mediated by adenosine. Further experiments are required to determine to what extent degradation or induction of adenosine release from postjunctional sources is involved. Breakdown of NAD to adenosine would be due to the enzyme 5'-nucleotidase, located exclusively on the extracellular face of the plasma membrane. Breakdown of adenosine in rat tissues is known to be an extremely rapid process and is potentiated markedly by inhibitors of adenosine deaminase such as pentostatin (Henderson *et al.*, 1977; Padua *et al.*, 1990; Klohs & Kraker, 1992). Burnstock & Hoyle (1985) reported a significant degradation of NAD to adenosine during passage over a preparation of the guinea-pig taenia coli. In that preparation, as in the present study, NAD was found to act at P_1-purinoceptors at least partly following breakdown to adenosine, while the actions of NADP were similar to those of a P_2-purinoceptor agonist (Burnstock & Hoyle, 1985). In the guinea-pig taenia coli, dipyridamole, a purine nucleoside uptake inhibitor, markedly potentiated responses to NAD, confirming an involvement of adenosine (Burnstock & Hoyle, 1985). Dipyridamole could not be used in the present study since it caused direct relaxation of the mesenteric arterial vasculature at operant concentrations. A necessity for NAD to be broken down to adenosine before acting on P_1-purinoceptors has been claimed in human fibroblasts (Bruns, 1980a). On the other hand, compared to the mononucleotides, adenine dinucleotides are known to have relatively long half-lives in plasma and in physiological solu-

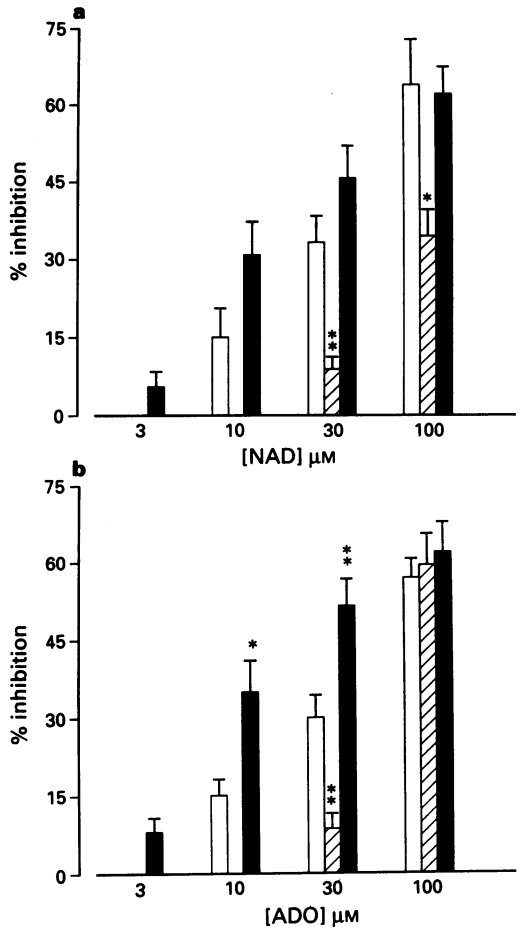

Figure 4 Graphs showing inhibitory effects of (a) nicotinamide adenine dinucleotide (NAD, $n = 4-8$) and (b) adenosine (ADO, $n = 4-8$) on vasoconstriction to electrical field stimulation of the rat mesenteric arterial bed at basal tone. There was no inhibition of sympathetic constrictor responses by NAD and adenosine at 3 μM in the absence of pentostatin and at 10 μM in the presence of 8-*para*-sulphophenyltheophylline (8-*p*SPT). Open columns, NAD or adenosine alone; hatched columns, NAD or adenosine in the presence of 8-*p*SPT (3 μM); solid columns, NAD or adenosine in presence of pentostatin (1 μM).

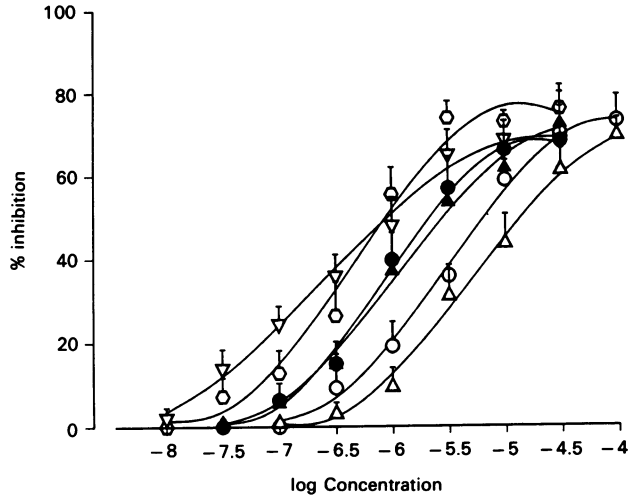

Figure 5 Concentration-dependent inhibition of sensory-motor vasodilatation of the rat mesenteric arterial bed in the presence of: nicotinamide adenine dinucleotide (NAD) alone (▲, $n = 10$); adenosine alone (●, $n = 6$); NAD in the presence of 8-*para*-sulphophenyltheophylline (8-*p*SPT) (3 μM) (△, $n = 6$); adenosine in the presence of 8-*p*SPT (3 μM) (○, $n = 6$); NAD in the presence of pentostatin (1 μM) (▽, $n = 7$); adenosine in the presence of pentostatin (1 μM) (○ $n = 5$).

tion (Luthjie & Ogilvie, 1988; Busshardt *et al.*, 1989; Ogilvie, 1992). Until more comprehensive data on NAD breakdown are available it is not possible to determine to what extent the action of NAD is dependent on the formation versus the release of adenosine in rat mesenteric arteries.

Interestingly, 8-*p*SPT was a more potent antagonist of NAD than adenosine both for postjunctional vasodilatation and for inhibition of sympathetic constriction at 100 μM of these purine compounds. These results suggest that NAD and adenosine are acting on different receptors. One possibility is that NAD may be acting on a distinct dinucleotide receptor, which would still be classified as a P_1-purinoceptor by virtue of antagonism by 8-*p*SPT. It is also possible that NAD and adenosine are acting at different currently recognised subtypes of the P_1-purinoceptor. A greater antagonism by 8-PT of responses to NAD compared to those of adenosine has been reported previously in the guinea-pig taenia coli, where the relative antagonism of NAD was an order of magnitude greater than that of adenosine (Burnstock & Hoyle, 1985; Hoyle & Edwards, 1992). The data from those studies showed an even greater difference in the inhibitory effect of 8-PT on NAD and adenosine than seen with 8-*p*SPT in the present study, supporting the concept of activation of different receptors. Postjunctional actions of NAD contributed significantly to its inhibitory effect on sympathetic constriction, and it is likely that this is also the case for adenosine which was equipotent as a vasodilator. The greater inhibition of NAD-mediated compared to adenosine-mediated sympathetic inhibition by 8-*p*SPT may be a reflection of the greater inhibition by 8-*p*SPT of the postjunctional effects of NAD.

Both NAD and adenosine were relatively weak vasodilators of the rat mesenteric arterial bed and it is likely that their more important extracellular role in rat mesenteric vessels is prejunctional modulation of sympathetic and sensory-motor neurotransmission. The greater potency of NAD and adenosine as inhibitors of sensory-motor compared to sympathetic neurotransmission (10 μM of these agents was approximately threshold for inhibition of sympathetic neurotransmission, but caused maximum inhibition of sensory-motor neurotransmission) may be because higher parameters of stimulation were used to elicit sympathetic vasoconstriction compared to sensory-motor nerve-mediated vasodilatation. This would also explain why a higher concentration of 8-*p*SPT was required to produce a significant attenuation of the inhibitory effects of adenosine and NAD on sympathetic compared to sensory-motor neurotransmission. Sensory-motor vasodilatation of the rat mesenteric arterial bed is mediated by the release of CGRP from capsaicin-sensitive afferents (Kawasaki *et al.*, 1988). Prejunctional modulation of sympathetic and sensory-motor neurotransmission by adenosine is mediated via P_1-purinoceptors of the A_1-subtype (Jackson, 1987; Rubino *et al.*, 1994); however, it is possible that different subtypes of A_1-purinoceptors are present on sympathetic and sensory-motor nerve terminals, or that different prejunctional inhibitory transduction mechanisms are involved.

Within the cell, ADP-ribose may regulate the activities of a number of enzymes, either by covalent attachment to, or by physical association with these enzymes (Hussain *et al.*, 1989). ADP-ribosylation of specific proteins is thought to occur in almost all forms of life and almost all compartments of the cell, and has been implicated in the control of a number of biological events (Hayaishi & Ueda, 1977; Ueda & Hayaishi, 1985). However, the extracellular actions of ADP-ribose, particularly on vascular preparations, have received little attention. This study shows that this molecule can act as

as a vasodilator, possibly at P_2-purinoceptors of the P_{2Y} subtype, but not at P_1-purinoceptors since vasodilatation was not inhibited by 8-*p*SPT. In contrast to rat mesenteric arteries, relaxant effects of ADP-ribose in the guinea-pig taenia coli are mediated by both P_1 (A_2)- and P_{2Y}-purinoceptors (Hoyle & Edwards, 1992). ADP-ribose does not appear to be an agonist at P_{2X}-purinoceptors (it did not cause constriction), in agreement with results obtained in the vas deferens (Fedan *et al.*, 1986; Hoyle & Edwards, 1992). It was not possible to test directly the involvement of P_2-purinoceptors since available compounds with P_{2Y}-purinoceptor antagonist activity (for instance suramin and reactive blue 2) (Burnstock & Warland, 1987; Dunn & Blakeley, 1988) produce a substantial drop in tone of the raised-tone mesenteric arterial preparation at operant concentrations.

The length of the polyphosphate chain and hence its negative charge (each phosphate carries a single negative charge) has been proposed as crucial for postjunctional activity of adenine dinucleotides; four or more phosphates/negative charges are required for vasoconstrictor activity while three or less phosphates/negative charges are required for vasodilator action (Ralevic *et al.*, 1995). According to the criteria established in that study of structure-activity relationships of the adenine dinucleotides (Ralevic *et al.*, 1995), NAD, having a pyrophosphate chain and two negative charges, qualifies as a vasodilator and not a vasoconstrictor, as was indeed found. Further support for the hypothesis that it is the charge carried by the molecule which is crucial for activity, rather than the number of phosphates *per se*, is the fact that ADP-ribose, carrying a maximum of two negative charges, is a vasodilator but not a vasoconstrictor, whereas ADP, having the same number of phosphates but carrying a maximum of three negative charges is a vasoconstrictor via P_{2X}-purinoceptors as well as being a potent vasodilator. However, steric hindrance presented by the ribose moiety may also be implicated in the weaker potency of ADP-ribose as a vasodilator compared to ADP.

Some interesting observations can be made from the structure-activity relationships of ADP, ADP-ribose and NAD, which highlight an important role of the ribose and nicotinamide moieties in determining the pharmacological profile of the molecule. Addition of ribose to ADP abolishes the P_{2X}-purinoceptor activity inherent in ADP and the resulting compound, ADP-ribose, is also less potent than ADP as a vasodilator at P_{2Y}-purinoceptors. The pharmacology of ADP-ribose is also different from that of ATP, from which it differs only in that the terminal phosphate is replaced by a ribose moiety, since ATP is a vasoconstrictor as well as a vasodilator at P_{2X}- and P_{2Y}-purinoceptors respectively. Further addition of nicotinamide, which had no actions *per se*, to ADP-ribose has a dramatic effect on agonist profile since it produces NAD, having P_1-, but not P_2-purinoceptor activity. Hence, moieties which are inert themselves, can crucially determine agonist potency as well as subtype of purine receptor that is activated by the molecule into which they are substituted. Mechanism(s) by which nicotinamide and/or ribose substitutions can produce profound effects on agonist profile may include steric hindrance at the receptor binding site or alterations in the strength and distribution of the negative charge of the pyrophosphate chain.

Vasodilatation by NADP was not mediated via P_1-purinoceptors since it was not antagonized by 8-*p*SPT, and is likely to be via P_2-purinoceptors, as in the guinea-pig taenia coli (Burnstock & Hoyle, 1985). These findings indicate that NAD and NADP act on separate classes of receptors with different mechanisms of action and is consistent with the fact that phosphorylation of the 2'-hydroxyl group is essential for P_1-purinoceptor activation (Londos & Wolff, 1977; Bruns, 1980b). Further, the 2'-phosphate group renders NADP resistant to cleavage by 5'-nucleotidase (Burger & Lowenstein, 1970). P_{2Y}-purinoceptors may be involved since vasodilatation to NADP is dependent on an intact endothelium (Ralevic *et al.*, 1995). An implication of the lack of P_1-purinoceptor activity of NADP is that it is unlikely to be able to modulate prejunctionally sympathetic or sensory-motor neurotransmission.

Nicotinamide mononucleotide was ineffective as a vasodilator, whereas AMP, equivalent to NAD without nicotinamide mononucleotide, is a weak vasodilator of the rat mesenteric arterial bed (unpublished observations), indicating that the purine moiety is important for vasodilator activity. None of the remaining analogues of NAD and NADP had significant postjunctional vasoconstrictor or vasodilator actions, which indicates that structural modifications of these molecules can profoundly affect their pharmacological actions.

In conclusion, these results show that in rat mesenteric arteries NAD can act prejunctionally as an inhibitory modulator of sympathetic and sensory-motor neurotransmission via P_1-purinoceptors, partly via adenosine. It is also a weak postjunctional vasodilator. In contrast, P_1-purinoceptor activity was absent in ADP-ribose (equivalent to NAD without nicotinamide) which was a relatively potent vasodilator, probably at P_2-purinoceptors. Additional studies are warranted to examine whether these molecules, traditionally associated with intracellular events, are released from cells under physiological or pathophysiological conditions to locally modulate vascular tone.

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