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Actions of adenine dinucleotides in the guinea-pig taenia coli: NAD acts indirectly on P_1-purinoceptors; NADP acts like a P_2-purinoceptor agonist

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1 The actions of the adenine dinucleotides β-nicotinamide adenine dinucleotide (NAD) and β-nicotinamide adenine dinucleotide phosphate (NADP) were examined on the carbachol-contracted taenia coli of the guinea-pig.

2 Both were capable of inducing full relaxations in a concentration-dependent manner; NADP was 21.4 times more effective than NAD at EC_{50}; the threshold for NADP was approximately 0.1 μM and for NAD approximately 1.0 μM.

3 The P_1-purinoceptor antagonist, 8-phenyltheophylline (10 μM), produced a large parallel rightward shift in the NAD concentration-response curve; in contrast it produced a small parallel leftward shift in the NADP concentration-response curve.

4 Dipyridamole (0.2 μM), a purine nucleoside uptake inhibitor, markedly potentiated responses to NAD and slightly potentiated NADP. 8-Phenyltheophylline antagonized the dipyridamole potentiation of both NAD and NADP.

5 By use of high performance liquid chromatography it was shown that the action of NAD involves a breakdown to adenosine.

6 Apamin, a K^+ channel blocker, which antagonizes P_2-purinoceptor activation in the intestine, abolished responses to NADP but not to NAD.

7 The α- and β-adrenoceptor antagonists, phentolamine (1 μM) and propranolol (1 μM), did not affect responses to NAD or NADP.

8 Tetrodotoxin, a neurotoxin, did not abolish responses to either NAD or NADP.

9 It is concluded that NAD acts largely indirectly as a P_1-purinoceptor agonist following its breakdown to adenosine by ectoenzymes, while NADP acts in a similar manner to a P_2-purinoceptor agonist.

Introduction

Purine nucleosides and nucleotides have potent actions on many tissues (Drury & Szent-Györgyi, 1929; Gaddum & Holtz, 1933; Green & Stoner, 1950; Burnstock, 1972). Subclasses of receptors for these compounds were proposed and on the basis of differential rank order of agonist potency, differential antagonism and differential qualitative responses, the receptors were termed P_1- and P_2-purinoceptors (Burnstock, 1978).

Purine dinucleotides also have potent actions on diverse tissues, for example: β-nicotinamide adenine dinucleotide (NAD) and β-nicotinamide adenine dinucleotide phosphate (NADP) and their reduced forms induce hyperpolarization of the smooth muscle membrane of the guinea-pig taenia coli (Romanenko, 1980; Romanenko *et al.*, 1980) as does flavine adenine dinucleotide (FAD) (Romanenko *et al.*, 1981); NAD, NADP and FAD mimic the inhibitory effect of adenosine on excitatory synaptic transmission in the rat vas deferens (Stone, 1981); NAD and NADP have inhibitory effects on the slow action potentials of guinea-pig atrial muscle (Schrader *et al.*, 1975); NAD depresses synaptic activity in the rat hippocampal dentate gyrus (Richards *et al.*, 1983a, b). Characteristics of NAD-binding to rat brain synaptic membranes have been evaluated (Khalmuradov *et al.*, 1983) and

NAD binding sites have been demonstrated autoradiographically in many rat brain structures (Candy *et al.*, 1984).

In the present experiments the actions of NAD and NADP have been studied on the carbachol-contracted guinea-pig taenia coli. 8-Phenyltheophylline (8-PT) which is a P_1-purinoceptor antagonist (Griffith *et al.*, 1981), apamin, which antagonizes P_2-purinoceptor activation in this preparation (Vladimirova & Shuba, 1978) and dipyridamole, a purine nucleoside uptake inhibitor (Kolassa *et al.*, 1970), were tested on NAD and NADP action. The aim of these experiments was to determine whether or not NAD and NADP acted on different classes of receptors and whether or not they acted with characteristics of purinoceptor agonists.

Methods

Guinea-pigs of either sex (300–450 g) were killed by a blow to the head and exsanguination. The abdomen was opened, the ventral and medial taenia coli were dissected free from the caecum together with the underlying myenteric plexus. Segments 1–2 cm long were suspended in 10 ml organ-baths containing a saline solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 7.7 (Bülbring, 1953). The organ-baths and reservoirs of saline solution were maintained at 36–37°C and constantly gassed with 95% O_2/5% CO_2.

The strips of muscle were initially stretched to 0.5–1 g tension. Recordings were made through Grass FTOC3 or Dynamometer UF1 isometric transducers output to a Grass 79D polygraph. Preparations were left to equilibrate for at least 45 min before any drugs were added. Carbachol (50 nM) was used to induce a sustained submaximal contraction upon which the relaxant effects of the test drugs were demonstrable. After maximal relaxation due to applied drug had been observed, the organ-bath was washed twice with fresh saline and 10 min were allowed before the next addition of carbachol.

NAD and NADP were added to the organ bath to produce final concentrations in multiples of 1 and 3 of decades of concentration units. Maximum responses to NAD and NADP were 100% relaxations. Concentration-response curves were constructed in the 20%–80% response range by determining from individual curves the concentrations required to give the responses of 20% and at increments of 10% to 80%. The mean ± s.e.mean was calculated and plotted to give the final, summed, concentration-response curve for each group.

When dipyridamole or 8-PT was used, the agent was added to the stock of Krebs solution and preparations were allowed to equilibrate for 30 min. Apamin was added directly to the organ bath and allowed 10–15 min to equilibrate.

The slope for individual concentration-response curves was calculated from the regression of % maximal response on log (agonist concentration) and the mean ± 95% confidence limits was determined for each group. Values of pD_2 were calculated from the negative logarithm of the EC_{50}.

NAD and NADP were tested against the α- and β-adrenoceptor antagonists phentolamine and propranolol. NAD, NADP and noradrenaline were added to the organ bath at concentrations from which the EC_{50} could be determined before and after 30 min incubation with phentolamine (1 μM) and propranolol (1 μM).

High performance liquid chromatography (h.p.l.c.) was used to detect adenosine in a series of experiments in which NAD was superfused over guinea-pig taenia coli. Four segments of taenia coli approximately 3 cm long were tied together at their ends, in parallel. One end of this bundle was attached to a rigid support and the other end to a Grass FTOC3 force-displacement transducer, which allowed isometric recording of mechanical activity. Dipyridamole (0.2 μM) was routinely included in the Krebs solution for these experiments. The preparation was superfused at a rate of 1 ml min^{-1} at 36 ± 0.5°C. After equilibrating for 1 h the superfusate was changed to one including carbachol (50 nM) in order to contract the taeniae to a standard tone. During a steady state of contracture (after approximately 2 min) the superfusate was changed to one which additionally contained NAD (10,100 or 1000 μM). During the maximal relaxation induced by NAD, a 1 ml sample of superfusate was collected. The sample was then frozen and assayed by h.p.l.c. three days later. Control samples of all superfusates were collected after being passed over the tissue as well as samples that had not been passed over the tissue. Chromatography was carried out on 100 μl samples using a μ-Bondapak reverse phase C18 column (Waters) and a u.v. detector equipped with a 254 nm filter. The mobile solvent consisted of 0.05 M ammonium phosphate buffer (pH 5.9) containing 8% (v/v) methanol. Identification and quantification of adenosine in the samples was achieved by comparison of the elution times and peak heights with adenosine standards of known concentrations. The minimum concentration of adenosine that was detectable using this system was 50 nM. The adenosine elution time was approximately 8.5 min and the adenosine was well separated from NAD which eluted much earlier at approximately 2.5 min.

Statistical analyses used the appropriate Student's *t* test, paired or unpaired, taking the 5% level of significance.

Drugs used

β-Nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide phosphate (NADP), tetrodotoxin (TTX), carbamyl choline (carbachol) were all from Sigma. Apamin was obtained from Serva, dipyridamole (Persantin) from Boehringer Ingelheim, phentolamine (Rogitine) from CIBA, propranolol (Inderal) from ICI and 8-phenyltheophylline (8-PT) from Calbiochem. 8-PT was dissolved in 80% v/v methanol/20% molar NaOH to produce a stock of 10^{-2} M; subsequent dilutions were aqueous. All other drugs were made up in aqueous dilutions to produce stock solutions such that additions to the 10 ml organ-bath were 30 μl or 100 μl.

Results

NAD

Results for NAD are summarized in Figure 1 and Table 1. NAD (1 μM – 1000 μM) produced concentration-dependent relaxations in the carbachol-contracted taenia coli with a pD_2 of 4.18 ± 0.110 ($n = 21$).

8-PT (10 μM) produced a parallel rightward shift in the NAD concentration-response curve (Figure 1, Table 1) reducing the NAD pD_2 value from 4.29 ± 0.170 to 3.04 ± 0.106 ($n = 11$) ($P < 0.001$, paired t test). The antagonism was so potent that the EC_{50} for NAD in the presence of 8-PT (10 μM) was estimated by extrapolation of the concentration-response curves.

Dipyridamole (0.2 μM) produced a non-parallel leftward shift in the NAD concentration-response curve and raised the NAD pD_2 value from 3.92 ± 0.160 to 5.25 ± 0.113 ($n = 7$) ($P < 0.001$, paired t test) (Figure 1, Table 1).

In the presence of dipyridamole (0.2 μM), 8-PT (10 μM) produced a parallel rightward shift in the NAD concentration-response curve obtained in the presence of dipyridamole alone, reducing the pD_2 value from 5.41 ± 0.189 to 3.94 ± 0.073 ($n = 6$) ($P < 0.001$, paired t test).

Apamin (0.03 μM), in the presence of dipyridamole (0.2 μM), did not produce a significant change in either the slope or pD_2 of the NAD concentration-response curves, which was 5.27 ± 0.195 for controls and 5.33 ± 0.159 in the presence of apamin ($n = 7$) ($P > 0.05$, paired t test).

NADP

Results for NADP are summarized in Figure 2 and Table 1. NADP (0.1 μM – 100 μM) produced a concentration-dependent relaxation of the carbachol-contracted taenia coli (Figure 2) with a pD_2 value of 5.51 ± 0.062 ($n = 25$).

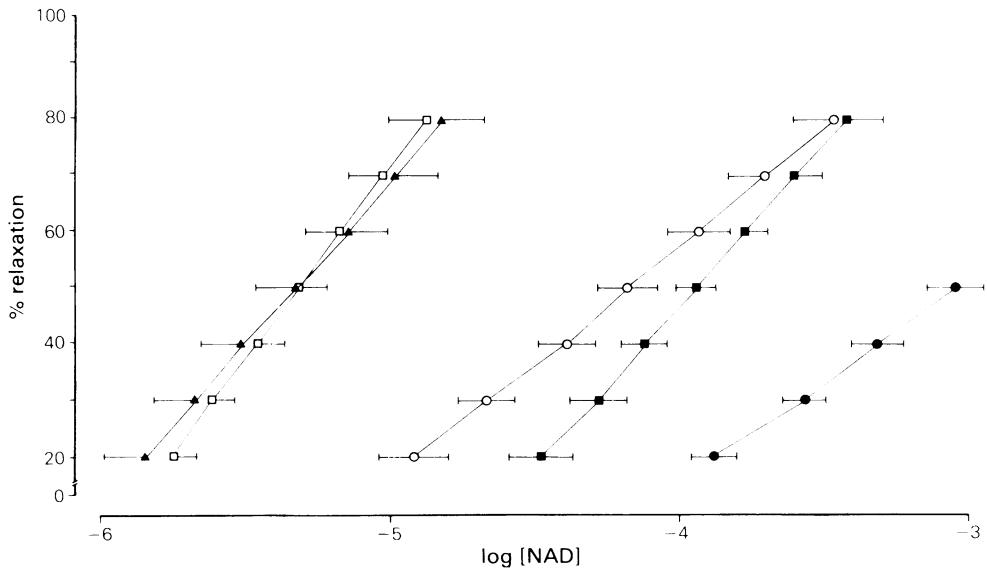

Figure 1 β-Nicotinamide adenine dinucleotide (NAD) concentration-response curves in carbachol-contracted guinea-pig taenia coli. Points represent the mean and horizontal lines s.e.mean. NAD (○, $n = 21$); in the presence of 8-phenyltheophylline (8-PT, 50 μM) (●, $n = 11$); in the presence of dipyridamole (0.2 μM) (□, $n = 13$); in the presence of dipyridamole (0.2 μM) and 8-PT (50 μM) (■, $n = 6$) and in the presence of diyridamole (0.2 μM) and apamin (0.03 μM) (▲, $n = 7$).

Table 1 Carbachol-contracted guinea-pig taenia coli: pD_2 values and slopes of concentration-response curves for NAD and NADP and in presence of 8-phenyltheophylline (10 μM, 8-PT), dipyridamole (0.2 μM, Dip), or apamin (0.03 μM, Apa)

	*pD2 ± s.e. (n)**	Slope ± 95% c.l.+	*Relative antagonism or potentiation***
NAD	4.18 ± 0.110 (21)	45.6 ± 7.21	
NAD + 8-PT	3.04 ± 0.106 (11)	41.8 ± 10.88	−17.8
NAD + Dip	5.32 ± 0.104 (13)°	74.4 ± 14.11	+21.4
NAD + Dip + 8-PT	3.94 ± 0.073 (6)	70.3 ± 33.54	−29.5
NAD + Dip + Apa	5.33 ± 0.139 (7)°	62.8 ± 17.02	1.0
NADP	5.51 ± 0.062 (25)++	76.7 ± 10.63	
NADP + 8-PT	5.68 ± 0.100 (12)	77.8 ± 12.35	+ 1.8
NADP + Dip	5.81 ± 0.056 (13)++	90.7 ± 17.94	+ 1.6
NADP + Dip + 8-PT	5.45 ± 0.104 (6)	78.3 ± 39.13	− 3.0
NADP + Apa	– (6)	–	

* Paired statistical analysis is discussed in the text
** + = potentiation, − = antagonism
° Not significantly different, or pD_2 values are significantly different from each other ($P < 0.05$, unpaired t tests)
+ Statistical differences in slopes are discussed in the text
++ $P < 0.05$, unpaired t test

8-PT (10 μM) did not antagonize NADP but did produce a small, parallel, leftward shift in the NADP concentration-response curve, raising the pD_2 value from 5.43 ± 0.105 to 5.68 ± 0.100 ($n = 12$) ($P < 0.05$, paired t test).

Dipyridamole (0.2 μM) produced a parallel leftward shift in the NADP concentration-response curve, raising the NADP pD_2 value from 5.52 ± 0.034 to 5.73 ± 0.086 ($n = 7$) ($P < 0.02$, paired t test).

In the presence of dipyridamole (0.2 μM), 8-PT (10 μM) produced a parallel rightward shift in the NADP concentration-response curve, reducing the NADP pD_2 value from 5.92 ± 0.050 for the control group in the presence of dipyridamole to 5.45 ± 0.104 ($n = 6$) ($P < 0.005$, paired t test).

Apamin (0.03 μM) abolished the relaxant effect of NADP (0.1 μM–300 μM) which included abolishing responses to NADP at concentrations up to 100 times greater than those which produced 100% relaxation in control preparations. Dipyridamole (0.2 μM) did not affect responses to NADP (0.1–100 μM) in the presence of apamin.

The neurotoxin, tetrodotoxin (TTX) (1 μM), did not abolish responses to NADP or NAD ($n = 2$).

Based on pD_2 values, the potency of NADP relative to that of NAD was 21.4 times greater; in the presence of dipyridamole (0.2 μM) this was reduced to 3.1; in the presence of 8-PT it was raised to 436.5; and in the presence of both agents the relative potency was 32.4.

Effect of adrenoceptor antagonists

In the presence of phentolamine (1 μM) and propranolol (1 μM) the pD_2 for noradrenaline was reduced from 7.39 ± 0.024 (mean ± s.e. $n = 4$) to 5.64 ± 0.078 ($P < 0.005$, paired t test) while for NAD the pD_2 was not significantly altered, from 4.59 ± 0.117 (4) to 4.75 ± 0.245 and similarly, the pD_2 for NADP was not significantly changed from 5.93 ± 0.003 (4) to 5.91 ± 0.013.

Breakdown of NAD to adenosine

Adenosine was undetected (i.e. < 50 nM) both in all control samples which had not been passed over the tissue and in all NAD-free samples which had been passed over the tissue.

Adenosine was detected in all samples containing

NAD which had been passed over the tissue (Table 2). These results imply that NAD is broken down to adenosine or that NAD induces release of adenosine from the tissue and supports the results from antagonism by 8-PT and potentiation by dipyridamole, showing that the dominant mediator of NAD-induced relaxations is adenosine.

Although the concentrations of adenosine measured in superfusates were too low to account for the simultaneous relaxations, the concentration of adenosine in the biophase would be much higher since the superfusate sample must also contain solution which did not directly contact the tissues.

Table 2 Conversion of NAD to adenosine in the guinea-pig taenia coli

NAD (μM)	*Adenosine* (nM) mean ± s.e. (*n*)	*Relaxation* (%)
10	150 ± 5.8 (3)	54.8 ± 0.33
100	370 ± 63.9 (3)	100
1000	440 ± 18.6 (3)	100

Adenosine was not detected (<50 nM) in any control sample, all adenosine detected was as a result of NAD passing over the tissue

Discussion

The results show that 8-PT is a potent antagonist of NAD, but not NADP, and that the relative antagonisms of the NAD EC_{50} in the absence or presence of dipyridamole are an order of magnitude greater than that of 1.6 reported for adenosine in the same preparation (Burnstock *et al.*, 1984). The lack of antagonism of NADP by 8-PT indicates that the NADP response is not due to activation of a P_1-purinoceptor. The small, but significant, potentiation may be due to a non-specific effect of 8-PT, evident in that the carbachol concentration had to be raised from 50 nM to 55 or 60 nM in order to produce control levels of contracture after equilibration in the presence of 8-PT.

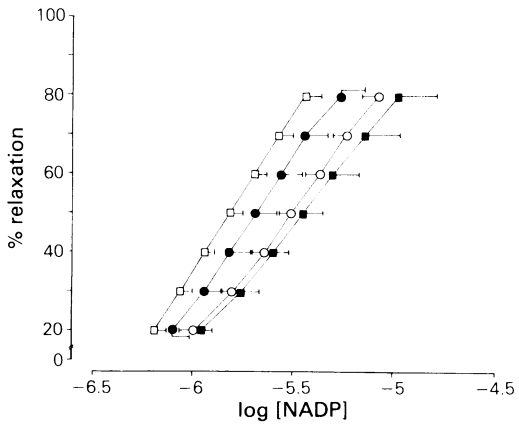

Figure 2 β-Nicotinamide adenine dinucleotide phosphate (NADP) concentration-response curves in carbachol-contracted guinea-pig taenia coli. Points represent the mean and horizontal lines s.e.mean. NADP (○, $n = 25$); in the presence of 8-phenyltheophylline (8-PT, 50 μM) (●, $n = 12$); in the presence of dipyridamole (0.2 μM) (□, $n = 13$) and in the presence of dipyridamole (0.2 μM) and 8-PT (50 μM) (■, $n = 6$). Apamin (0.03 μM) abolished responses of NADP (up to 300 μM).

Dipyridamole (0.2 μM) enhanced the action of NAD with a relative potentiation similar to that reported for adenosine, yet the slope of the NAD concentration-response curve was significantly steepened in the presence of dipyridamole, an effect not seen with adenosine (Satchell & Burnstock, 1975). The potentiation of NAD by dipyridamole indicates that NAD was degraded to an adenosine moiety which would form part of the NAD response. This increase in the slope of the response curve could occur if NAD had a direct action on putative dinucleotide receptors in addition to having had an indirect action on P_1-purinoceptors via breakdown to adenosine: in the presence of dipyridamole the action of adenosine is potentiated and the summed action of NAD and adenosine could yield a steeper concentration-response curve (see Waud, 1975). The breakdown of NAD to adenosine would be due to the enzyme 5′-nucleotidase which is located exclusively on the extracellular face of the plasma membrane of various mammalian tissues including smooth muscle (De Pierre & Karnovsky, 1974). The necessity for NAD to be broken down before acting on a P_1-purinoceptor has been demonstrated in human fibroblasts (Bruns, 1980a) and that appears to be the case here also, since adenosine was detected in superfusates passed over the taenia coli as a result of NAD being present in the Krebs solution.

NADP was potentiated slightly by dipyridamole and this potentiation was totally inhibited by 8-PT. NADP is resistant to cleavage by 5′-nucleotidase because of its 2′-phosphate group (Burger & Lowenstein, 1970); the 2′-phosphate group has further significance since a 2′-hydroxyl group is obligatory for P_1-purinoceptor activation (Londos & Wolff, 1977; Bruns, 1980b). The potentiation of NADP by dipyridamole was sensitive to 8-PT and therefore is unlikely to be due to an inhibition of NADP uptake. NADP may induce release of purines from the muscle itself; the effect of such an action could be potentiated by dipyridamole but if purine release contributed to the NADP relaxant response, then antagonism or at least no change in the NADP responses would be the

expected effect of 8-PT. Dinucleotides have been shown to release purines from some tissues (Stone, 1981).

The lack of effect of apamin against NAD in contrast to its very potent antagonism of NADP is further evidence for NAD and NADP having different mechanisms of action. Although apamin antagonizes P_2-purinoceptor agonists (Vladimirova & Shuba, 1978) it is not specific since it blocks K^+ channels (Banks *et al.*, 1979; Maas & Den Hertog, 1979; Maas *et al.*, 1980), and, importantly, apamin does not antagonize adenosine activity in the guinea-pig taenia coli (Brown & Burnstock, 1981). The photoaffinity analogue of ATP, $ANAPP_3$, and tachyphylaxis by α, β-methylene ATP have been used effectively as P_2-purinoceptor antagonists. However, while $ANAPP_3$ antagonizes excitatory responses to ATP in the guinea-pig vas deferens (although not competitively) it does not antagonize ATP-induced relaxations in guinea-pig fundic strips (Frew & Lundy, 1982): in the guinea-pig taenia coli $ANAPP_3$ does not appear to be specific for the P_2-purinoceptor since AMP as well as ATP is antagonized; further, $ANAPP_3$ does not antagonize the relaxant response to ATP at concentrations of ATP greater than 10^{-4}M (Westfall *et al.*, 1982). Similarly, α, β-methylene ATP desensitizes excitatory P_2-purinoceptors in the guinea-pig urinary bladder (Kasakov & Burnstock, 1983) and vas deferens (Meldrum & Burnstock, 1983), but the inhibitory P_2-purinoceptors of the guinea-pig taenia coli are not susceptible to α, β-methylene ATP tachyphylaxis (Burnstock, unpublished observations). Therefore, at present there is no suitable specific ATP antagonist against which NADP can be tested in this preparation.

These results show that NAD and NADP act on the smooth muscle via separate classes of non-adrenoceptors with different mechanisms of action. NADP is not a P_1-purinoceptor agonist and although it is effectively antagonized by apamin, this is not evidence to classify it as a P_2-purinoceptor agonist. On the other hand, NAD acts on P_1-purinoceptors probably largely indirectly, following its conversion to adenosine.

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