Br. J. Pharmac. (1975), 55, 439-445

ADENOSINE AND DIPYRIDAMOLE ACTIONS AND INTERACTIONS ON ISOLATED CORONARY ARTERY STRIPS OF CATTLE

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1 The actions and interactions of adenosine and dipyridamole were investigated on isolated strips of coronary arteries of beef cattle. It was found that small diameter arteries (about 0.5-1.0 mm o.d.), raised to a moderate level of tone with potassium, responded with relaxation to low concentrations of adenosine.

2 Dipyridamole, over a broad concentration range (6.0×10^{-8}-2.0×10^{-5}M), enhanced these responses, shifting the adenosine concentration-response curve (3.7×10^{-8}-1.1×10^{-4}M) considerably to the left. In contrast, inhibitory concentration-response curves to sodium nitrite and to noradrenaline were not materially altered by dipyridamole.

3 Studies of the uptake of [^{3}H]-adenosine revealed a rapid uptake of the nucleoside by coronary artery strips, which was inhibited by dipyridamole (6.0×10^{-8}-2.0×10^{-5}M); but this may not be sufficient to account fully for the observed sensitization.

4 It is concluded that the regulation of adenosine responses and the action of dipyridamole in the heart involve a more direct association with coronary vascular tissue than has been previously appreciated.

Introduction

The proposal has been made that adenosine, a product of adenine nucleotide utilization in cardiac tissue with vasodilator activity, is a regulator of coronary artery diameter (Berne, 1963). Major evidence in support of this hypothesis is the finding of elevated levels of adenosine in the heart, pericardial fluid and venous effluent during hypoxic conditions and, more recently, the observed enhancement of reactive hyperaemia in the presence of dipyridamole, an agent known to sensitize responses to exogenous adenosine (Miura, Tominaga & Hashimoto, 1967; Bittar & Pauly, 1971; Juhran, Voss, Dietmann & Schaumann, 1971; Parratt & Wadsworth, 1972a). Dipyridamole blocks the uptake of adenosine into red blood cells and into heart muscle as well, and the increased amounts of adenosine reaching the coronary vessels, in the presence of this inhibitor, are presumed to account for the magnified response. In addition, inhibition of adenosine inactivation has been invoked by some to explain, at least partially, the direct dilator effects of dipyridamole (Stafford, 1966; Nott, 1970).

The possibility that the uptake of adenosine into coronary vascular tissue itself, is a factor regulating responses to this agent has not been explored, nor has the possibility of a direct interaction between dipyridamole and adenosine at the level of the coronary vasculature been examined. In the present experiments, in which an *in vitro* preparation of bovine coronary artery strips was used to eliminate extrinsic factors, it was found that [^{3}H]-adenosine is taken up by vascular tissue and that dipyridamole inhibits this uptake and also directly and specifically sensitizes coronary artery responses to adenosine.

Methods

Hearts were removed within 10 min of slaughter of the cattle and immersed in oxygenated Krebs solution and transported to the laboratory. The total elapsed time was approximately 20 minutes. The left circumflex and descending coronary arteries or branches of the left descending coronary artery were dissected out, cleaned of visible fat and adherent tissue and cut into spiral strips of about 23 x 2.5 mm and 23 x 1.5 mm, respectively. The strips were suspended under 2 g

tension (or in the case of the branch vessels 1 g tension) in 15 ml muscle chambers at 37° C containing Krebs-Henseleit solution of the following composition (mM): NaCl 115.3, KCl 4.6, $CaCl_2$ 1.8, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, glucose 7.8; to this medium disodium ethylenediamine tetraacetic acid (EDTA) (0.03 mM) was added to retard heavy metal catalyzed oxidation of catecholamines. A 60 min period was allowed for equilibration before eliciting drug responses. Isotonic contractions were recorded by means of frontal writing levers on a slowly moving kymograph drum (about 1.8 mm/min) with a lever magnification of 6.8-fold as described previously (Kalsner, 1974a).

All drug concentrations are expressed in terms of molarity. Potassium chloride, sodium nitrite, adenosine and dipyridamole were dissolved to the appropriate concentration in distilled, demineralized water. (−)-Noradrenaline was diluted to the desired concentration in 0.9% w/v NaCl solution containing 0.01 N HCl. Control and treated strips for each experimental protocol were taken from the same preparations. This was made necessary by the variation in the absolute response magnitudes to adenosine of strips from different preparations. Strips were used to obtain only one dose-response curve to a given agonist and then discarded.

[^{3}H]-adenosine uptake protocol

Adenosine [2,8-^{3}H] (30 mCi/μM) (New England Nuclear) was diluted to a stock concentration of 30.0 μCi/ml (1 μM) in water and stored frozen in 1 ml aliquots under nitrogen gas. Aliquots were thawed only once, immediately prior to use. For uptake studies strips of coronary artery were prepared as described above and pre-incubated individually at 37° C for 30 min in test tubes containing 10 ml Krebs solution. Aeration with 95% O_2 and 5% CO_2 was maintained throughout. The strips were then incubated for 10 min with [^{3}H]-adenosine (300 nCi/ml). The final concentration of [^{3}H]-adenosine was 1×10^{-8} M or 5×10^{-6} M; the latter achieved by dilution with non-radioactive adenosine. Dipyridamole when used, was added 15 min prior to incubation with the tritiated adenosine.

After incubation with [^{3}H]-adenosine for 10 min, the strips were blotted gently, weighed, chopped and placed in scintillation vials containing 2 ml of solubilizer (protosol, New England Nuclear) and 0.2 ml of distilled water to promote dissolution of the tissue. The vials were then maintained overnight in a water bath at 50° C to speed solubilization. Fifteen ml of scintillation solution (4 g of PPO and 50 mg of POPOP per litre of toluene) was added to each vial and the samples counted to a 2% error in a Beckman LS-150 system using automatic external standardization, to determine efficiency. Uptake of radioactivity is expressed as d/min per g of tissue after correction for extracellular space, assumed to be 0.35 ml/g tissue (Somlyo & Somlyo, 1968). The concentration of adenosine in the bathing medium was confirmed for each experiment by counting the radioactivity in 0.1 ml aliquots. The tissue to medium ratio was calculated as d/min per g of tissue (without extracellular space correction) divided by the d/min per ml of bathing medium.

Data analysis

Mean values of all data are shown with their standard errors and were compared by Student's *t*-test for unpaired data. Differences with *P* values of 0.05 or less were considered significant. Changes in response sensitivity at the ED_{10mm} level are expressed as the ratio of geometric mean values (Fleming, Westfall, De La Lande & Jellett, 1972). Each ED_{10mm} was converted to its log and the mean for each group recorded. The antilog of each mean log is presented as the geometric mean. Due to the lack of parallelism of certain of the dose-response curves, comparisons at other response levels would yield somewhat different values (Kalsner, 1947b), and this is evident from an examination of Figures 1 and 2.

Results

Responses of coronary arteries to adenosine

In preliminary experiments it was found that the large epicardial coronary arteries (o.d. 1-5 mm) did not respond to adenosine with any material relaxation when tested over a wide concentration range. This is in agreement with the finding of Schnaar & Sparks (1972) using dog vessels of similar dimensions. For this reason, smaller branch vessels of the left descending coronary artery (approximately 0.5-1.0 mm o.d.) were used for the present work. Artery strips first were constricted with potassium chloride (30 mM) to provide sufficient tone to obtain inhibitory dose-response curves to adenosine. After the contractions to potassium achieved a stable plateau level of response, e.g., a mean of 91.6 ± 7.6 mm in a typical set of 12 control strips (Figure 1a), cumulatively increasing concentrations of adenosine were added to the muscle chambers. Sufficient time was allowed between additions of the nucleoside for responses to plateau fully. The mean inhibitory concentration-response curves to adenosine (3.7×10^{-8}-1.1×10^{-4} M), obtained in four separate groups of control strips are presented

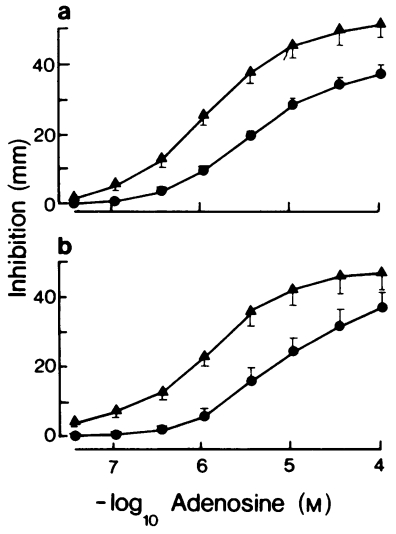

Figure 1 Effects of dipyridamole on the cumulative concentration-response curves to adenosine in coronary artery strips. Controls (•), dipyridamole-treated (▲). Concentrations of dipyridamole are 2.0×10^{-5} M and 6.0×10^{-6} M in (a) and (b). Number of control and treated values are 12 and 13 in (a) and 5 and 5 in (b). Mean values are shown; vertical lines show s.e. mean. Details are provided in text.

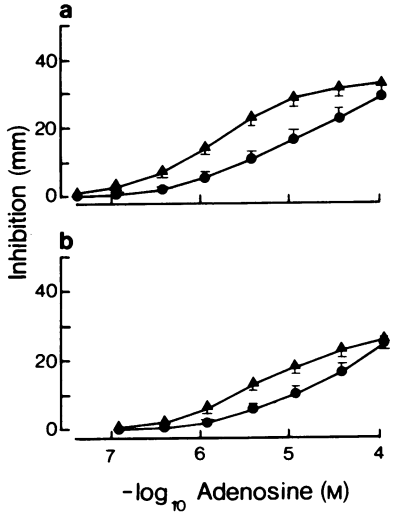

Figure 2 Effects of low concentrations of dipyridamole on the adenosine concentration-response curve. Controls (•), dipyridamole-treated (▲). Concentrations of dipyridamole are 6.0×10^{-7} and 6.0×10^{-8} M in (a) and (b). Number of control and treated values are 11 and 12 in (a) and 6 and 11 in (b). Mean values are shown; vertical lines show s.e. mean. Details are given in text.

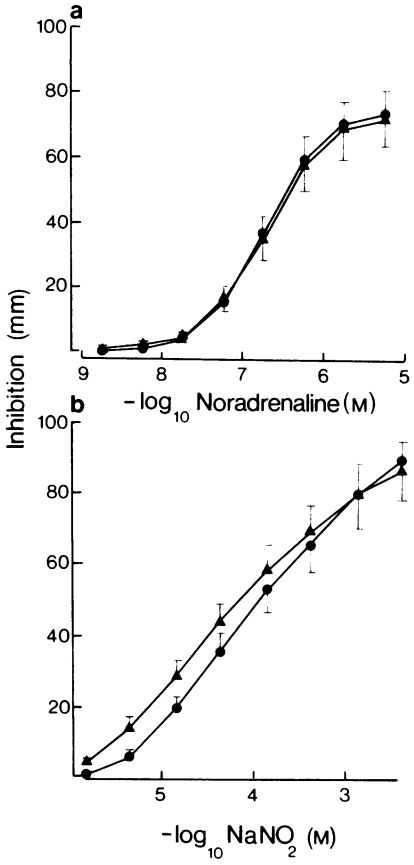

Figure 3 Effects of dipyridamole on the cumulative concentration-response curves to noradrenaline and sodium nitrite in coronary artery strips. Controls (•), dipyridamole-treated (▲) (2.0×10^{-5} M). Number of control and treated values are 11 and 13 in (a) and 11 and 11 in (b). Mean values are shown; vertical lines show s.e. mean. Details in text.

in Figures 1 and 2. The capacity of the strips to relax further is not usually exhausted after the response to adenosine 1.1×10^{-4} M is fully achieved, since sodium nitrite, a non-competitive inhibitor of muscle tone, additionally relaxed them to their pre-potassium baselines. However, concentrations of adenosine above 1.1×10^{-4} M were not routinely employed in the present experiments since an abrupt and apparently non-specific depression of tone was evident at the high concentration of 3.7×10^{-4} M.

Effects of dipyridamole on adenosine responses

To explore the effects of dipyridamole on the adenosine concentration-response curve, coronary artery strips taken from the same preparations as

controls were contracted by potassium and exposed to dipyridamole (6.0×10^{-6} or 2.0×10^{-5} M) and 15 min later, without washout, responses to adenosine were obtained (Figure 1a and b). Dipyridamole, itself, in these high concentrations occasionally exerted a direct depressant effect on coronary artery tone but it was only a small percentage of the total potassium contraction amplitude; e.g., a mean inhibition of $6.1 \pm 1.8\%$ in 13 strips after dipyridamole (2.0×10^{-5} M). The adenosine concentration-response curves were shifted considerably to the left by both concentrations of dipyridamole (Figure 1a and b). The increase in sensitivity measured as the ratio of geometric means (control vs treated) at the ED_{10mm} response level was 9.8 and 7.2 after 6.0×10^{-6} and 2.0×10^{-5} M, respectively. These values did not differ significantly from each other.

In separate experiments again with control and treated strips taken from the same preparations to minimize variability, lower concentrations of dipyridamole (6.0×10^{-7} and 6.0×10^{-8} M) were also found to enhance significantly responses to adenosine (Figure 2a and b). The ratio of geometric means at the ED_{10mm} level (control/treated) was 6.0 and 3.7 respectively. In other experiments it was determined that a further 10-fold reduction in the concentration of dipyridamole, to 6.0×10^{-9} M, did not alter significantly the shape or position of the adenosine concentration-response curve when compared to matching control strips ($P > 0.2$).

To provide information on the specificity of the enhancing effects of dipyridamole, strips were contracted by potassium, as above, and complete inhibitory concentration-response curves to noradrenaline (1.8×10^{-9} to 6.0×10^{-6} M) and sodium nitrite (1.4×10^{-6} to 1.4×10^{-2} M) were obtained in the presence and absence of dipyridamole (2.0×10^{-5} M). Noradrenaline relaxes bovine coronary artery strips through β-adrenoceptor activation as described previously (Kalsner, 1974a). As indicated in Figure 3(a) and (b) responses to these agonists were not increased materially, even by this high concentration of dipyridamole, although a slight shift to the left of the lower portion of the $NaNO_2$ curve did occur. However, this shift was statistically significant only at the single agonist concentration of 4.3×10^{-6} M. The direct dilator effect of dipyridamole exerted on some preparations, and referred to above, may explain this slight enhancement, confined to the lower portion of the concentration-response curve. Since the catabolism of adenosine leads to the formation of inosine and hypoxanthine, the direct effects of these agents on coronary vascular tone was also studied. Over a wide concentration range (3.7×10^{-8} to 1.1×10^{-4} M and 7.4×10^{-8} to 2.2×10^{-4} M) neither inosine nor hypoxanthine inhibited potassium-induced tone.

Uptake of [^{3}H]-adenosine into coronary arteries

Direct evidence for the uptake of adenosine into coronary artery strips and its inhibition by dipyridamole was obtained by incubation of strips with [^{3}H]-adenosine at 1×10^{-8} and 5×10^{-6} M in the presence and absence of the sensitizing agent,

Table 1 Uptake of [^{3}H]-adenosine into coronary artery strips and the effects thereon of dipyridamole

	No. of strips	*Dipyridamole conc. (M)*	*Uptake (d/min x 10^{-3} per g)*	P value*	*% Inhibition of uptake*
[^{3}H]-Adenosine (1×10^{-8} M)	8	–	3146 ± 435	–	–
	6	6×10^{-9}	3390 ± 516	>0.5	–
	5	6×10^{-8}	1775 ± 210	<0.001	43.6
	7	6×10^{-7}	654 ± 85	<0.001	79.2
	7	6×10^{-6}	534 ± 23	<0.001	83.0
	4	2×10^{-5}	342 ± 16	<0.001	89.1
[^{3}H]-Adenosine (5×10^{-6} M)†	9	–	1048 ± 108	–	–
	10	6×10^{-7}	924 ± 97	>0.3	11.9
	13	6×10^{-6}	736 ± 87	<0.05	29.8
	9	2×10^{-5}	466 ± 122	<0.01	55.6

* Comparisons are with corresponding control group without dipyridamole treatment. † Diluted with non-radioactive adenosine as detailed in methods section. Means are presented with their standard errors. Strips were exposed to [^{3}H]-adenosine in the presence or absence of dipyridamole, and the accumulation of radioactivity after a 10 min incubation period is expressed after correction for extracellular space. Details are provided in text.

over a wide concentration range. With the lower concentration of adenosine, the vascular tissue rapidly accumulated tritium to reach a tissue/medium ratio of 4.3 after a 10 min incubation and this accumulation was strikingly inhibited by dipyridamole (6.0×10^{-8} M-2.0×10^{-5} M) (Table 1). However, in those strips exposed to a higher concentration of [^{3}H]-adenosine, one which produced a moderate response of the vascular strips, the tissue/medium ratio achieved was only 1.2 after 10 min and dipyridamole was much less effective in reducing uptake (Table 1). In other experiments it was found that strips of the left circumflex artery, although unresponsive to adenosine, also accumulated the nucleoside at the low concentration of 1×10^{-8} M, but did so less avidly than smaller, responsive, vessels. Control uptake gave a tissue/medium ratio of 2.3 and this was inhibited by dipyridamole as effectively as in responsive preparations of smaller arteries; a reduction of 69, 75 and 78% after treatment with 6×10^{-8}, 6×10^{-7} and 6×10^{-6} M of the antagonist respectively.

Discussion

Considerable evidence has been advanced to support the hypothesis that adenosine, an endogenously formed metabolite of adenine nucleotides in myocardial cells, functions as a regulating mechanism to adjust coronary vessel diameter, and hence coronary blood flow, to myocardial needs (Rubio, Berne & Katori, 1969; Berne, Rubio, Dobson & Curnish, 1971; Rubio, Berne & Dobson, 1973; Berne & Rubio, 1973). It has been proposed that adenosine is released continuously from myocardial cells into the interstitial fluid where it contacts the resistance vessels, in amounts directly relaxed to cellular metabolic rates (Scott, Daugherty, Dabney & Haddy, 1965; Katori & Berne, 1966; Rubio & Berne, 1969). Important impetus for this hypothesis has come from reports that dipyridamole, an inhibitor of adenosine uptake into erythrocytes and myocardial tissue (Koss, Beisenherz & Maerkisch, 1962; Gerlach & Deuticke, 1963; Bunag, Douglas, Imai & Berne, 1964; Pfleger, Voikmer & Kolassa, 1969; Kolassa, Pfleger & Rummel, 1970; Hokins, 1973), enhances vasodilator responses to exogenous adenosine in perfused heart preparations (Bretschneider, Frank, Bernard, Kochsiek & Scheler, 1959; Stafford, 1966; Moir & Downs, 1972; Sano, Sato & Hashimoto, 1972; Parratt & Wadsworth, 1972b) and the hyperaemic response to coronary artery occlusion, as well (Miura *et al.*, 1967; Bittar & Pauly, 1971; Juhran *et al.*, 1971; Parratt & Wadsworth, 1972a). However, this latter finding is not universally accepted (Kubler, Spieckermann & Bretschneider, 1970) and is interpreted by some to indicate a role for adenosine only in the more severe hypoxic stresses (Parrat & Wadsworth, 1972a; Moir & Downs, 1972).

The present experiments were undertaken to determine if coronary arterial tissue has the capacity to take up extracellular adenosine and to explore the possibility that adenosine and dipyridamole interact directly at the level of the coronary vasculature rather than solely indirectly by an action of the inhibitor at sites remote from the site of action of adenosine. For this reason, preparations of coronary artery tissue suspended *in vitro* in Krebs medium were utilized.

Dipyridamole, (6×10^{-8}-2×10^{-5} M) exerted a direct action on vascular tissue to enhance responses to adenosine and to shift the entire concentration-response curve to the left. This action was specific for adenosine. Responses to noradrenaline and to sodium nitrite were not materially increased by the highest concentration of dipyridamole employed (2×10^{-5} M), providing no support for a previously reported sensitization of noradrenaline dilator responses by this agent, in perfused dog hearts (Hashimoto & Sano, 1968). This confirms the report of Parrat & Wadsworth (1972b) that dipyridamole is ineffective in enhancing noradrenaline dilator responses in intact heart preparations. In addition it was found that coronary vascular tissue has a considerable avidity for adenosine as determined by its uptake of [^{3}H]-adenosine and this accumulation was reduced by dipyridamole. Hopkins (1973) has pointed out that, in cardiac tissue much of the accumulated adenosine is retained intracellularly in the form of nucleotides, and this may be the case with vascular tissue as well.

While no definite statements can be made regarding the relationship between degree of sensitization and percentage inhibition of uptake due to limitations in our knowledge of the topography of the cell surface and specifically the relative locations of receptors and uptake sites, the lack of a close correlation between them is apparent. Responses to adenosine (5×10^{-6} M) were greatly enhanced by dipyridamole (6×10^{-7}-2×10^{-5} M) but uptake was not correspondingly reduced. A 50% and 65% inhibition of uptake is required to explain a 2 and 3-fold sensitization (dose-ratio); and uptake must be reduced by 90% to account for a 10-fold sensitization. It seems likely, therefore, that at least a component of dipyridamole sensitization may involve an action on vascular cells unrelated to inhibition of an uptake mechanism. In this

connection, Hopkins (1973) previously reported that the enhancing action of lidoflazine on the responses of guinea-pig atria to adenosine could not be accounted for simply by its known ability to block myocardial uptake of the nucleoside.

The specific sensitization of vascular responses coupled with the inhibition of adenosine uptake indicates that the action of dipyridamole and 'dipyridamole-like' compounds involves a more intimate association with coronary vascular tissue than has been previously appreciated. Berne has proposed that newly-formed adenosine leaves the heart muscle, enters the interstitial fluid, acts on resistance vessels and then has its action terminated by uptake into erythrocytes and to some extent into cardiac tissue, where it is either degraded to inosine or hypoxanthine, or incorporated into nucleotides via adenosine kinase (Berne, 1963). Dipyridamole would sensitize action by blocking this uptake thus diverting increased amounts of adenosine to the vascular receptors and maintaining them there for longer periods. The present findings warrant an alternative explanation.

The magnitude of the sensitization observed here indicates that effects on vascular cells probably contribute in a major way to the potentiation of adenosine responses observed in the intact heart. Parratt & Wadsworth (1972a, b) have reported that dipyridamole produces a 2-8 fold sensitization of adenosine dilator responses in the intact heart, and it has been shown here that an effect of this magnitude could involve, to a significant extent, an interaction at the level of vascular tissue, the immediate site of adenosine action in the heart.

It is important to note that, although the amount of vascular tissue in the heart is an insignificant proportion of its total bulk, this is not the critical factor in determining the *in vivo* implications of the present finding. If adenosine reaches vascular sites of action in sufficient concentrations to elicit relaxation then the effects of dipyridamole on response magnitude will be similar to those observed in the present experiments; regardless of the absolute amounts of adenosine involved. In addition, regulation of coronary dilator responses to adenosine by other endogenous influences *in vivo*, also can be envisaged to occur through alterations in its rate of uptake into vascular cells. The present data therefore provide a new aspect for consideration in the currently controversial picture of the role of adenosine in the dynamics of coronary blood flow regulation.

The author thanks Mr Mohamad Suleiman for excellent technical assistance. This investigation was supported by grants from the Ontario Heart Foundation and the Medical Research Council of Canada.

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(Received June 24, 1975.)