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THE POTENTIATION OF THE ACTION OF ADENOSINE ON THE GUINEA-PIG HEART

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Abstract—Experiments were performed to demonstrate whether dipyridamole, hexobendine or lidoflazine potentiates the action of adenosine by inhibiting its uptake. Most of the [8-^{14}C]adenosine taken up into the guinea-pig isolated heart was rapidly incorporated into ATP and retained in this form. Dipyridamole or hexobendine (5×10^{-5} M) inhibited uptake and phosphorylation of adenosine. Assessment of the degree of potentiation of the action of adenosine in relation to the degree of inhibition of uptake of adenosine showed that dipyridamole and hexobendine behaved similarly, and block of uptake could explain potentiation of adenosines' action by these drugs. However, potentiation by lidoflazine could not be explained by either block of adenosine uptake or inhibition of adenosine deaminase.

ADENOSINE reduces the rate and force of contraction of atria, and dilates coronary vessels. Both these effects are augmented by dipyridamole.[1,2] The mechanism underlying this potentiation has been suggested to be inhibition of adenosine deaminase[3,4] or inhibition of the intracellular uptake of adenosine.[5–7] Two other drugs that powerfully dilate coronary vessels, hexobendine and lidoflazine, also potentiate the action of adenosine and limit its uptake by the heart.[5,8,9]

The following comparison of the potencies of dipyridamole, hexobendine and lidoflazine, both in potentiating the action of adenosine and in inhibiting its uptake by the heart, was designed to show whether there is a causal relationship.

METHODS

Animals. Guinea-pigs used in these studies weighed 380–420 g.

Uptake of [8-^{14}C]*adenosine.* Each guinea-pig was stunned, its heart removed and the coronary vessels perfused through an aortic cannula. The perfusion fluid was McEwen's[10] solution, warmed to 32° and saturated with 5% CO_2 in O_2. The flow rate was 6–7 ml/min. After 5–10 min, [8-^{14}C]adenosine (2·7 nmoles, 75 nCi) was injected at 14 sec intervals in five, 100 μl-lots. One min later, after most of the radioactivity had disappeared from the perfusate, the heart was removed from the cannula and the ventricle freed from obvious fat and fibrous tissue. In two experiments, atria from five hearts were pooled and then treated in the same way as the ventricles. The muscle was weighed and homogenized in 3 vol. 6% trichloracetic acid. The homogenate was centrifuged and radioactivity in the supernatant estimated by liquid scintillation counting. The counting mixture consisted of 15 ml of toluene–methanol (650:350) containing 0·4% 2,5-bis-2-(5-tertbutylbenzoxazolyl)-thiophene (BBOT, Packard) to which was added 0·1 ml of acid supernatant. In studies of inhibition of adenosine uptake, dipyridamole, hexobendine or lidoflazine was included in the perfusion fluid

in concentrations ranging between 10^{-9} and $2{\cdot}6 \times 10^{-5}$ M. Owing to the limited solubility of lidoflazine it was not possible to study it at higher concentrations. Five hearts were treated in the absence of these drugs and served as controls.

Two hearts were perfused for 10 min prior to analysis, the perfusate from each heart being collected in 10 lots and assayed for radioactivity.

Adenosine uptake was estimated as nmoles of adenosine per gram wet weight of tissue. In estimating adenosine concentration it was assumed that each dose of adenosine (0·5 ml) injected into the perfusion fluid during 1 min was diluted to a total volume of 7 ml (i.e., that the average flow rate was 6·5 ml/min). Uptake of adenosine in the presence of dipyridamole, hexobendine or lidoflazine was estimated as a percentage of the control uptake, and plotted against the logarithm of the concentration of the antagonist.

Metabolism of [8-^{14}*C*]*adenosine.* The chemical nature of the ^{14}C-label retained in control hearts was compared with that in hearts treated with 10^{-5} M dipyridamole or hexobendine. Conditions for perfusion of each heart were as described above except that a dose of 10 nmoles of adenosine (280 nCi) was used. After perfusion the ventricle was quickly blotted, frozen in liquid nitrogen and weighed. The tissue was then ground to a fine powder and extracted with 3 vol. 0·3 M perchloric acid. The solution was maintained at 4° throughout preparation for chromatography. After centrifugation 0·12 ml 3% EDTA was added to 1 ml of supernatant. This solution was then neutralized with 3 M KOH. After 1 hr the mixture was centrifuged and 100 μl was spotted onto a sheet of Whatman chromatography paper (4 MM) measuring 30 × 20 cm. Carrier ATP, ADP, AMP, inosine and adenosine were added to the chromatogram. The chromatogram was run in 2 dimensions, first in isobutyric acid–H_2O–NH_3–0·1 M EDTA (100:46:14:1·6) pH 4·6, and second in 1 M ammonium acetate including 0·01 M EDTA–90% ethanol (30:70). The positions of the carriers were located under u.v. light. The paper in each of these areas was cut into 3 × 3 mm pieces and suspended in 0·4% BBOT in toluene to which had been added Thixotropic-gel (Packard). The amount of ^{14}C-label was then estimated.

Potentiation of the action of adenosine. Isolated atria were set up in an organ bath containing McEwen's solution maintained at 32° and bubbled with 5% CO_2 in O_2. Spontaneous contractions were recorded with an isotonic microdynamometer (Ugo Basile, Milan) writing on a smoked drum. Adenosine was added to the bath at 2 min intervals to give concentrations ranging from 0·4 to 4 nmoles/ml. The bath fluid was replaced 30 sec later. After a reproducible dose–response curve had been established for adenosine, dipyridamole, hexobendine or lidoflazine was added to the bath each time it was refilled and a new dose–response curve was established to adenosine. The potentiation of the action of adenosine was measured at only one or two dose levels of the potentiating drug on each pair of atria.

Degree of potentiation was determined by plotting the percentage decrease in the amplitude of contraction against the logarithm of the adenosine concentration. Scatter in the points did not generally allow estimates of potentiation in terms of more than one significant figure. During these experiments it was necessary to reduce the concentrations of adenosine used down to one-hundredth of the initial concentration range.

Adenosine deaminase. The effect of lidoflazine (10^{-5} M) on adenosine deaminase from guinea-pig heart was measured. The method involved obtaining a 100,000 *g*

supernatant from a homogenate of the tissue, incubation with [8-^{14}C]adenosine as substrate and separation and counting of the ^{14}C-labelled product. Details of the method are published elsewhere.[7] The 100,000 *g* supernatant was stored at −20° for 6 months and was devoid of adenosine kinase activity.

Drugs used. Adenosine, AMP, ADP, ATP, inosine, (Sigma), [8-^{14}C]adenosine (28 mCi/mmole, Radiochemical Centre, Amersham), dipyridamole (2,6-*bis*-(diethanolamino)-4,8-dipiperidinopyrimidine as Persantin, Boehringer Ingelheim), hexobendine (*N,N'*-dimethyl-*N,N'-bis*-(3-(3',4',5'-trimethoxybenzoxy)-propyl) ethylenediamine-dihydrochloride, Österreichische Stickstoffwerke), and lidoflazine (1-(4,4-di(4-fluorophenyl)-butyl)-4-(2,6-dimethylanilino-carbonyl)methyl-piperazine, Janssen Pharmaceutica).

RESULTS

Uptake of [8-^{14}C]*adenosine.* Serial collections of the perfusates from hearts perfused for 10 min demonstrated that 1 min following injection of [8-^{14}C]adenosine, over 90 per cent of radioactivity in the hearts could not easily be washed out (Fig. 1).

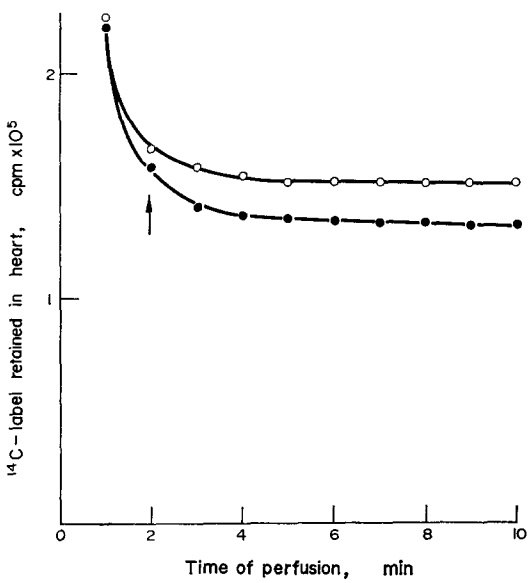

FIG. 1. Loss of ^{14}C-label from 2 perfused guinea-pig hearts after injection of [8-^{14}C]adenosine (2·5 nmoles, 70 nCi) during the first minutes. The arrow indicates the time at which ventricles were collected for assay in all other experiments.

The amount of radioactivity in control ventricles from hearts perfused for 1 min following injection of [8-^{14}C]adenosine corresponded to 0·39 ± 0·02 nmoles of adenosine per g wet wt of tissue (mean ±S.E. of 5 observations). This represents about 25 per cent of the injected adenosine. In pooled atria the concentration was 0·33 nmoles/g. The level of radioactivity retained by the ventricles in the presence of dipyridamole, hexobendine or lidoflazine is shown in Fig. 2. It can be seen that each of the three drugs reduced the amount of radioactivity retained by the ventricles.

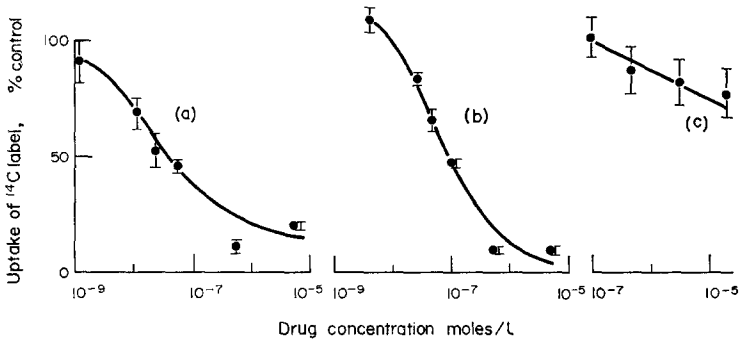

FIG. 2. Uptake of adenosine by perfused guinea-pig hearts in the presence of hexobendine (a), dipyridamole (b) and lidoflazine (c) after injection of [8-^{14}C]adenosine (2·5 nmoles, 70 nCi). Each point represents uptake expressed as a percentage of uptake by control hearts, and the S.E. is indicated by the bar.

Pooled atria from hearts perfused with dipyridamole (5×10^{-6} M) contained radioactivity equivalent to 0·05 nmoles of adenosine per g.

Metabolism of [8-^{14}*C*]*adenosine.* In control hearts practically all the ^{14}C-label was found to be in the form of adenosine phosphates, and predominantly as ATP (Table 1). Dipyridamole or hexobendine (10^{-5} M) had little effect on the retention of ^{14}C-labelled adenosine and inosine, however the concentrations of adenosine phosphates were markedly reduced by these drugs.

TABLE 1. CHEMICAL NATURE OF THE COMPOUNDS LABELLED WITH ^{14}C AFTER INJECTION OF [8-^{14}C]-ADENOSINE INTO PERFUSED HEARTS

	ATP	ADP	AMP	Inosine	Adenosine
Control	2600 ± 20	500 ± 50	120 ± 8	63 ± 6	31 ± 9
Dipyridamole	88 ± 5*	33 ± 3*	28 ± 4*	49 ± 7	38 ± 6
Hexobendine	60 ± 14*	32 ± 8*	20 ± 8*	40 ± 6*	46 ± 6

[8-^{14}C]Adenosine (10 nmoles, 280 nCi) was injected into perfused guinea-pig hearts over a period of 1 min, in the absence and in the presence of dipyridamole 10^{-5} M or hexobendine 10^{-5} M. One min later ventricles were collected and assayed. Results are expressed as pmoles of labelled nucleotide or nucleoside per g of ventricle. Each result is the mean, with S.E., of four observations.

* A significant difference between the drug-treated and control hearts was observed ($P < 0·05$).

Potentiation of the action of adenosine. In isolated atria, adenosine reduced the rate and force of spontaneous contractions. Log dose–response lines were obtained over a narrow range of concentrations, typically 0·5–2 nmoles/ml. Dipyridamole, hexobendine and lidoflazine each potentiated the action of adenosine on isolated atria. The effects of these drugs on the sensitivity to adenosine are shown in Table 2. At concentrations of 5×10^{-6} M, dipyridamole and hexobendine reduced the rate of spontaneous contractions without an effect on the amplitude. In three of the four experiments at this concentration marked deviations from a parallel shift in the log dose–response lines were observed.

TABLE 2. POTENTIATION OF THE ACTION OF ADENOSINE ON ISOLATED GUINEA-PIG ATRIA

Drug	Concentration	Adenosine potency ratio*
Dipyridamole	5×10^{-8} M	2,2
	5×10^{-7} M	14,12
	5×10^{-6} M	≃80†, ≃80†
Hexobendine	3×10^{-8} M	2,2,2
	5×10^{-8} M	4,4
	5×10^{-7} M	8,8
	5×10^{-6} M	≃40†, 14
Lidoflazine	$2{\cdot}5 \times 10^{-7}$ M	2,2,2
	$2{\cdot}5 \times 10^{-6}$ M	10,10

* The inverse ratio of the concentration of adenosine in the presence of the potentiating drug to that yielding the same response when alone. Figures shown are the results of individual experiments.
† Shift in log dose–response curve was not parallel.

Adenosine deaminase. Since it emerges from this study (see Discussion) that inhibition of uptake of adenosine by lidoflazine cannot fully explain adenosine's potentiation, it was questioned whether lidoflazine inhibits the breakdown of adenosine. Lidoflazine has been shown not to inhibit adenosine deaminase from the blood of

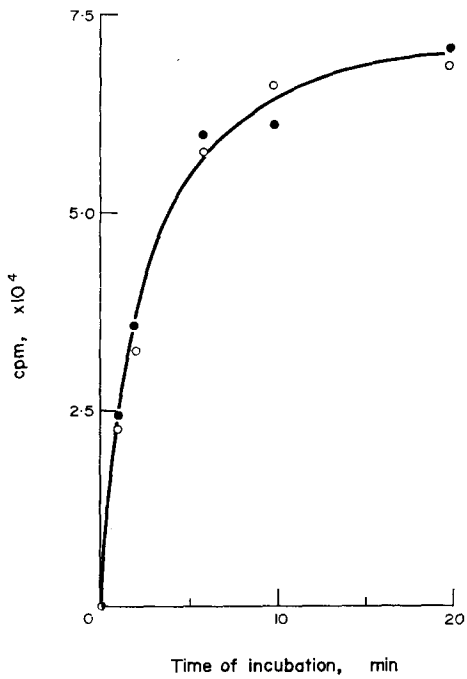

FIG. 3. The formation of ^{14}C-labelled inosine from ^{14}C-labelled adenosine by guinea-pig heart adenosine deaminase in the absence (○——○) and in the presence (●——●) and of lidoflazine 10^{-5} M.

man or dog.[11] However, the nature of adenosine deaminases is known to vary between species and between the organs of the same species.[12] It was therefore considered necessary to observe the effect of lidoflazine on the enzyme from guinea-pig heart. It was found (Fig. 3) that lidoflazine (10^{-5} M) did not affect the rate of inosine production.

DISCUSSION

In these experiments it was demonstrated that 1 min after injection of [8-^{14}C] adenosine, over 90 per cent of the radioactivity in the perfused guinea-pig heart had an extremely slow rate of turnover to the perfusion fluid. Under these conditions, retention of radioactivity by the heart has suggested dependence on the action of a membrane carrier.[7] Therefore it is concluded that after 1 min perfusion the level of radioactivity retained by a heart represents the degree of uptake of adenosine into an intracellular compartment. Adenosine was observed to be rapidly phosphorylated to ATP.

Dipyridamole and hexobendine each produced a block of adenosine uptake of about 90 per cent at concentrations above 5×10^{-7} M. The remaining 10 per cent was probably the fraction available to washout. This conclusion is supported by the observation that the total levels of adenosine and inosine were unaffected by dipyridamole or hexobendine. Block of uptake occurred as a reduction of adenosine phosphates only. It has been suggested that uptake of adenosine involves membranal phosphorylation.[7,13] However, in red blood cells, retention of nucleosides appears to involve facilitated diffusion followed, in the case of adenosine, by phosphorylation.[14] Such may be the case in heart. The present results demonstrate that dipyridamole and hexobendine are very potent inhibitors of this process and that lidoflazine is a relatively weak inhibitor.

Interpretation of potentiation of the action of adenosine in isolated atria, in terms of degree of blockade of uptake observed in ventricles, is based on the assumption that both tissues behave similarly in respect to block of adenosine uptake. In the control and dipyridamole-treated atria from hearts perfused with [8-^{14}C]adenosine, levels of radioactivity were similar to those in the ventricles. Furthermore, estimation of potentiation involved up to 100-fold changes in adenosine's concentration. An analysis of the results in terms of block of uptake of adenosine demands that no significant changes occurred in the degree of block of uptake over the concentration range of adenosine used. On the basis of a Michaelis constant of 1×10^{-6} M for the uptake of adenosine by guinea-pig heart,[7] it may be estimated that a change of about 1 per cent would have occurred.

When degree of potentiation is plotted against block of adenosine uptake (Fig. 4) it is observed that dipyridamole and hexobendine behaved almost identically. At low concentration, hexobendine was almost twice as potent as dipyridamole in potentiating the action of adenosine. This is in keeping with the observations made on block of adenosine uptake with these drugs. A 2-fold increase in sensitivity to adenosine was associated with 40–50 per cent inhibition of adenosine uptake by dipyridamole or hexobendine. However, lidoflazine produced a 2-fold increase in sensitivity at a concentration which did not significantly affect adenosine uptake. Nor could this effect be attributed to inhibition of adenosine deaminase since it was shown that the

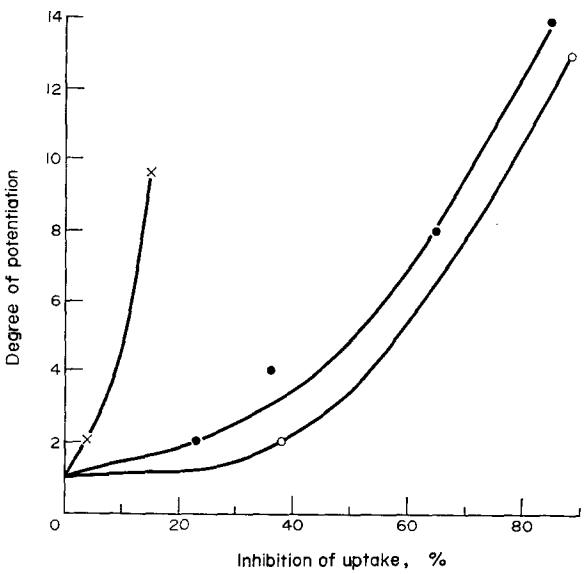

FIG. 4. The relationship between the percentage block of adenosine uptake and the degree of potentiation of the action of adenosine observed with lidoflazine (×——×), hexobendine (●——●) and dipyridamole (○——○). This graph is a combination of the results presented in Fig. 2 and Table 1.

enzyme from guinea-pig heart was not inhibited by lidoflazine. Afonso *et al.*[9] suggested that lidoflazine increases the sensitivity of the smooth muscle of the coronary vessels in the dog to the action of adenosine. Such an action could explain the present findings. It is interesting to speculate whether the adenosine receptor is an allosteric enzyme, lidoflazine mimicking some other physiological mediator.

In general, the results do not fully support the conclusion of Pfleger *et al.*[5] that the actions of these three drugs in potentiating the action of adenosine can be fully explained in terms of block of uptake. They suggest that, in the heart, block of adenosine uptake is the mode of action of dipyridamole or hexobendine, however lidoflazine acts to potentiate adenosine in some other way.

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