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THE ACCUMULATION OF ADENOSINE IN RABBIT INTESTINAL MUSCLE

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1 Strips of longitudinal muscle from rabbit intestine accumulated radioactivity when exposed to [^{3}H]-adenosine.

2 Accumulation of radioactivity was not sodium-dependent or ouabain-sensitive, but was reduced by cooling, zero glucose plus bubbling with N_2, 2,4,dinitrophenol, dipyridamole, hexobendine and lidoflazine.

3 After 7 min exposure to [^{3}H]-adenosine, the tissue was found to contain radioactive adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine itself in the approximate ratio 13 : 6 : 4 : 1.

4 In the presence of dipyridamole, hexobendine or lidoflazine (each 1 μM), the amounts of radioactive ATP, ADP, AMP and adenosine were reduced with the concentration of adenosine not significantly different from controls.

5 It is concluded that energy-dependent uptake of adenosine does not occur in the longitudinal muscle of rabbit intestine. Adenosine enters the tissue by a passive process and rapidly becomes phosphorylated giving rise to apparently high tissue : medium ratios.

6 The drugs dipyridamole, hexobendine and lidoflazine appear to reduce the accumulation of radioactivity by preventing the formation of adenosine phosphate derivatives.

Introduction

The drugs dipyridamole, hexobendine and lidoflazine have been reported to potentiate the actions of adenosine and reduce adenosine uptake in isolated heart (Kraupp, Wolner, Adler-Kastner, Chirikdjian, Ploszczanski & Tuish, 1966; Afonso, O'Brien & Crumpton, 1968; Kolassa, Pfleger & Rummel, 1970; Hopkins & Goldie, 1971). Interest in these compounds has been further stimulated by the suggestion (Burnstock, Campbell, Stachell & Smythe, 1970) that adenosine triphosphate (ATP) is the transmitter liberated by the non-cholinergic, non-adrenergic intramural inhibitory nerves of mammalian intestine. Burnstock (1972) has proposed that, by analogy with energy-dependent choline uptake and noradrenaline uptake in cholinergic and adrenergic nerves respectively, the adenosine moiety is taken up by a similar process in purinergic nerves. In the absence of specific antagonists to the action of ATP, the possibility of potentiating the effects of exogenous ATP and of intramural stimulation by the use of the drugs dipyridamole, hexobendine and lidoflazine provides an opportunity for a crucial test of the purinergic nerve hypothesis.

Stafford (1966) reported that the effects of adenosine in rabbit intestine were potentiated by dipyridamole and Satchell, Lynch, Bourke & Burnstock (1972) have claimed that the effects of adenosine, ATP and of intramural stimulation in guinea-pig taenia coli are potentiated by dipyridamole and by hexobendine. However, we have failed to confirm potentiation of the effects of adenosine, ATP and intramural stimulation in the presence of dipyridamole, hexobendine and lidoflazine, and consider that the data presented by Stafford (1966) and by Satchell *et al.* (1972) were inadequate to allow the claim that potentiation of inhibitory responses had occurred (Hulme & Weston, 1974).

Close examination of the work of Kraupp *et al.* (1966), Afonso *et al.* (1968), Kolassa *et al.* (1970), and Hopkins & Goldie (1971), reveals that these workers have not shown that an adenosine uptake process exists (see Iversen, 1970) but merely that tissues accumulate radioactivity following exposure to [^{3}H]-adenosine. In view of this and of the disparity between the work of Stafford (1966) and Satchell *et al.* (1972) and that of Hulme & Weston

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(1974), it was decided to determine the characteristics of adenosine accumulation in rabbit intestine. In addition, the effects of dipyridamole, hexobendine and lidoflazine were examined to discover whether these compounds affected adenosine uptake itself and/or the subsequent metabolism of adenosine within the tissue.

Methods

Preparation of longitudinal muscle strips

In most experiments, 2 cm lengths of the longitudinal muscle layer were removed from rabbit duodenum and intestine as previously described (Weston, 1971). In other experiments, strips free from Auerbach's plexus were prepared by the method described for guinea-pig ileum (Paton & Zar, 1968).

Measurement of ^3H accumulation after exposure to [^3H]-adenosine

Muscle strips were divided into groups of six. Each group was placed in a test tube containing 10 ml Krebs solution at 37°C and bubbled with 5% CO_2 in O_2. After a 30 min equilibration period, the tissues were subjected to a variety of conditions as detailed below.

Time course. [^{3}H]-adenosine (1 μCi) was added to each tube and 5, 10, 20, or 30 min later a group of strips was removed. Each was blotted, weighed and then transferred to a test tube containing 4 ml 0.4 N perchloric acid after which it was homogenized (Ultra-Turrax) and the resulting suspension centrifuged for 5 min at 4000 *g* (Griffin-Christ). One ml of the supernatant was taken for scintillation counting.

Dependence on adenosine concentration. After the 30 min equilibration period, each group of strips was exposed to 4 μCi [^{3}H]-adenosine and to 50 μM, 25 μM, 10 μM, 5 μM or 1 μM adenosine. Two minutes later, each strip was blotted, weighed, extracted in perchloric acid and centrifuged as described under *Time course.* One ml of the supernatant was taken for scintillation counting.

Effect of cooling, metabolic inhibition, dipyridamole, hexobendine and lidoflazine. After the 30 min equilibration period, groups of strips were exposed to the following conditions for the times indicated: cooling to 0°C (1 h); ouabain, 100 μM (1 h); [Na^+], 25 mM (1 h); 2,4,dinitrophenol, 1 mM (1 h); zero glucose (2.5 h) followed by bubbling with 5% CO_2 in N_2 (30 min); dipyridamole, 10 nM, 100 nM or 1 μM (30 min); hexobendine, 10 nM, 100 nM or 1 μM (30 min); lidoflazine, 10 nM, 100 nM or 1 μM (30 minutes). These conditions were then maintained and 1 μCi [^{3}H]-adenosine was added to each tube. Seven minutes later, the tissues were extracted as described under *Time course* and 1 ml of the resulting supernatant was taken for scintillation counting.

Separation of [^3H]-adenosine and its metabolites

Groups of strips were prepared as described previously and placed in test tubes containing 5 ml Krebs solution. After equilibration, test tissues were exposed to dipyridamole, hexobendine or lidoflazine (each 10 nM, 100 nM or 1 μM) for 30 min, after which 20 μCi [^{3}H]-adenosine was added. Seven minutes later, each tissue was removed, blotted and added to a tared tube containing 3 ml ice cold 0.4 N perchloric acid. After homogenization, weighing and centrifugation, 2 μl of the resulting supernatant was applied to a thin-layer chromatography plate spread with cellulose F, thickness 0.1 mm (Merck). The solvent system was 14 ml 1 M acetic acid, 86 ml 1 M sodium citrate, pH 6. To the radioactive spot was added 5 μl of a mixture of ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine (each 10 mM). After 5 h, spots were located under a u.v. lamp and scraped from the plates into vials prior to liquid scintillation counting.

Radioassays

One ml of aqueous sample was added to 10 ml phosphor in a counting vial. Samples were counted (Packard) for 10 min or until 50,000 counts had accumulated. Media were counted for 1 min or until 200,000 counts had accumulated. The channels ratio method using automatic external standardization was used to correct for quenching.

Statistical analysis

The output from the scintillation counter was processed by a digital computer (Data General, Nova) which also performed the statistical analyses on the data by Student's *t* test.

Drugs and solutions

The following drugs were used: [^{3}H]-adenosine, generally labelled, 17 Ci/mmol (Amersham); this material showed only one peak of radioactivity corresponding in position with that of adenosine after thin-layer chromatography with cellulose F

plates (Merck) as described previously; adenosine, AMP, ADP and ATP (Boehringer); 2,4,dinitrophenol (Sigma); dipyridamole (Boehringer); hexobendine (Linz); lidoflazine (Janssen); ouabain (Sigma).

The phosphor used for liquid scintillation counting had the following composition: 0.1 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (DMPOPOP, Nuclear Enterprises), 5.5 g diphenyloxazole (PPO, Packard), 333 ml Triton X-100 (B.D.H.), 667 ml toluene (B.D.H.).

Acetic acid and sodium citrate for use as the solvent system in the thin-layer chromatography experiments was obtained from B.D.H. In the experiments with $[Na^+]$, 25 mM, the sodium chloride was omitted from the Krebs solution and replaced with 220 mM sucrose solution (B.D.H.).

The Krebs solution had the following composition (mM): Na^+ 143.0, K^+ 5.9, Ca^{++} 2.5, Mg^{++} 1.2, Cl^- 125.0, HCO_3^- 25.0, SO_4^{--} 1.2, $H_2PO_4^-$ 1.2, dextrose 11.1. The pH of this solution was 7.4 while bubbling with 5% CO_2 in O_2.

Results

Accumulation of ^3H from [^3H]-adenosine

Strips of rabbit intestinal muscle accumulated radioactivity against an apparent concentration gradient. Seven minutes after exposure to [^{3}H]-adenosine, the apparent tissue : medium (T/M) ratio was 7.1 ± 0.24 ml/g (mean of 30 experiments with s.e.). There was no significant difference between the radioactivity accumulated by duodenal strips and that accumulated by strips removed from the lower ileum.

The accumulation of radioactivity in strips with and without Auerbach's plexus was compared. In these experiments, the T/M ratio for strips with Auerbach's plexus was 7.2 ± 0.49 ml/g and for strips without the plexus 6.19 ± 0.45 ml/g (each 15 experiments with s.e.). The difference between these means is not significant ($P = 0.063$).

The accumulation of radioactivity following exposure to adenosine was found to be a saturable process. In these experiments, there was a 2 min exposure time to [^{3}H]-adenosine since, at this time, the increase in apparent T/M ratio was linear even for the highest concentration of adenosine used (50 μM). From these experiments, it was possible to calculate a Michaelis constant (K_m) of 800 nM. However, the results of subsequent experiments show that the use of this term is almost certainly inappropriate in this instance (see discussion section).

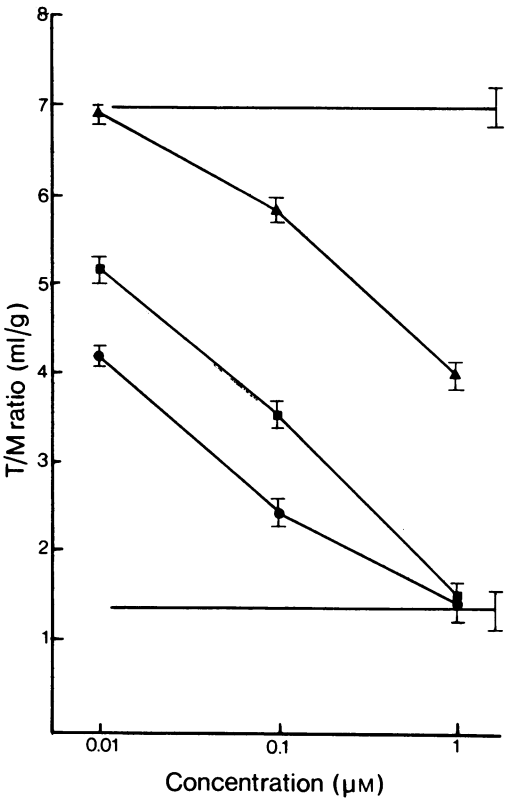

Fig. 1 Effects of cooling, dipyridamole, hexobendine and lidoflazine on accumulation of radioactivity from [^{3}H]-adenosine. Prior to addition of [^{3}H]-adenosine, 1 μCi, controls were incubated in Krebs solution for 30 min and other tissues were exposed to dipyridamole (●), hexobendine (■) or lidoflazine (▲) for 30 min or cooled to 0° C for 1 hour. Seven minutes later, extracts were prepared for liquid scintillation counting. Upper horizontal line shows T/M ratio in Krebs solution alone (control), lower horizontal line shows T/M ratio after cooling. Each value is derived from six experiments and standard errors are shown.

Effects of dipyridamole, hexobendine, lidoflazine and cooling on tissue labelling

These results are summarized in Figure 1. Dipyridamole, hexobendine and lidoflazine were each effective in reducing the accumulation of ^{3}H in the tissue. Concentrations greater than 1 μM were not employed since, at higher concentrations, these drugs showed agonist activity in tissue bath experiments (Hulme & Weston, 1974). Cooling the tissue reduced ^{3}H accumulation to levels not significantly different from those produced by dipyridamole and hexobendine, each 1 μM.

Dependence of ^3H accumulation on energy metabolism

These results are summarized in Table 1. This table shows that [Na^+], 25 mM and ouabain, 100 μM had no effect on ^3H accumulation whereas 2,4,dinitrophenol, 1 mM and zero glucose plus bubbling with 5% CO_2 in N_2 greatly reduced the accumulation.

Analysis of ^3H-labelled compounds after exposure to [^3H]-adenosine

The ^3H-labelled constituents of tissue homogenates were separated by thin-layer chromatography and these results are summarized in Table 2. In control experiments, the ratio of labelled ATP : ADP : AMP : adenosine was approximately 13 : 6 : 4 : 1, the total radioactivity corre-

Table 1 Effect of changes in the incubation medium on accumulation of radioactivity from [^3H]-adenosine

Incubation medium	*T/M ratio (ml/g)*
Krebs solution	6.95 ± 0.21
Low Na^+ solution (25 mM)	6.55 ± 0.38
Ouabain, 100 μM	6.41 ± 0.31
2,4,dinitrophenol	1.93 ± 0.11*
Glucose-free solution bubbled with 5% CO_2 in N_2	1.64 ± 0.12*

* Indicates a significant difference between value when treated with drug and that when incubated in Krebs solution alone ($P < 0.05$).
Incubation in Krebs solution was maintained for 30 minutes. Glucose-free conditions were maintained for 2.5 hours. Bubbling with 5% CO_2 in N_2 lasted 30 minutes. All other conditions were maintained for 1 h before addition of [^3H]-adenosine. [^3H]-adenosine (1 μCi) was in contact with the tissue for 7 min, during which time pre-incubation conditions were maintained. Each value is derived from six observations and standard errors are shown.

Table 2 Analysis of radioactive tissue constituents after exposure to [^3H]-adenosine and the effects of dipyridamole (Dip), hexobendine (Hex) and lidoflazine (Lid)

	ATP	*ADP*	*AMP*	*Adenosine*	*T/M ratio (ml/g)*
		(pmol/g)			
Control	790 ± 88	364 ± 71	249 ± 32	65 ± 15	6.16 ± 0.33
Dip					
10 nM	560 ± 16	212 ± 14	118 ± 25	73 ± 10	4.0 ± 0.16
100 nM	230 ± 48	136 ± 22	74 ± 11	81 ± 18	2.18 ± 0.16
1 μM	108 ± 12	63 ± 10	40 ± 6	49 ± 7	1.10 ± 0.18
Hex					
10 nM	625 ± 48	254 ± 25	131 ± 17	62 ± 6	4.54 ± 0.18
100 nM	293 ± 27	137 ± 15	75 ± 8	47 ± 11	2.37 ± 0.17
1 μM	94 ± 8	61 ± 15	53 ± 12	55 ± 14	1.13 ± 0.15
Lid					
10 nM	760 ± 83	390 ± 51	212 ± 37	58 ± 7	6.03 ± 0.1
100 nM	680 ± 43	231 ± 28	133 ± 17	33 ± 10	4.98 ± 0.12
1 μM	394 ± 51	204 ± 26	104 ± 21	45 ± 8	3.38 ± 0.13

Before addition of [^3H]-adenosine, 20 μCi, controls were incubated in Krebs solution for 30 minutes. Other tissues were exposed to dipyridamole, hexobendine or lidoflazine for 30 minutes. Seven minutes later, extracts were prepared for thin-layer chromatography. The values for T/M ratio were calculated by adding together the radioactivity present in the ATP, ADP, AMP and adenosine spots. About 85% of the added radioactivity was recovered from these spots with the result that the values for T/M ratio are somewhat lower than those shown in Figure 1. Each value is derived from the mean of five experiments and standard errors are shown.

sponding to a T/M ratio of 6.16 ± 0.33 ml/g. Values for T/M ratios are lower than shown in Fig. 1 since only 85% of the radioactivity was recovered from the ATP, ADP, AMP and adenosine spots. In the presence of dipyridamole, hexobendine and lidoflazine, each 10 nM, 100 nM, or 1 μM, there was a concentration-dependent reduction in the total amount of radioactivity in the tissue homogenates resulting from a decrease in the amounts of radioactive ATP, ADP and AMP. The amount of radioactive adenosine was unaffected. In the presence of dipyridamole or hexobendine, each 1 μM, the ratio of radioactive ATP : ADP : AMP : adenosine was approximately 2 : 1 : 1 : 1. The values for T/M ratios shown in Table 2 and derived from the thin-layer chromatography experiments bear close resemblance to the values shown in Figure 1.

Discussion

The results demonstrate that rabbit intestinal muscle is able to accumulate large amounts of ^3H following exposure to [^3H]-adenosine. This observation is in agreement with the results of previous workers who have shown that heart muscle accumulates radioactivity after exposure to labelled adenosine (Kolassa *et al.*, 1970; Kraupp *et al.*, 1966; Afonso *et al.*, 1968). In the absence of further experiments, these groups of workers have usually referred to the accumulation of radioactivity as 'adenosine uptake'. The present experiments have demonstrated that such a term, if used in the sense described by Iversen (1970), does not apply to the accumulation of radioactivity in rabbit duodenum.

The cooling experiments showed the extent (about 20%) to which passive processes could account for the ^3H content of the tissues. The ability of 2,4,dinitrophenol and of zero glucose plus bubbling with nitrogen to reduce tissue radioactivity seems to favour an energy-dependent process accounting for the remaining 80% of radioactivity. In addition, the ability of adenosine to reduce tissue ^3H levels after exposure to [^3H]-adenosine (apparent K_m = 800 nM) also favours an energy-dependent process. However, the presence of such a process is not supported by the inability of ouabain and of reduced [Na^+] to affect ^3H accumulation. Evidence from the thin-layer chromatography experiments and from the effects of the drugs dipyridamole, hexobendine and lidoflazine strongly suggests that no energy-dependent process is responsible for transfer of adenosine into the tissue.

Control experiments showed that ATP, ADP and AMP accounted for most of the tissue radioactivity. Dipyridamole, hexobendine and lidoflazine reduced the total amount of tissue radioactivity by selectively reducing the amounts of ATP, ADP and AMP present in the tissue. Similar findings were reported recently from studies of the action of dipyridamole and hexobendine in guinea-pig heart (Hopkins, 1973). This suggests that the three drugs do not prevent entry of adenosine into the tissue but rather that they inhibit the phosphorylation of adenosine and/or AMP and/or ADP. Calculations have shown (Table 2) that this effect on the synthesis of AMP, ADP and ATP accounts for the reduction in total tissue radioactivity and explains the ability of cooling to reduce ^3H accumulation by about 80%. Paradoxically, the ability of 2,4,dinitrophenol and of abolition of oxidative and glycolytic ATP synthesis to reduce tissue radioactivity is also consistent with the passive entry of adenosine into the tissue. These procedures limit cellular ATP synthesis, less [^3H]-adenosine is therefore required, and the adenosine concentration gradient between inside and outside rapidly diminishes reducing the entry of further [^3H]-adenosine. The K_m of 800 nM is almost certainly a reflection of the characteristics of the rate-limiting step in adenosine phosphorylation rather than a characteristic of any adenosine uptake process.

Lidoflazine was found to be the least potent of the three drugs formerly regarded as adenosine uptake blocking agents. However, there was no evidence that the mechanism by which this drug prevented ^3H accumulation differed from that of dipyridamole or hexobendine.

The accumulation of radioactivity by strips from which Auerbach's plexus had been removed was not significantly different from that in strips with the plexus although there was a definite trend towards lower T/M ratios in strips from which the plexus had been removed. Considering the weight of the plexus relative to that of the longitudinal muscle, it is perhaps not surprising that no difference between strips with and without the plexus could be demonstrated. It is also possible that the thinness of the strips without the plexus and perhaps greater damage during their preparation might alter their ability to accumulate radioactivity.

In view of the finding that energy-dependent adenosine uptake does not occur in rabbit intestinal longitudinal muscle, it is also possible that the process does not exist, as previously claimed, in those tissues in which only accumulation of radioactivity after exposure to radioactive adenosine has been demonstrated (Kolassa *et al.*, 1970; Kraupp *et al.*, 1966; Afonso *et al.*, 1968). Furthermore, the analogy based on these observations between adenosine uptake in purinergic nerves and

choline and noradrenaline uptake in cholinergic and adrenergic nerves respectively (Burnstock, 1972) may now be incorrect.

The present experiments have shown that dipyridamole, hexobendine and lidoflazine prevent adenosine accumulation by reducing its phosphorylation rather than by affecting an energy-dependent uptake process. This does not affect their potential in testing the purinergic nerve hypothesis although it must be remembered that the results of the present experiments and of all other experiments to date refer to the accumulation of adenosine by muscle and not by nerves.

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