POTENTIATION BY DILAZEP OF THE NEGATIVE INOTROPIC EFFECT OF ADENOSINE ON GUINEA-PIG ATRIA

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1 Dilazep, a coronary dilator, has been reported to potentiate the negative inotropic and negative chronotropic responses of guinea-pig atria to adenosine. Studies were made on the mechanism of the potentiating action of dilazep with special reference to the degradation and uptake of adenosine.

2 The negative inotropic actions of adenosine and adenine nucleotides, such as ATP, ADP, AMP and cyclic AMP, on guinea-pig atria were selectively and dose-dependently augmented by dilazep at concentrations insufficient to produce any effect alone (0.01 to 1 μM).

3 Incubation of atrial tissue with 8.8 nM adenosine, containing 0.1 μCi of [^{3}H]-adenosine, resulted in accumulation of [^{3}H]-adenosine in the tissue; dilazep (0.01 to 1 μM) inhibited this accumulation.

4 Adenosine (10 μM to 10 mM) was degraded to inosine and hypoxanthine during incubation with atrial tissue; dilazep (0.1 to 10 μM) retarded the disappearance of adenosine and the formation of inosine and hypoxanthine.

5 These results suggest that dilazep potentiates the negative inotropic effect of adenosine on guinea-pig atria by preventing both its accumulation by atrial tissue and degradation by deaminase.

Introduction

Myocardial ATP is degraded to adenosine under hypoxic conditions caused by reduction in the oxygen content of arterial blood or increase in the oxygen consumption of myocardial tissue. The adenosine thus formed is released into the intracellular space and probably plays an important role in regulating coronary blood flow (Berne, 1963; Bunag, Douglas, Imai & Berne, 1964; Rubio, Berne & Katori, 1969; Rubio & Berne, 1969).

It has been shown that the coronary vasodilator, dipyridamole, potentiates the coronary vascular response to adenosine, possibly by preventing degradation of adenosine in the tissue or erythrocytes (Jacob & Berne, 1960; Bunag *et al.*, 1964), or by inhibiting the uptake of adenosine into erythrocytes (Koss, Beisenherz & Maerkisch, 1962) and tissue (Afonso & O'Brien, 1967; Pfleger, Volkmer & Kolassa, 1969; Kalsner, 1975) or by inhibiting the phosphorylation of adenosine (Hopkins & Goldie, 1971) and thus preventing its uptake.

Like dipyridamole and hexobendine (Kraupp, Wolner, Alder-Kastner, Ploszczanski & Tuisl, 1965; Raberger & Kraupp, 1971), dilazep has been reported to have a coronary dilator effect (Hensel, Bretschneider, Kettler, Knoll, Kochsiek, Reploh, Spiecker-

mann & Tauchert, 1972; Lenke, Brock & Zechel, 1972; Sano, Katsuki & Kawada, 1972), and also to potentiate the coronary dilator effect of adenosine (Spieckermann, Hellberg, Kettler, Reploh & Strauer, 1969; Buyniski, Losada, Bierwagen & Gardiner, 1972). In addition, dilazep potentiates the negative inotropic effect of adenosine on guinea-pig atria (Buyniski *et al.*, 1972).

The present experiments were undertaken to clarify the mechanism of the potentiating action of dilazep on the negative inotropic effect of adenosine on guinea-pig atria, with special reference to the uptake of adenosine and degradation of adenosine by deaminase.

Methods

Guinea-pigs of either sex weighing about 400 g were used. Spontaneously beating atria were isolated from surrounding tissue and mounted in a 10 ml organ bath filled with Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.7, CaCl$_2$ 1.8, MgCl$_2$ 1.8, NaH$_2$PO$_4$ 0.42, NaHCO$_3$ 11.9 and glucose 11.2. The bath was maintained at 37°C and bubbled with O$_2$.
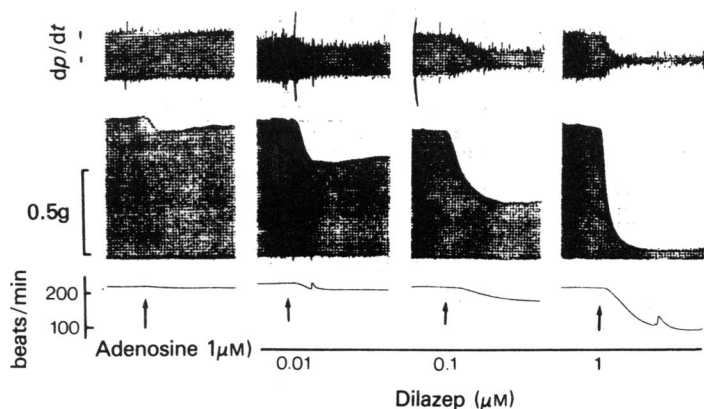
Figure 1 Potentiating effect of dilazep on the inhibition by adenosine of spontaneously beating guinea-pig atrium. Records show (a) dp/dt, (b) contraction force and (c) rate of beating.

Contractions were recorded isometrically on an oscillograph (Nihon Kohoden SM 8) through a strain gauge transducer (SB-1T-H). An initial tension of approximately 0.5 g was applied to the atria. The input was further amplified through a d.c. amplifier to drive a heart rate tachometer (Nihon Kohoden RT-5) and differentiator (Nihon Kohoden RPD-5).

For construction of dose-response curves, agonists were added cumulatively to the organ bath in a volume of 0.1 to 0.2 ml. The apparent activities of adenine derivatives in the absence and presence of dilazep are expressed as negative logarithms of the molar concentrations producing 50% inhibition of the maximal response (negative log ED_{50} values, pD_2 values). When tested, dilazep was added 10 min before adenine derivatives.

Adenosine uptake

Segments of atria weighing about 10 mg were equilibrated for 60 min at 37°C with Tyrode solution bubbled with O_2, and then transferred to 10 ml of the same solution containing 8.8 nM adenosine with 0.1 μCi [^3H]-adenosine. Dilazep was added to the medium both during preincubation for 60 min and during incubation with [^3H]-adenosine. At various times during incubation, segments were removed, rinsed twice with Tyrode solution and blotted. Pieces were weighed, transferred to scintillation vials and digested with Soluene 350. Radioactivity of the digests was measured in 15 ml scintillating solution (2,5-diphenyl-oxazole) (PPO) 4 g, 1,4-di-2-(4-methyl-5-phenyloxazolyl)benzene (POPOP) 0.1 g, toluene 1 l in a scintillation spectrophotometer (Aloka LSC-602), with automatic external standardization to determine efficiency. Tritium uptake was expressed as the ratio of the activity in the tissue to that in the medium (T/M ratio).

Degradation of adenosine

Samples of 50 mg of atrial segments were incubated with 0.5 ml of 10 μM adenosine containing 10 μCi

Table 1 Negative inotropic actions of adenine derivatives on guinea-pig atria in the presence and absence of dilazep

| | Concentration of dilazep (μM) | | | |
	0	0.01	0.1	1.0
Adenosine	5.06 ± 0.02	5.42 ± 0.09	6.13 ± 0.08	6.60 ± 0.09
ATP	4.72 ± 0.18	5.13 ± 0.12	5.62 ± 0.18	6.25 ± 0.17
ADP	4.50 ± 0.14	5.24 ± 0.03	6.05 ± 0.10	6.29 ± 0.10
AMP	4.61 ± 0.09	5.19 ± 0.08	5.84 ± 0.07	6.25 ± 0.09
Cyclic AMP	3.42 ± 0.15	3.79 ± 0.18	4.49 ± 0.12	4.85 ± 0.10
2'-Deoxy ade.	2.81 ± 0.06	3.01 ± 0.06	2.93 ± 0.18	3.57 ± 0.11
IMP	2.53 ± 0.01	2.62 ± 0.16	2.93 ± 0.18	3.57 ± 0.11

Activities are expressed as negative logarithms of ED_{50} values. Values are means (±s.e.) of those for atria from 8 animals.
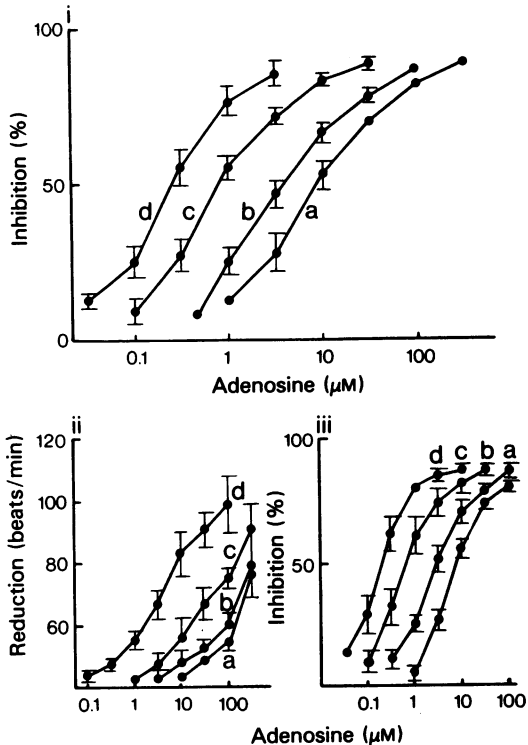
Figure 2 Potentiation by dilazep of adenosine-induced negative inotropic response (i), negative chronotropic response (ii) and dp/dt (iii) of spontaneously beating guinea-pig atria. The ordinates in (i) and (iii) show the inhibitions as percentages, taking the original responses as 100%, and the ordinate scale in (ii) shows the decrease in rate of beating. (a): control; (b) with 0.01 μM dilazep; (c) with 0.1 μM dilazep; (d) with 1 μM dilazep. Values were obtained with atria from 8 animals; vertical lines show s.e. mean.

[³H]-adenosine (0.88 μM) as a tracer. Small samples taken at intervals were mixed with an equal volume of 10 mM solution of authentic adenosine, inosine or hypoxanthine and 5 μl aliquots were applied to a thin layer chromatography (t.l.c.) plate (Cellulose F, 0.1 mm thickness, Merck). The plate was developed with 1 M sodium citrate: 1 M acetic acid (86:14, v/v) under cooling. Spots were located under u.v. light, scraped off the plate into vials and suspended in 1 ml of water using a sonicator. Then scintillator fluid was added and radioactivity was counted as described above.

The drugs used were adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine 3',5'-cyclic monophosphate (cyclic AMP), adenosine, inosine, inosine monophosphate, adenine, hypoxanthine, xanthine, 2'-deoxyadenosine, dilazep (1,4-bis-(3-(3,4,5-tri-

methoxybenzoyl)-propyl)-perhydro-1,4-diazepine), dipyridamole, verapamil, nifedipine and [2-³H]-adenosine (specific activity 13.2 Ci/mmol, New England Nuclear).

Results

Effect of dilazep on negative inotropic effect of adenosine

Dilazep at concentrations too low to have any effect alone dose-dependently and selectively augmented the negative inotropic effect of adenosine on isolated guinea-pig atria. As shown in Figure 1, in the presence of 0.01 μM, 0.1 μM and 1 μM dilazep, the inhibition by 1 μM adenosine increased from control values of 11% to 30%, 63% and 94%, respectively, with prolongation of the duration of activity. The dose-response curve for adenosine was shifted to the left with increasing concentrations of dilazep, indicating potentiation of the negative inotropic effect of adenosine. The pD₂ value for adenosine increased from the control value of 5.06 to 5.24, 6.13 and 6.60, in the presence of 0.01 μM, 0.1 μM and 1 μM dilazep, respectively (Table 1). The effect of dilazep was long lasting, complete recovery taking about 40 min to 90 min depending on the concentration used. A concentration of over 10 μM dilazep had a negative inotropic effect by itself. The inhibitory effect of adenosine gradually disappeared with time even though the adenosine remained in the bath.

In addition to enhancing the effect of adenosine, dilazep also prolonged duration of action of adenosine: 0.01 μM dilazep prolonged the negative inotropic effect of 1 μM adenosine to 15 min from the control time of 5 min, whilst with increase in the concentration of dilazep to 1 μM, the inhibition by adenosine became almost irreversible, persisting for more than 4 h. Dilazep augmented not only the negative inotropic effect but also the negative chronotropic effect and −dp/dt caused by adenosine (Figures 1 and 2).

The adenine derivatives tested had essentially similar effects to adenosine. However, ATP, ADP and AMP were about 3 to 5 times less potent than adenosine, and cyclic AMP, 2'-deoxyadenosine and inosine monophosphate were about 1/10, 1/300 and 1/500 as active of adenosine. Dilazep did not potentiate the effect of inosine, but it potentiated the effects of the other adenine nucleotides to a similar extent to that of adenosine. Adenine, xanthine and hypoxanthine at concentrations above 1 mM had no effect even in the presence of dilazep. The dose-response curves for these adenine derivatives are shown in Figure 3. The order of potencies of these compounds for inducing negative inotropic effects in the absence and presence of dilazep was: adenosine = ATP = ADP = AMP >
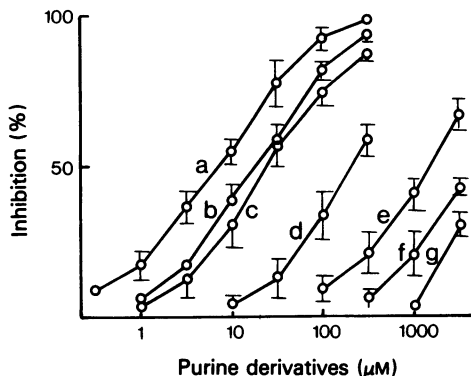
Figure 3 Dose-response curves for the effects of purine derivatives on the spontaneous beating of guinea-pig atria. The ordinate scale shows the response as a percentage of the maximal response and the abscissa scale shows the concentrations of purine derivatives. (a) adenosine; (b) ATP; (c) AMP; (d) cyclic AMP; (e) 2'-deoxyadenosine; (f) inosine monophosphate; (g) inosine. Values (means) were obtained with atria from 8 animals; vertical lines show s.e. mean.

Figure 4 Effect of dilazep on the accumulation of tritium activity in atria during incubation with 8.8 nM adenosine containing 0.1 μCi [³H]-adenosine. (a) Control; (b) with 0.01 μM dilazep; (c) with 0.1 μM dilazep; (d) with 1 μM dilazep. Accumulation of tritium activity is expressed as the ratio of the activity in the tissue (T) to that in the medium (M). Values (means) were obtained with atria from 8 animals; vertical lines show s.e. mean.

cyclic AMP > 2'-deoxyadenosine > inosine monophosphate.

Dilazep was about 10 times more potent than dipyridamole in augmenting the negative inotropic effect of adenosine, the pD_2 values of dilazep and dipyridamole being 7.64 ± 0.16 and 6.49 ± 0.11, respectively.

Effect of dilazep on adenosine uptake

Next we examined whether the effect of dilazep was due to prevention of adenosine uptake by atria. Exposure of atrial tissue to 8.8 nM [³H]-adenosine (0.1 μCi) resulted in accumulation of ³H activity in the tissue; the apparent T/M ratio gradually increased with time, being 1.28 ± 0.21, 2.30 ± 0.41, 3.75 ± 0.27 and 6.60 ± 0.26 after 2, 5, 10 and 20 min, respectively (Figure 4). Incubation of the atria with 0.01 μM dilazep for 60 min reduced the T/M ratio for accumulation of ³H activity in 10 min to 2.07 ± 0.38 from 3.75 ± 0.27. Dilazep at concentrations of 0.1 μM and 1 μM decreased the T/M ratio further to 1.23 ± 0.12 and 0.88 ± 0.02, respectively. The simultaneous addition of unlabelled adenosine at a concentration of 10 μM (which caused a 50% inhibition of spontaneous contractions of atria) reduced the T/M ratio after 10 min and 20 min incubation to 58.1% and 55.8%, respectively, of the values with [³H]-adenosine alone (8.8 nM, 0.1 μCi). In this case, 0.1 μM dilazep also reduced the uptake of adenosine by the tissue in 10 min and 20 min to 53.6% and 51.0%, respectively.
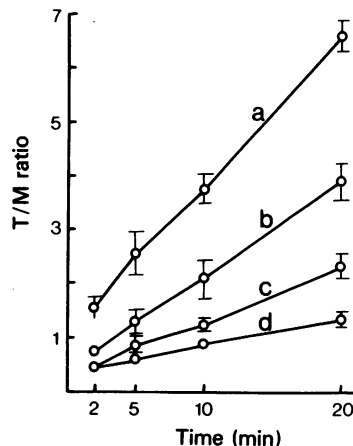
Dilazep was about 10 times more potent than dipyridamole in inhibiting the uptake of [³H]-adenosine.

Effect of dilazep on degradation of adenosine

To see whether dilazep also affected adenosine degradation, we examined its effect on the time course of the disappearance of adenosine and the formation of adenosine metabolites. A preliminary experiment, in which samples of 100 mg of atrial tissue were incubated with 1 ml of 10 mM adenosine and then its metabolites were identified by their R_F values on t.l.c. plates, showed that adenosine gradually decreased in the incubate with increased levels of inosine and hypoxanthine, and that 10 μM dilazep inhibited these changes by about 50%.

The amounts of metabolites were measured using [³H]-adenosine as tracer. Samples of 50 mg of atrial muscle were incubated in 0.5 ml of medium containing 10 μM adenosine with 10 μCi of [³H]-adenosine, and samples of medium taken at various intervals were developed on t.l.c. plates. Then the radioactivities in areas corresponding to adenosine, inosine and hypoxanthine were counted.

The ³H-activity located in the position of adenosine decreased with the time of incubation while the [³H]-activities in the positions of inosine and hypoxanthine increased with the disappearance of adenosine (Figure 5). After incubation for 30 min the ratio
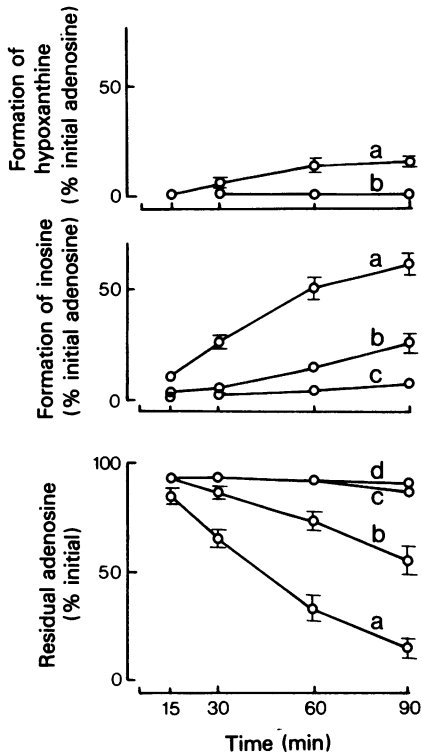
Figure 5 Degradation of adenosine, and formation of inosine and hypoxanthine during incubation of atria with 10 μM adenosine containing 10 μCi [³H]-adeno-sine. The amount of adenosine remaining, and the formation of inosine and hypoxanthine are expressed as percentages of the initial concentration of adenosine (10 μM). (a) Control; (b) with 0.01 μM dilazep; (c) with 0.1 μM dilazep; (d) with 1 μM dilazep. Values (means) were obtained with atria from 6 animals; vertical lines show s.e. mean.

of adenosine:inosine:hypoxanthine was 66.4:26.7:6.9. Dilazep caused dose-dependent decrease in degradation of adenosine: at a concentration of 0.1 μM the ratio of adenosine:inosine:hypoxanthine after 30 min incubation was 95.3:3.1:1.6.

Effects of calcium antagonists

To see whether the effect of dilazep is due to a calcium antagonistic action, we tested the effects of verapamil and nifedipine on the negative inotropic effect of adenosine. Pretreatment of atria with verapamil (0.01 to 0.1 μM) or nifedipine (0.01 μM) at concentrations insufficient to produce a negative inotropic effect alone did not affect the action of adenosine.

Reduction of the calcium content of the medium to 0.9 mM (1/2 the control) decreased the amplitude of contractions, but did not appreciably affect the extent of augmentation by dilazep of the negative inotropic effect of adenosine. Furthermore, the uptake of [³H]-adenosine was scarcely affected by verapamil (0.01 to 1 μM), nifedipine (0.01 to 1 μM) or by reduction in the calcium content of the medium to 0.32 mM (1/6 the control).

Effect of dilazep on ileal muscle

In guinea-pig ileal longitudinal muscle, the uptake of [³H]-adenosine (8.8 nM, 0.1 μCi) was prevented by 0.1 μM dilazep, the T/M ratio for accumulation of [³H]-activity in 10 min being reduced to 1.41 ± 0.95 from control value of 6.65 ± 1.29. However, dilazep did not potentiate the inhibitory effect of adenosine on the twitch response induced by transmural stimulation (0.1 Hz, 1 ms, 25 V).

Discussion

The present experiments showed that the negative inotropic effects of adenosine and adenine derivatives, such as ATP, ADP, AMP and cyclic AMP, on guinea-pig atria were selectively potentiated by low concentrations of dilazep. This is analogous to the observation that dilazep potentiates the negative chronotropic effect of adenosine on guinea-pig atria (Buyniski *et al.*, 1972) and the coronary vasodilatory effect of adenosine (Buyniski *et al.*, 1972; Sano *et al.*, 1972).

The present results also showed that the essential structure for producing the negative inotropic effect on atria is adenine-ribose, and that an energy-rich phosphate group is not required. This conclusion is based on the following findings: (1) adenosine, which has no phosphate group, was more effective than ATP, ADP or AMP. (2) Unlike adenosine, adenine, with no ribose moiety, did not reduce contractions. (3) Inosine, which lacks the 6-amino group of adenosine, had only low activity (about 1/1000 that of adenosine). (4) The oxygen atom in the ribose moiety was necessary for activity, because 2'-deoxyadenosine with no oxygen had very low activity (about 1/300 that of adenosine). Therefore in the purine compounds tested, ribose, the oxygen atom in the ribose moiety and the 6-amino group were necessary for activity.

In the present work the possible mechanisms of the potentiating effect of dilazep considered were: (1) inhibition of adenosine uptake by the tissue; (2) prevention of adenosine degradation and (3) calcium antagonistic action.

It has been suggested that dipyridamole potentiates the action of adenosine by inhibiting adenosine uptake by erythrocytes (Pfleger *et al.*, 1969) or tissue (Pfleger *et al.*, 1969; Hopkins, 1973b; Kalsner, 1975).

From the present results it seems likely that the brevity of the action of adenosine could be due to rapid uptake of adenosine by atrial tissue and that inhibition of this uptake by dilazep could result in augmentation and prolongation of the action of adenosine by increasing its effective concentration at the site of action. This idea was deduced from the finding that dilazep reduced the tissue uptake of [^{3}H]-adenosine. However, the question arises, as to whether the reduction of adenosine uptake by 1/10 the control value, resulted in a 3 to 10 fold increase in the negative inotropic response of the atria to adenosine. The total amount of [^{3}H]-adenosine taken up in 20 min was, at most, 6% of that in the medium and the concentration of [^{3}H]-adenosine used was about 1/10 the concentration used in the contractility studies.

Hopkins (1973b) and Kalsner (1975) were dubious from studies on atria and coronary arteries as to whether a 50% reduction of adenosine uptake could cause 5 to 10 fold increase in the inhibitory action of adenosine. If this were the case, the potentiation by dilazep could not be satisfactorily explained as being due to inhibition of adenosine uptake.

In guinea-pig ileal muscle, dilazep always prevented the uptake of [^{3}H]-adenosine, but usually did not augment the inhibitory action of adenosine on the twitch response induced by transmural stimulation. This might suggest that in guinea-pig atria inhibition of adenosine uptake may not be the cause of the potentiating action of dilazep, or even be related to it.

Adenosine in the medium gradually disappeared with the concomitant formation of inosine and hypoxanthine during incubation with atrial muscle. In the presence of dilazep, both the disappearance of adenosine, and the formation of inosine and hypoxanthine, were retarded. In addition, the time course of the disappearance of adenosine was almost parallel to the modification in the negative inotropic effect of adenosine. Therefore, it seems likely that the brevity of the action of adenosine is due to degradation of adenosine by an enzyme, probably adenosine deaminase, in the atria, and that potentiation by dilazep is due to inhibition of this degradation. This idea, and the present observations, are compatible with the proposal that the short duration of action of adenosine (Clarke, Davoll, Philips & Brown, 1952; Bunag *et al.*, 1964) is attributable to rapid deamination of adenosine by an enzyme in erythrocytes, and that dipyridamole prevents the degradation of adenosine.

The effects of some coronary dilators are due to a calcium antagonistic action (Fleckenstein, Tritthart,

Fleckenstein, Herbest & Grün, 1969; von Vater, Kroneberg, Hoffmeister, Kaller, Meng, Oberdorf, Puls, Schlossmann & Stoepel, 1972; Kanazawa, Suzuki, Ino-oka, Takahashi, Mori, Maruyama, Ashikawa & Koiwa, 1974). The question therefore arises as to whether dilazep-induced potentiation results from a reduction of calcium in or near the membrane. In our experiments, it appears that the potentiation by dilazep is not due to its calcium antagonistic action, because other calcium antagonists, such as verapamil and nifedipine, did not augment the negative inotropic effect of adenosine or prevent its uptake and degradation.

It is still unknown whether ATP, ADP, AMP and cyclic AMP act directly or via some breakdown product of adenosine. Since ATP had an essentially similar effect to adenosine, it may be degraded to adenosine and thus act on the same site. If this is so, dilazep should potentiate the effect of ATP by preventing the uptake or degradation of adenosine, not by preventing the uptake of ATP because ATP is reported not to be taken up by tissue (Burnstock, 1972; Hopkins, 1973a). This idea is compatible with the present finding that adenosine was about 3 times more potent than ATP. However, judging from the time course of degradation of ATP to adenosine, the onset of the negative inotropic effect of ATP is too rapid to be due to formation of sufficient adenosine to have an inhibitory action.

It has been reported that, after adenosine has been taken up, it is converted to adenosine nucleotides (Jacob & Berne, 1960; Su & Bevan, 1970; Kuchii, Miyahara & Shibata, 1973; Hulme & Weston, 1974) or degraded to inosine or hypoxanthine (Koss *et al.*, 1962; Bunag *et al.*, 1964; Deuticke & Gerlach, 1966). In view of these reports and the present observations, dilazep may not affect the uptake of adenosine, but rather its subsequent phosphorylation to nucleotides by adenosine kinase or its degradation in the cell. These possibilities must be examined by further analysis of the metabolites accumulated in the tissue.

In summary, it is suggested from our results that dilazep potentiates the negative inotropic effects of adenosine on guinea-pig atria by preventing both adenosine degradation and its accumulation in the tissue.

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