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Effect of Diltiazem and its Metabolites on the Uptake of Adenosine in Blood: an In-vitro Investigation*

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Abstract—Using whole blood from man and rabbits, the effect of diltiazem, its metabolites, and other calcium antagonists on the uptake of adenosine has been described. The uptake and metabolism of adenosine was extremely rapid with a half-life in plasma of less than 30 s. Adenosine is rapidly and extensively metabolized to hypoxanthine. Metabolites of diltiazem, deacetyl diltiazem and deacetyl *O*-desmethyl diltiazem were considerably more potent than the parent drug. Diltiazem was one-tenth as active as verapamil, but more active than nifedipine or amlodipine. Dipyridamole was the most potent uptake-inhibitor tested (IC50 < 1 μM), whereas the angiotensin converting enzyme inhibitor enalapril was virtually devoid of any inhibitory activities (IC50 > 1000 μM). The results obtained from both man and rabbit were similar.

The importance of adenosine in regulating many biological functions has been increasingly recognized, especially in its effects on the cardiovascular system (Sollevi 1986). It is known that adenosine is released from the myocardium during ischaemia (Sollevi et al 1985a). Thus adenosine or its metabolites could be used as biochemical markers to quantify myocardial ischaemia (Sollevi 1986; DeJong 1988). There is also evidence which suggests that uptake and metabolism of adenosine may differ in patients with ischaemic heart disease and healthy subjects (Sollevi 1986). When given i.v. to patients during anaesthesia, adenosine increased cardiac output and reduced blood pressure and whole body oxygen consumption (Sollevi et al 1985b). In conscious, normal healthy subjects, however, i.v. adenosine has been shown to increase systolic blood pressure, heart rate, and plasma adrenaline and noradrenaline concentrations. This was thought to be caused by activation of the sympathetic nervous system (Biaggioni et al 1986). When given directly into a coronary artery, adenosine produced a dose-dependent increase in coronary sinus blood flow in conscious patients, and a typical chest pain which was not associated with electrocardiographic (ECG) or echocardiographic indications characteristic of ischaemia (Marzilli et al 1989). The intravascular effect of adenosine is short lived because it is rapidly taken up by erythrocytes and metabolized to inosine and hypoxanthine (Plagemann et al 1985).

Many therapeutic agents act by altering the normal physiological functions of adenosine (Clanachan et al 1987). For example, dipyridamole exerts its vasodilating effect by inhibiting the uptake of extracellular adenosine thereby prolonging the effect of adenosine (Belloni et al 1987; Conradson et al 1987). On the other hand, theophylline and other methylxanthines are competitive inhibitors of the binding of adenosine to its receptors and hence they antagonize the effects of adenosine and dipyridamole (Mustafa & Askar 1986; Sollevi 1986). There is also evidence to suggest that the calcium antagonists such as verapamil, nifedipine and diltiazem may inhibit the uptake of adenosine, although they differ considerably in their potencies (Ford et al 1985; Belloni et al 1987).

Diltiazem is a benzothiazepine calcium antagonist widely used in the treatment of angina and related disorders (Kendall & Okopski 1986). It is extensively metabolized to a host of metabolites some of which have potent pharmacological activities (Kiyomoto et al 1983; Yabana et al 1985; Sugawara et al 1988). Studies from our laboratory and others have shown that some of the metabolites of diltiazem, such as deacetyl diltiazem (M1) and deacetyl *N*-monodesmethyl diltiazem (M2) have considerably longer effective half-lives than the parent diltiazem (Boyd et al 1989; Yeung et al 1990). Thus these metabolites may contribute significantly to the overall therapeutic and adverse effects of the administered drug. Apart from very limited data which suggest that the anti-platelet and vasodilating effects of some of the diltiazem metabolites were comparable with those of diltiazem (Kiyomoto et al 1983; Yabana et al 1985; Schoemaker et al 1987), little is known of other pharmacological properties of these metabolites. The present studies describe the effect of diltiazem, its metabolites and other calcium antagonists on the uptake of adenosine in whole blood.

Materials and Methods

Chemicals

Diltiazem hydrochloride (Cardizem) was supplied by Nordic Laboratories (Quebec, Canada). The metabolites of diltiazem were received as gifts from the Tanabe Seiyaku Co. (Osaka, Japan) via Dr P. Leonard of Nordic Laboratories. The chemical names of the metabolites and their abbreviations are listed in Table 1. The other therapeutic agents were supplied by their respective manufacturers: nifedipine (Miles Inc., CT, USA), amlodipine (Pfizer Inc., CT, USA), verapamil hydrochloride (Knoll Pharmaceuticals Canada, Ont., Canada), alprazolam (UpJohn Co., MI, USA), dipyrida-

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Table 1. Chemical names and their abbreviations of the diltiazem metabolites.

Chemical name	Abbreviation
N-Monodesmethyl diltiazem	MA
Deacetyl diltiazem	M1
Deacetyl *N*-monodesmethyl diltiazem	M2
Deacetyl *O*-desmethyl diltiazem	M4
Deacetyl *N*, *O*-didesmethyl diltiazem	M6
Deacetyl diltiazem *N*-oxide	M1NO
Deacetyl *O*-desmethyl diltiazem *N*-oxide	M4NO

mole (Boehringer Ingelheim Canada Ltd, Ontario, Canada), and enalapril maleate (Merck Frosst Canada Inc., Quebec, Canada). Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) was supplied by Burroughs Wellcome Co. (Research Triangle Park, NC, USA).

[2-^{3}H]Adenosine (27 Ci mmol^{-1}) was purchased from Amersham Canada Ltd (Ontario, Canada). "Stopping Solution", prepared in normal saline, contained 26 μM EHNA, 100 μM dipyridamole and 4 mM EDTA (Gewirtz et al 1987). All other solvents and chemicals were reagent grade, and were used without purification. Thin layer chromatography sheets were aluminium-backed Silica Gel 60 F_{254}, 0·2 mm thickness (Merck, Germany). The developing solvent was a mixture of isobutyric acid: water: ammonia (66:33:1).

Adenosine uptake protocol

Fresh whole blood obtained from healthy volunteers and New Zealand White rabbits was used. Blood from volunteers was collected via an antecubital vein in heparinized evacuated glass tubes (Vacutainers, Becton Dickinson, Ontario, Canada). Blood from rabbits was collected under halothane anaesthesia by cardiac puncture. The blood was anticoagulated with heparin in a solution of isotonic phosphate buffered pH 7·4 saline (PBS-heparin) (9 mL of blood + 1 mL of PBS containing 100 int. units of sodium heparin). PBS (0·05 mL) containing various concentrations (0·01–1000 μM) of drug or metabolite was added to 0·2 mL of the anticoagulated whole blood. The mixture was vortexed (Multi-Tube Vortexer, Canlab, New Brunswick, Canada), and 0·1 mL of a working [2-^{3}H]adenosine tracer solution containing approximately 10000 counts min^{-1} was added. The tracer solution was prepared by diluting the stock solution (1 mCi mL^{-1}) with non-radioactive adenosine so that addition of 10000 counts min^{-1} of the tracer produced a final concentration of 6 μM of adenosine in the incubation medium. Uptake of adenosine was terminated after 2 s incubation by rapid addition of 0·2 mL of the "Stopping Solution". The mixture was vortexed, and centrifuged at 4°C for 10 min (1760 *g*). A sample of the supernatant (0·2 mL) was mixed with 10 mL of scintillation fluid and the radioactivity was determined in a liquid scintillation counter (Model 3133T, Beckman Instrument (Berkeley, CA, USA). The uptake of adenosine at each drug or metabolite concentration was determined in quadruplicate.

Separate experiments were also carried out in which [2-^{3}H]adenosine was incubated in whole human blood for 0, 2, 5, 10, 60, 120 and 300 s before the reaction was terminated. The "0" time incubation was prepared by adding the "Stopping Solution" to the whole blood before the tracer. The experimental procedure was as described above except that after centrifugation, both erythrocyte and plasma fractions were treated with 0·1 mL of perchloric acid (3·5 M). After centrifugation (1760 *g*, 10 min, 4°C) and neutralization with potassium carbonate, the supernatants were lyophilized and the dry residues of both erythrocyte and plasma fractions were analysed by thin layer chromatography (TLC). The developing solvent completely resolved adenosine and its metabolites (ATP, ADP, inosine, and hypoxanthine). The TLC sheet was viewed under an ultraviolet light, and the spots corresponding to each chemical species were scraped off and placed in plastic scintillation vials (Fisher Scientific, Nova Scotia, Canada). The powder was extracted with 1 mL of a mixture of methanol: water (1 : 1) for 5 min. To the mixture was added 10 mL of scintillation fluid, and the radioactivity corresponding to each spot determined.

Data analysis

The data obtained from the uptake experiment were presented graphically as uptake of adenosine measured at 2 s of incubation vs log concentrations of drug or metabolite. The uptake value determined at each drug or metabolite concentration was expressed as a percentage of the control value (i.e. uptake of adenosine in the absence of drug or metabolite). The inhibitory concentrations at which there was a 50% inhibition of uptake of adenosine (IC50) were determined by linear regression analysis of the log concentration-response curve. The half-life ($t_{\frac{1}{2}}$) of adenosine in plasma was determined by linear regression analysis of the log transformation of the radioactivity remaining in the plasma fraction after 0, 2 and 5 s of incubation.

Results

The effectiveness of the "Stopping Solution" in inhibiting the uptake and metabolism of adenosine was instantaneous after addition and prolonged for at least 30 min under the described experimental conditions (results not shown). The radioactive adenosine added was not metabolized such that over 90% of the radioactivity in the plasma corresponded to adenosine. Approximately 15% of the radioactivity added to the whole blood was found in the erythrocytes at 0 time (Fig. 1A), which could mainly be due to residual radioactivity from the trapped plasma which was not completely removed by centrifugation. More than 80% of the radioactivity in the erythrocytes was intact adenosine, and the remaining radioactivity corresponded to inosine and hypoxanthine.

The uptake of adenosine by human erythrocytes was extremely rapid. The $t_{\frac{1}{2}}$ of adenosine in plasma was less than 40 s when estimated by total radioactivity remaining in the plasma fraction. It was less than 30 s when radioactivity corresponding to adenosine was used in obtaining the parameter. Diltiazem at 100 μM prolonged the $t_{\frac{1}{2}}$ of adenosine considerably (Table 2). Adenosine was also rapidly and extensively metabolized in blood such that after 1 min incubation, the radioactivity remaining as intact adenosine was less than 10 and 20% of the total radioactivity in erythrocytes and plasma, respectively. The major part of the radioactivity was attributable to hypoxanthine. In erythrocytes, ATP constituted the second major component whereas it was only a minor constituent in plasma (Fig. 1).

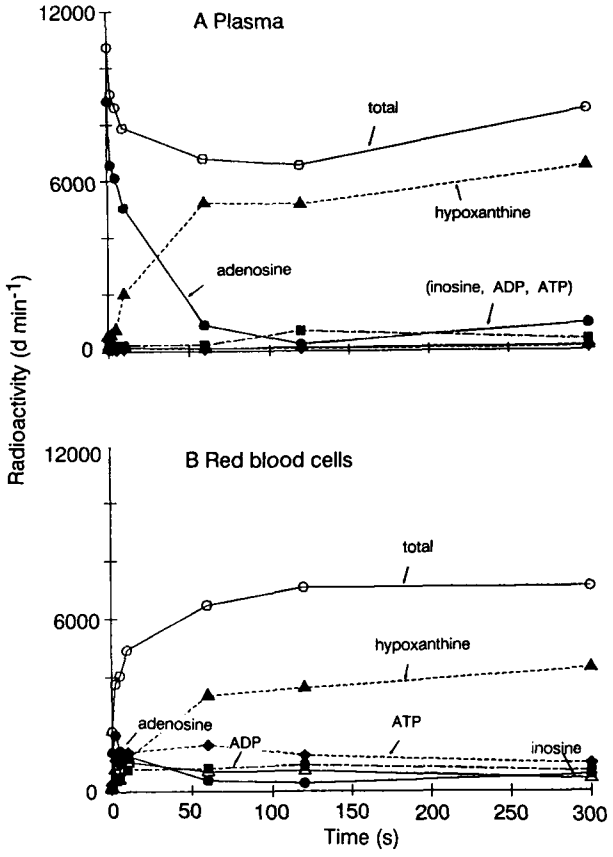

FIG. 1. Uptake and metabolism of adenosine in whole blood. A, plasma and B, erythrocytes.

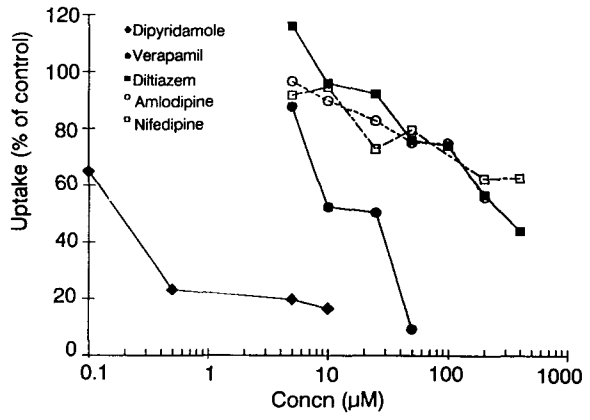

FIG. 2. Log concentration-response curves for dipyridamole and calcium antagonists.

Table 2. Plasma half-life of adenosine in human blood.

Condition	$t_{\frac{1}{2}}$(s)[a]	$t_{\frac{1}{2}}$(s)[b]
Drug-free	23·0	38·2
Diltiazem (100 μM)	37·5	111·3

[a] $t_{\frac{1}{2}}$ values were calculated from the radioactivity corresponding to adenosine determined at 0, 2, and 5 s of incubation by linear regression analysis of the log radioactivity vs time data.
[b] Values were calculated from total radioactivity.

Table 3. Effect of diltiazem and its metabolites on the uptake of adenosine in blood[a].

Drug or metabolite	Man	Rabbit
Diltiazem	300·6	93·7
MA	537·6	306·8
M1	92·5	87·2
M2	>1000	>1000
M4	185·5	38·8
M6	>1000	180·3
M1NO	>1000	>1000
M4NO	>1000	>1000

[a] Values are inhibitory concentrations (μM) at which there was 50% inhibition of uptake of adenosine (IC50).

Table 4. Effect of calcium antagonists and related compounds on the uptake of adenosine in blood[a].

Drugs	Man	Rabbit
Dipyridamole	0·2	0·3
Verapamil	15·8	29·6
Alprazolam	47·9	29·6
Diltiazem	300·6	93·7
Amlodipine	653·1	138·3
Nifedipine	721·4	912·3
Enalapril	>1000	>1000

[a] Values are inhibitory concentrations (μM) at which there was 50% inhibition of uptake of adenosine (IC50).

Compared with parent diltiazem, the metabolites, M4 and M1 (Table 1) were more potent in inhibiting the uptake of adenosine, whereas M2, M6 and the *N*-oxide metabolites (M1NO and M4NO) were relatively inactive. Similar rank orders of potency were obtained in both man and rabbit blood although it appeared that diltiazem and its metabolites were more active in rabbit blood (lower IC50 values) (Table 3). Relative to other calcium antagonists, diltiazem was considerably less potent than verapamil but more potent than nifedipine and amlodipine (Fig. 2). Among all the agents tested, dipyridamole was the most potent (IC50 = 0·2 μM) followed by verapamil, alprazolam, diltiazem, amlodipine, and then nifedipine. Enalapril was virtually devoid of any activity. Close similarity in the rank order of potencies of the agents tested was again observed between the human and rabbit blood (Table 4).

Discussion

The results obtained in this study confirm the data reported earlier by others (Plagemann et al 1985) that uptake and metabolism of adenosine by erythrocytes is extremely rapid. The concentration of adenosine in erythrocytes was maximum at the first sampling time (2 s after addition), and declined rapidly with a corresponding rise in the concentrations of hypoxanthine and ATP. An attempt was made to determine the rate of uptake by using the data obtained between 0 and 5 s for calculating the plasma $t_{\frac{1}{2}}$ since at this

time, the major part of the adenosine had not been metabolized. However, because of the rapidity of these processes, the $t_{\frac{1}{2}}$ values as determined in this study were hybrid parameters reflecting both uptake and metabolism of adenosine.

The results reported in this study confirm those by others that calcium antagonists inhibit the uptake of adenosine (Ford et al 1985). It is interesting to note that verapamil was 10 times more potent than diltiazem, which was in turn more than twice as active as either nifedipine or amlodipine (Table 4). These activities differ from their activities in relaxing smooth muscles, in which the dihydropyridine calcium antagonists were more potent than verapamil or diltiazem (Mustafa & Askar 1986), and suggest that the mechanism of receptor mediated relaxation of smooth muscle is different from that of inhibition of erythrocyte uptake of adenosine. This is not surprising as the action of adenosine on smooth muscle is thought to be receptor-mediated while only adenosine transporters are present on erythrocytes. The well-known adenosine uptake inhibitor dipyridamole (Afonso 1970) was the most potent agent tested in this study, but alprazolam, which has been shown to possess calcium channel blocking activities (Rampe & Triggle 1986), also showed an appreciable inhibitory effect on the uptake of adenosine. Thus there might be a relationship between calcium antagonism and uptake or metabolism of adenosine. Preliminary experiments carried out in our laboratory have shown that calcium enhanced the metabolism of adenosine in erythrocytes (unpublished results), and it is yet to be determined whether or not calcium antagonists would inhibit this role in the metabolism of adenosine.

Of the metabolites of diltiazem available for evaluation, M4 and M1 were more potent than diltiazem in inhibiting the uptake of adenosine (Table 3). Other studies have indicated that the anti-platelet activity of M4 and M1 were also more potent than diltiazem (Kiyomoto et al 1983). Thus the mechanism of the effect of these agents on the uptake of adenosine and platelet aggregation may be similar. Clinically, M1 but not M4 is a major metabolite of diltiazem in plasma (Boyd et al 1989; Yeung et al 1990); at steady state, plasma concentrations of M1 could reach significant levels in patients (Hung et al 1988; Höglund & Nilsson 1989). Thus the biological effects of M1 may contribute significantly to the overall therapeutic or adverse effect of diltiazem.

Because of the importance of adenosine in the normal physiology and pathophysiology of the coronary circulation, drugs which alter the normal availability of adenosine will have significant effects on coronary tone. Adenosine, depending on regional concentration, can enhance vasodilation, increase coronary flow (Klassen & Armour 1990) and decrease ischaemia. On the other hand, it may also dilate healthy regions and decrease perfusion pressure to adjacent regions subtended by a coronary stenosis. This may redistribute coronary blood flow away from an ischaemic region and actually precipitate ischaemia. This phenomenon underlies the use of dipyridamole as a test to detect myocardial ischaemia when vessel obstruction is significant (Picano 1989). It is known that nucleoside transport inhibitors (e.g. dipyridamole, dilazep) block both influx and efflux of adenosine (Meghji et al 1988). Thus anginal pain may also be related to extensive blockade of adenosine efflux during ischaemia.

The calcium antagonists are therapeutic agents widely used in the treatment of ischaemic heart disease and yet clinical experience with the efficacy of these agents following myocardial infarction is controversial (Yusuf et al 1988a, b). The variations in response could be due to patient heterogeneity such that only certain groups of patients would benefit from the calcium antagonists. It is also possible that the inherent differences among the calcium antagonists in their ability to alter the normal physiological role of adenosine as demonstrated in this study may account for some of the variations. Further studies are needed to evaluate the clinical relevance of inhibition of adenosine uptake for the calcium antagonists and other therapeutic agents used in the treatment of myocardial ischaemia.

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