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NUCLEOSIDE INFLUX AND EFFLUX IN GUINEA-PIG VENTRICULAR MYOCYTES

INHIBITION BY ANALOGUES OF LIDOFLAZINE

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Abstract—Adenosine influx and formycin B influx and efflux were characterized in guinea-pig ventricular myocytes at 22°. Transport by both modes was saturable and inhibited by nitrobenzylthioinosine (NBMPR), indicating the presence of an equilibrative NBMPR-sensitive nucleoside transporter in the cardiomyocytes. The kinetic constants for influx and efflux of formycin B, a non-metabolized nucleoside, were similar, suggesting that the nucleoside transporter exhibits symmetrical kinetics (apparent K_m 490 ± 160 and 700 ± 140 μM; V_{max} 6.5 ± 1.7 and 3.5 ± 0.3 nmol/10^6 cells per min for influx and efflux, respectively). No evidence was found of either NBMPR-insensitive equilibrative nucleoside transport or sodium-dependent concentrative nucleoside transport. Inhibition of adenosine influx (apparent K_m100 ± 33 μM), by lidoflazine and the analogues mioflazine, soluflazine and R73-335, gave average K_i values of 730, 100, 64 and 2.9 nM, respectively. These compounds also inhibited formycin B efflux with a similar potency to that of adenosine influx. NBMPR-sensitive nucleoside transport was associated with high affinity binding of NBMPR (apparent K_d~1 nM; 9.6 × 10^5 sites/cell). Specific binding of NBMPR was also inhibited by lidoflazine and its analogues. Mioflazine and soluflazine were 20–30-fold more potent at inhibiting NBMPR-sensitive nucleoside influx in guinea-pig erythrocytes than ventricular myocytes, indicating that the potency of some of the compounds studied is tissue dependent.

Key words: adenosine transport; nitrobenzythioinosine; formycin B; cardiomyocytes; Na^+/nucleoside transporter (SNST1)

Reperfusion injury following a period of myocardial ischemia is produced by a variety of influences, including inadequate perfusion and free radical mediated injury. The resulting metabolic and functional abnormalities and depressed ATP levels may persist for hours or days [1]. Adenosine produced during ischemia by the enhanced breakdown of ATP passes into the interstitial space and may reach the vascular lumen via intercellular clefts or at high concentrations by passing through the endothelial cells [2]. Adenosine released in this way or exogenously applied enhances the recovery of ATP levels [3–5], and reduces the metabolic demands of the myocardium [1]. More importantly, at high concentrations, adenosine inhibits neutrophil adherence to endothelium and superoxide anion release [6]. This not only attenuates tissue damage but also inhibits the blockage of capillaries and thereby the development of the no-reflow phenomenon [7].

Interstitial adenosine is rapidly cleared by uptake into the surrounding tissues via nucleoside transporters [8] or by washout on reperfusion [2]. Studies on rat and guinea-pig myocyte preparations have revealed a saturable nucleoside transport system inhibitable by NBMPR†, dilazep and dipyridamole, suggesting the presence of an NBMPR-sensitive facilitated diffusion nucleoside transporter [9–11]. However, the apparent affinity for adenosine uptake in these myocyte preparations ranged from 6 to 146 μM. The susceptibility of adenosine uptake in myocytes to inhibition by other compounds is unknown. In addition, no studies on the efflux of nucleosides from myocytes have been conducted. At least four other nucleoside transport systems, in addition to the NBMPR-sensitive carrier, have been demonstrated in mammalian cells. These include an equilibrative system resistant to inhibition by NBMPR, and three Na^+-dependent co-transporters with unique substrate specificities but all accepting adenosine as a permeant [12–15]. On the basis of northern blots a recently cloned rabbit renal Na^+/nucleoside transporter (SNST1) was reported to be abundantly present in heart [16]. Studies aimed at a direct demonstration of the kinetic presence of this Na^+-dependent system in heart have not been performed.

Lidoflazine and its derivatives are inhibitors of equilibrative nucleoside transport and specific [^{3}H]-NBMPR binding in a variety of tissues and cells from different species [17–20]. Moreover, some of these compounds have been shown to enhance myocardial adenosine concentrations during ischemia and to potentiate interstitial adenosine levels on reperfusion resulting in an increase in ventricular function [2, 21, 22]. A theoretical model has been proposed to explain the elevation of interstitial adenosine by dipyridamole, which acts symmetrically

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† Abbreviations: NBMPR, nitrobenzylthioinosine, {6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine}; BSA, bovine serum albumin.

on efflux and influx [23], but increases in interstitial adenosine levels could be optimised by using nucleoside transport inhibitors selective for influx compared to efflux. The aim of this study therefore was to investigate the mechanisms of nucleoside influx and efflux in guinea-pig cardiac myocyte preparations. In addition, a comparison of the effects of lidoflazine and analogues on influx and efflux was performed using adenosine and the non-metabolized inosine analogue formycin B, respectively.

MATERIALS AND METHODS

Solutions

Modified Krebs Henseleit solution (KHS). 127 mM NaCl, 4.6 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, 8.3 mM glucose, 20 mM taurine, 10 mM creatine, 2 mM Na pyruvate, 0.5 mM uric acid, 1 mM L-arginine, L-glutamic acid and D-ribose, pH 7.4, and gassed with 95% (v/v) O_2/5% (v/v) CO_2.

Assay medium (AM). 137 mM NaCl, 2.7 mM KCl, 6.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10.0 mM glucose, 0.5 mM EGTA and 5% (w/v) polyvinylpyrrolidone (PVP40), pH 7.4

Digestion medium (DM). 50 mL KHS plus 80 μM $CaCl_2$, 0.10% (w/v) collagenase 200 U/mg (Worthington Type 1), 0.012% (w/v) dispase (Boehringer Mannheim), 0.012% (w/v) trypsin 1 : 250 (Gibco) and 0.1% (w/v) BSA.

Myocyte preparation

Ventricular myocytes were prepared by a combination of enzymic digestion and mechanical dissociation [24, 25]. Briefly, heparinized (600 U/100 g) male and female guinea-pigs, body weight 250–500 g, were anaesthetized with 24 mg of nembutal per 100 g body weight, and the heart was isolated and mounted on a Langendorff perfusion column at 37°. The heart was perfused with KHS plus 750 μM $CaCl_2$ for 4 min, then with KHS plus 100 μM EGTA for 4 min and finally with DM on recirculating perfusion for 40 min. The ventricles were then cut away from the rest of the myocardial tissue, chopped coarsely and resuspended in the same protease solution for 30 min, with gentle gassing from below to maximize cell dissociation. Following filtration through nylon mesh, 200 μm pore size, the cells were pelleted at 37 g for 1 min. The pellet was resuspended in AM at 22°. Cell viability was determined by the proportion of cells with rod-like morphology and found to be equivalent to values obtained by trypan blue dye exclusion.

Nucleoside transport in myocyte preparations

Myocytes ($0.1–0.04 \times 10^6$ viable cells) were preincubated, in AM, in the presence or absence of inhibitor solution for 15 min prior to assay, in a total volume of 1 mL. The cells were then gently spun out and the inhibitor solution was replenished. Influx at 22° was initiated by the addition of 50 μL of radiolabelled nucleoside to 1 mL of cells and terminated by spinning 1 mL of the solution through silicone oil (density 1.02 g/mL). The aqueous and silicone oil layers were removed by suction, the tubes were wiped, and the pellet was digested with 0.1 N NaOH before determination of radioactivity. Blank values due to entry into the extracellular space of the pellet were obtained by processing cell samples treated with 20 μM dilazep and simultaneously exposed to radioactively labelled nucleoside at 0°. Transport rates were calculated after subtraction of the blank.

For efflux experiments, cells were preloaded for at least 1 hr in the presence of varying concentrations of [^{3}H]formycin-B, the last 15 min in the presence or absence of test compound. Efflux was initiated by the addition of 1 mL AM solution containing the appropriate test compounds to 50 μL cells, and terminated as for influx. Non-mediated efflux of [^{3}H]formycin B was determined by measuring the rate of formycin B efflux into medium containing 20 μM NBMPR.

The intracellular volume of the cell suspensions was measured using [^{3}H]H_2O as a marker and [^{14}C]-inulin to correct for extracellular H_2O in the cell pellet (1 and 0.2 μCi/mL final sp. radioactivity, respectively). The average intracellular water space was 20.6 ± 2.9 μL/10^6 cells (±SEM) (N = 34).

Metabolism

Cells were treated as for transport assays and spun through the silicone oil into 50 μL of 3 N perchloric acid to terminate metabolism. The acid extract was neutralized with 5 N KOH and the radioactivity associated with the possible metabolites of adenosine determined by TLC as described previously [26].

Cardiac membrane preparation

Heparinized male and female guinea-pigs, body weight 250–500 g, were anaesthetized with nembutal as above. Hearts were removed and dissected free of other tissue. Crude membrane fractions (15,000 g pellet) were prepared as described previously [27] and stored at −70° in 50 mM Tris–HCl (pH 7.4 at 22°).

NBMPR binding

Steady-state binding of [^{3}H]NBMPR to crude cardiac membranes and intact cells was determined at 22° by filtration and centrifugation through oil, respectively [27, 28]. Specific binding is defined as the difference in the amount bound in the presence and absence of 10 μM unlabelled NBMPR.

Uridine influx by guinea-pig erythrocytes

Washed (0.2 mL portions, 10% haematocrit) [18] were incubated at 22° with 1 mL of the test compound for 15 min. The cells were collected by centrifugation (10 sec, 12,000 g), 1 mL of medium was removed and the cells were resuspended in a final volume of 0.2 mL of the test compound. Uridine influx was initiated by the addition of 0.2 mL of [^{3}H]uridine and after 5 min, a time interval that allowed initial rates of uridine influx to be measured [29], the incubations were terminated by addition of 1 mL ice-cold iso-osmotic NaCl medium containing 10 μM NBMPR [18, 29]. The cells were washed four times with 1 mL of the ice-cold stop medium using a microcentrifuge (10 sec, 12,000 g). Radioactivity associated with the cell pellet and estimates of non-

mediated uridine permeation were determined as previously described [18, 29].

Data analysis

All measurements were in triplicate, and each experiment was performed at least three times. For inhibition data, non-linear regression analysis was carried out using the computer program EBDA which provides an estimate of the IC_{50} value and the slope factor. Inhibition constants (K_i) were obtained by the application of the Cheng and Prussoff equation [30]. The concentration dependence data of transport and [^{3}H]NBMPR binding were analysed using the computer packages ENZFITTER and P.FIT and the simple Michaelis–Menten equation.

Chemicals

[2,5′,8-^{3}H]Adenosine (58 Ci/mmol) and inulin [^{14}C]carboxylic acid (10.7 mCi/nmol) were purchased from Amersham (U.K.) and ICN (High Wycombe, U.K.), respectively, and [G-^{3}H]formycin-B (7 Ci/mmol) and [^{3}H]NBMPR (23–36 Ci/mmol) were obtained from Moravek Radiochemicals (Brea, CA, U.S.A.). The substituted piperazine flazine analogues (R73-335; soluflazine, R64-719; miofla-zine, R51-469 and lidoflazine, R7904) and dilazep were generous gifts from Dr H. Van Belle, Janssen Pharmaceutics (Belgium) and Hoffman-La Roche and Co. (Switzerland), respectively. NBMPR and eserine were obtained from Sigma Chemical Co. (Poole, U.K.).

RESULTS

In preliminary experiments, adenosine uptake (20 and 200 μM) by guinea-pig ventricular myocytes at 22° was linear for up to 150 sec. Initial rates of total adenosine uptake were 0.24 and 2.3 nmol/10^6 cells/min at 20 and 200 μM adenosine, respectively, and 20 μM NBMPR inhibited 82% of the total uptake at 20 μM adenosine. The major metabolites of accumulated radioactivity following an incubation period of 1 min at 50 μM extracellular [^{3}H]adenosine were adenine nucleotides. The intracellular adenosine concentration was 4.3 ± 0.8 μM (N = 3). At such low intracellular concentrations compared to the external concentration, little backflux of adenosine will occur. In other experiments, the time course for the uptake of 50 μM [^{3}H]adenosine in the absence or presence of iodotubericidin (10 μM), an inhibitor of adenosine kinase, was similar. These results, together with the linearity of the time course studies for adenosine uptake, demonstrated that initial rates of adenosine influx at 22° could be measured over a 1 min time interval.

The concentration dependence of adenosine uptake by guinea-pig cardiac myocytes is shown in Fig. 1. Total uptake was fitted to a two-component system comprised of a saturable and a linear process, whereas uptake in the presence of 20 μM NBMPR was linear. The kinetic constants of the saturable component, which conformed to simple Michaelis–Menten kinetics, were determined by subtracting the linear component estimated in the presence of NBMPR from the total uptake values. Least squares fit of the data in Fig. 1 gave an apparent K_m of 71 ± 13 μM with a V_{max} of 1.8 ± 0.2 nmol/10^6 cells/min. The mean values from three experiments were 100 ± 33 μM for the apparent K_m with a V_{max} of 3.1 ± 0.84 nmol/10^6 cells/min (mean ± SEM, N = 3).

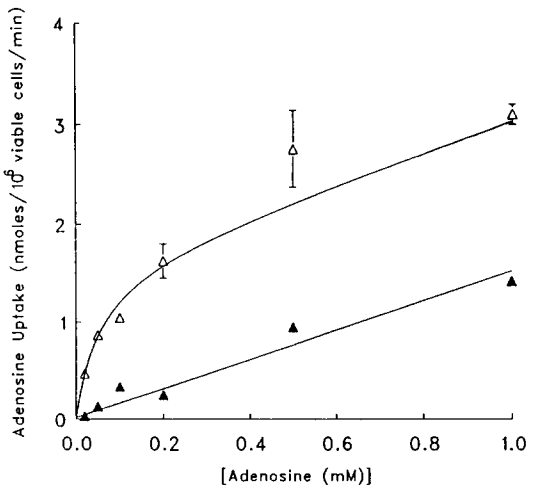

Fig. 1. Concentration dependence of adenosine uptake by guinea-pig cardiac myocytes. Ventricular myocytes were incubated for 1 min at 22° with graded concentrations of [^{3}H]adenosine in the absence (△) or presence (▲) of 20 μM NBMPR. The kinetic constants of uptake were determined by non-linear least squares fit of the Michaelis–Menten equation to the difference in uptake values in the presence and absence of NBMPR and gave a K_m value of 71 ± 13 μM with a V_{max} of 1.8 ± 0.2 nmol/10^6 cells per min. SEM bars are shown unless obscured by the symbols. Data are from a representative experiment.

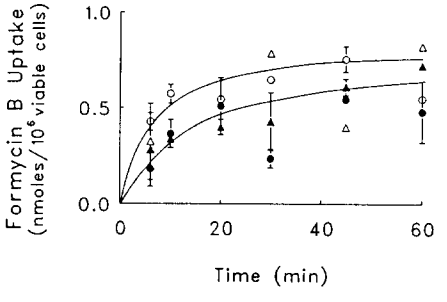

Fig. 2. Lack of sodium dependency of [^{3}H]formycin B uptake in guinea-pig ventricular myocytes. Myocytes were washed and resuspended in Na^+-containing AM medium (●, ○) or Na^+-free AM medium (NaCl and Na_2HPO_4 replaced by *N*-methyl D-glucamine and K_2HPO_4, respectively; (▲, △). Uptake of 50 μM [^{3}H]formycin B in the presence and absence of sodium ions was measured at 22° in the presence (solid symbols) and absence (open symbols) of 10 μM dipyridamole. Data points represent the average of triplicate values ±SEM.

The results of Fig. 1 demonstrate that as much as 50% of the total flux of adenosine at an extracellular concentration of 1 mM is insensitive to inhibition by

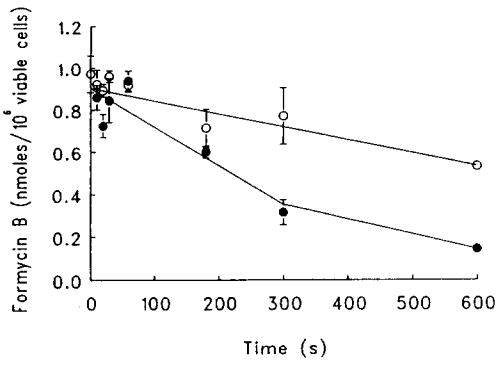

Fig. 3. Time course of formycin B efflux from guinea-pig ventricular myocytes. Cells were preloaded with 50 μM [^{3}H]formycin B and the efflux of radiolabel at 22° was determined in the absence (●) or presence (○) of 20 μM NBMPR and expressed as the amount of formycin B remaining with the cells. Values represent the average of triplicate estimates; SEM bars are shown unless obscured by the symbols. Data are from a representative experiment.

NBMPR. The linear nature of the NBMPR-resistant component of adenosine uptake suggests that it represents either simple diffusion or a mediated system with a low affinity for adenosine. To test whether a mediated NBMPR-insensitive nucleoside transporter was present, the ability of nucleosides and high concentrations of nucleoside transport inhibitors, such as dilazep and dipyridamole to inhibit the linear component of adenosine influx, was examined. No significant inhibition was observed. In further experiments, 50 μM [^{3}H]-formycin-B uptake into cardiac myocytes in the presence or absence of sodium ions was compared and found to be similar. Moreover, prolonged time course studies (up to 60 min) in the presence of 10 μM dipyridamole to block nucleoside efflux via the equilibrative nucleoside transporter, failed to detect a Na^+-dependent concentrative accumulation of formycin-B (see Fig. 2). Similarly, the uptake of 50 μM [^{3}H]adenosine at 37° in the presence of iodotubercidin (10 μM) was unaffected by replacing sodium ions with *N*-methyl D-glycamine ions. The above results suggest the presence of only an NBMPR-sensitive equilibrative nucleoside transporter in guinea-pig ventricular myocytes.

A requirement for efflux studies is that either the permeant must be non-metabolized or the cells must be rendered incapable of metabolizing the substrate. Formycin-B, an analogue of inosine, has been demonstrated to be only poorly phosphorylated in a variety of mammalian cells [31]. Influx of formycin-B by cardiac myocytes was saturable (apparent K_m 490 ± 160 μM; V_{max} 6.5 ± 1.7 nmol/10^6 cells/min; mean ± SEM; N = 3) and sensitive to inhibition by NBMPR. Moreover, formycin-B inhibited both adenosine influx and [^{3}H]NBMPR binding in guinea-pig cardiomyocytes (see Table 1). These results indicate that formycin-B is a permeant for the saturable adenosine influx pathway and as such should be a suitable model nucleoside for efflux studies in cardiomyocytes. Control studies demonstrated no significant metabolism of formycin-B (>95% of the intracellular radioactivity following a 2 hr incubation of guinea-pig cardiomyocytes with 50 μM [^{3}H]formycin-B cochromatographed with unlabelled formycin-B). Figure 3 shows the time course of 50 μM formycin B efflux in the presence or absence of 20 μM NBMPR by guinea-pig cardiomyocytes. Efflux was linear for up to 5 min and estimates of the NBMPR-sensitive component of efflux revealed that it was saturable and conformed to Michaelis–Menten kinetics (apparent K_m 700 ± 140 μM; V_{max} 3.5 ± 0.3 nmol/10^6 cells/min; mean ± SEM; N = 3) (Table 2).

The ability of a series of known nucleoside transport inhibitors to interact with the nucleoside transport mechanism in guinea-pig cardiomyocytes was studied by comparing the inhibitory potency of the compounds for adenosine influx and formycin-B efflux (Figs 4 and 5). Since many of the compounds tested are lipophilic and can be incorporated into the cell membrane, the potential problem of inhibitor depletion was minimized by the use of a large volume and/or a preincubation step (see Materials and Methods). Each analogue of lidoflazine exhibited a similar potency for inhibition of both adenosine influx and formycin-B efflux by the guinea-pig cardiomyocytes with R73-335 being the most potent and lidoflazine the least potent (Table 1). With the exception of lidoflazine, the dose–response curves of Figs 4 and 5 were monophasic with average slope values not significantly different from 1. NBMPR, dilazep and dipyridamole were potent inhibitors of adenosine influx by the cardiomyocytes (Table 1) and also gave monophasic dose–response curves. The initial rate of adenosine influx at 100 nM NBMPR was similar to that at 20 μM dilazep, confirming the absence of an NBMPR-insensitive nucleoside transporter in the cells. Lidoflazine, mioflazine, soluflazine and R73-335 were also shown to inhibit uridine influx via the NBMPR-sensitive nucleoside transporter in guinea-pig erythrocytes with an order of potency similar to that observed in the guinea-pig ventricular myocytes (Table 3).

Degradation of the analogues of lidoflazine by esterases has been reported, resulting in an underestimation of the apparent inhibition constants for blocking nucleoside transport activity [32]. Inhibition of adenosine influx by guinea-pig cardiomyocytes was therefore determined for soluflazine in the presence and absence of 10 μM eserine, a potent ester hydrolase inhibitor [32]. Eserine had no significant effect on the K_i values determined (49 ± 15 and 32 ± 3 nM in the presence and absence of eserine, respectively).

The concentration dependence of specific steady-state [^{3}H]NBMPR binding to guinea-pig ventricular myocytes and crude cardiac membranes was saturable (apparent K_d 1.2 ± 0.20 (N = 4) and 0.67 ± 0.27 (N = 3) nM; B_{max} 1.6 ± 0.6 ± mol/10^6 cells and 1.1 ± 0.4 pmol/mg protein, respectively). Scatchard plots of the specific binding data were linear, indicating a single population of high-affinity NBMPR binding sites. In a control study, the binding constants of [^{3}H]NBMPR binding to guinea-pig cardiac membranes prepared from freshly isolated

Table 1. Comparison of the affinities of inhibitors for the NBMPR-sensitive nucleoside transport system of guinea-pig ventricular myocytes derived from [^{3}H]NBMPR binding and transport studies

Inhibitor	Apparent K_i values		
	[^{3}H]NBMPR binding	Adenosine influx	Formycin B efflux
NBMPR	1.6 ± 0.7 nM	4.7 ± 3.1 nM	1.9 ± 0.6 nM
Dilazep	4.5 ± 2.3 nM	4.9 ± 2.4 nM	ND
Dipyridamole	44 ± 3 nM	20 ± 27 nM	ND
Lidoflazine	380 ± 60 nM	730 ± 400 nM	230 ± 30 nM
Mioflazine	41 ± 22 nM	100 ± 31 nM	180 ± 50 nM
Soluflazine	19 ± 5 nM	64 ± 21 nM	17 ± 4 nM
R73-335	15 ± 2 nM	2.9 ± 1.2 nM	12 ± 4.5 nM
Formycin B	1700 ± 110 μM	980 ± 320 μM	ND

Adenosine influx (50 μM), formycin B efflux (20 μM) and site-specific [^{3}H]NBMPR binding (1 nM) were determined as described in the text. The concentration of inhibitor that caused 50% inhibition (IC_{50} values) was determined from the dose–response inhibition curves using the Ligand data analysis package (see Figs 4 and 5). Where appropriate, K_i values were calculated from the equation $K_i = IC_{50}/(1 + [\text{Ligand}]/K_m)$ or K_d, where K_m was taken as 100 and 700 μM for adenosine influx and formycin B efflux, respectively, and K_d taken as 0.67 nM for NBMPR binding. Values are the average ± SE.

ND, Not determined.

Table 2. Comparison of the kinetic constants for NBMPR-sensitive nucleoside influx and efflux by guinea-pig cardiomyocytes

Transport mode	Apparent K_m (μM)	V_{max} (nmol/10^{6}cells per min)
Adenosine influx	100 ± 33(3)	3.1 ± 0.84(3)
Adenosine influx*	146	1.5
Formycin B influx	490 ± 160(3)	6.5 ± 1.7(3)
Formycin B efflux	700 ± 140(3)	3.5 ± 0.3(3)
2-Chloroadenosine influx*	36	0.70

* Data taken from Ref. 10.

The kinetic constants for adenosine and formycin B influx and formycin B efflux were determined as described in Materials and Methods. Values are means ± SE (N).

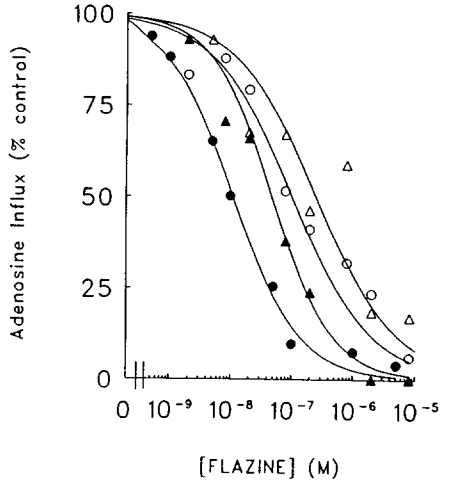

Fig. 4. Effect of lidoflazine, mioflazine, soluflazine and R73-335 on adenosine influx at 22° by guinea-pig ventricular myocytes. Cells were preincubated with lidoflazine (△), mioflazine (○), soluflazine (▲) and R73-335 (●) at 22° as described in Materials and Methods. Initial rates of 50 μM adenosine influx were determined using a 1 min incubation interval and the non-mediated flux was estimated with cells treated with 20 μM dilazep. Results are given as a percentage of the mediated control flux and are the average of triplicate estimates. For clarity, the SE are not plotted and the data are from representative experiments. Plotted lines represent best fitted curves produced by analysis of the data using the computer program EBDA.

hearts and hearts subjected to the collagenase digestion procedure were compared and found to be similar (data not shown). Specific NBMPR binding was sensitive to inhibition by formycin-B, dilazep, dipyridamole and the analogues of lidoflazine (see Table 2). The order of potency for inhibition of NBMPR binding was equivalent to that for inhibition of adenosine influx by guinea-pig cardiomyocytes. Average slope values (pseudo-Hill coefficients) were close to 1 for all compounds except lidoflazine, which gave an average value of 2 ± 0.2.

DISCUSSION

The present studies confirm and extend earlier observations that guinea-pig ventricular myocytes possess an NBMPR-sensitive nucleoside transport system. The kinetic constants for adenosine influx were similar to those determined by Heaton and Clanachan (see Table 2) [10]. Moreover, the maximal number of transporters, assuming that one NBMPR molecule binds to a single carrier site, was also

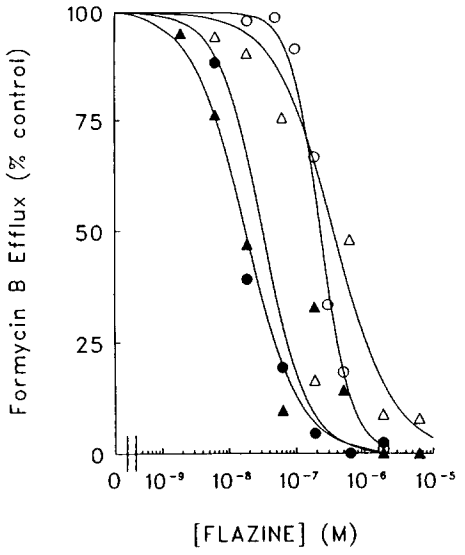

Fig. 5. Effect of lidoflazine, mioflazine, soluflazine and R73-335 on formycin B efflux from guinea-pig ventricular myocytes. The initial rates of formycin B efflux (50 μM) at 22° were determined as described in Materials and Methods following incubation with lidoflazine (○), mioflazine (△), soluflazine (●) and R73-335 (▲). The flux rates corrected for the non-mediated NBMPR-insensitive component are plotted as a percentage of the control flux. For clarity, the SE are not plotted. Plotted lines represent best fitted curves produced by analysis of the data using the computer program EBDA.

Table 3. Inhibition of NBMPR-sensitive uridine influx in guinea-pig erythrocytes

Inhibitor	Apparent K_i value (nM)
Lidoflazine	180 ± 41
Mioflazine	4.7 ± 1.2
Soluflazine	2.1 ± 0.5
R73-335	1.6 ± 0.6

Initial rates of NBMPR-sensitive uridine influx (200 μM, 22°, 5 min) by guinea-pig erythrocytes were determined as described in Materials and Methods following a 15 min preincubation with the test compounds. Apparent K_i values were determined as described in Table 1. Values are the average ± SE.

similar between the present study and that of Heaton and Clanachan [10]. Influx of formycin-B into guinea-pig myocytes was demonstrated to be via the NBMPR-sensitive nucleoside carrier but exhibited a lower affinity (K_m 490 ± 160 μM) with a higher capacity (V_{max} 6.5 ± 1.7 nmol/10^{6}cells/min) than that observed for adenosine uptake. Interestingly, the kinetic parameters of 2-chloroadenosine influx by guinea-pig cardiomyocytes showed the opposite relationship [10], that is a higher affinity but a lower maximum velocity compared to the parameters for adenosine influx (Table 2). Such a relationship is to be expected since previous studies with the NBMPR-sensitive equilibrative nucleoside transporter in mammalian erythrocytes have shown that it conforms to the simple carrier model [8, 14, 33]. Thus, the V_{max}/K_m ratio should be independent of the permeant, and within experimental error the present data demonstrate similar ratios.

The possible presence of other mediated nucleoside transport systems in our guinea-pig cardiac myocytes was tested and rejected. The absence of a Na^+-dependent nucleoside co-transporter was surprising in view of the reported presence of mRNA for the SNST1 Na^+/nucleoside transporter in rabbit heart [16]. Possible explanations for the apparent inconsistency between the northern blots and the direct uptake studies include species differences, the lack of translation of the mRNA, or the presence of the SNST1 transporter in cells other than myocytes. It should also be noted that the detection of SNST1 mRNA in rabbit heart was performed with a partial length cDNA, raising the possibility that a species of mRNA unrelated to the Na^+/nucleoside co-transporter was being detected on the northern blots.

A second route for adenosine entry into the guinea-pig ventricular myocytes was also demonstrated, which exhibited the properties of simple diffusion (not inhibitable and directly proportional to the adenosine concentration). This pathway is unlikely to play a significant role in the uptake of adenosine, as at physiological concentrations of adenosine (~100 nM), greater than 95% of adenosine influx into the myocytes will be via the NBMPR-sensitive nucleoside transporter.

The present study also demonstrated that formycin-B efflux from ventricular myocytes was inhibitable by NBMPR and saturable with kinetic constants similar to formycin-B influx. These data suggest that the NBMPR-sensitive nucleoside transporter in the myocytes exhibits symmetrical kinetics, a property that has been observed for other cell types [8, 14]. Further evidence of the symmetrical nature of the carrier came from the inhibition studies. The apparent K_i values for adenosine influx and formycin-B efflux by lidoflazine and its analogues were similar and not significantly different. Soluflazine and lidoflazine, however, demonstrated a slight trend to inhibit efflux selectively, whereas R73-335 appeared to exhibit a higher affinity for the influx mode of uptake.

Earlier studies have demonstrated that the sensitivity of the NBMPR-sensitive nucleoside transporter to inhibition by flazines is species dependent [17, 18, 20]. For example, in rabbit erythrocytes the four flazines tested here inhibited uridine influx with apparent K_i values in the low nM range (4–9 nM) [18]. In contrast, uridine influx in rat erythrocytes was up to 1000-fold more resistant to inhibition by the flazine analogues. Cells from guinea-pigs would appear to lie in between these extremes although variation between cell types was also observed. Thus, guinea-pig erythrocytes were highly sensitive to inhibition by the analogues tested, with the notable exception of lidoflazine. In particular, mioflazine and soluflazine were 20–30-fold more potent at inhibiting NBMPR-sensitive

nucleoside influx in guinea-pig erythrocytes compared to the similar process in guinea-pig ventricular myocytes (Tables 2 and 3). In contrast, R73-335 exhibited similar K_i values for both preparations. These data indicate that in addition to the potency of the analogues of lidoflazine being species dependent it is also tissue dependent.

The pseudo-Hill coefficients for inhibition of [^{3}H]-NBMPR binding by the flazine analogues were not significantly different from unity, with the exception of lidoflazine. Earlier studies with calf lung membranes had reported pseudo-Hill coefficients of greater than unity for these compounds [34]. This discrepancy has been partly resolved by Hammond [35], who demonstrated that provided the incubation time for the binding assay was sufficient to allow both [^{3}H]NBMPR and the competing compound to come to steady state, the pseudo-Hill coefficients for some of the flazine derivatives were no longer greater than unity. Alternatively, the data would be consistent with a recent proposal suggesting the presence of multiple interacting inhibitor binding sites on the transporter [35].

In conclusion, guinea-pig ventricular myocytes possess a single equilibrative nucleoside transporter that exhibits symmetrical kinetics and is inhibited by NBMPR. The analogues of lidoflazine, mioflazine, soluflazine and R73-335, blocked nucleoside influx as well as efflux symmetrically, suggesting that none of these compounds is selective for influx compared to efflux in guinea-pig myocytes. The data also indicate that the potency of soluflazine and mioflazine to inhibit nucleoside influx is tissue dependent.

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