Studies of the nucleoside transporter inhibitor, draflazine, in the human myocardium

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1 The aim of the present study was to determine the effect of the nucleoside transporter inhibitor, draflazine, on the force of contraction in human myocardium and the affinity of the compound for the nucleoside transporter. Nucleoside transport inhibitors, like draflazine, are of potential importance for cardiopreservation of donor hearts for heart transplantation.

2 Functional experiments were performed in isolated electrically driven (1 Hz, 1.8 mmol l⁻¹ Ca²⁺) human atrial trabeculae and ventricular papillary muscle strips. The affinity of draflazine for the myocardial nucleoside transporter was studied in isolated membranes from human ventricular myocardium and human erythrocytes in radioligand binding experiments using [³H]-nitrobenzylthioinosine ([³H]-NBTI). Dipyridamole was studied for comparison.

3 In membranes from human myocardium and erythrocytes, [³H]-NBTI labelled 1.18 pmol mg⁻¹ protein and 23.0 pmol mg⁻¹ protein, respectively, nucleoside transporter molecules with a K_D value of 0.8 nmol l⁻¹. Draflazine concentration-dependently inhibited binding of [³H]-NBTI to myocardial and erythrocyte membranes with a K_i-value of 4.5 nmol l⁻¹. The potency as judged from the K_i values was ten times greater than that of dipyridamole in both myocardial and erythrocyte membranes.

4 Draflazine, at concentrations up to 100 µmol l⁻¹, did not produce negative inotropic effects in atrial and ventricular myocardium. (–)-N⁶-phenylisopropyladenosine (R-PIA) and carbachol did not reduce force of contraction in ventricular myocardium, but exerted concentration-dependent direct negative inotropic effects in atrial myocardium.

5 The data provide evidence that draflazine specifically binds to the nucleoside transporter of the human heart and erythrocytes with high affinity. The compound does not produce negative inotropic effects at concentrations as high as 100 µmol l⁻¹.

6 Draflazine could be a useful agent for cardiopreservation because it does not produce cardiodepressant effects. Thus, it may be possible to perfuse explanted hearts directly with this agent without the hazard of cardiodepression.

Keywords: Nucleoside transporter; nucleoside uptake inhibition; adenosine; human myocardium; cardiac transplantation

Introduction

Adenosine is released from the heart during ischaemia (Fox *et al.*, 1974), increased cardiac workload (Foley *et al.*, 1978; McKenzie *et al.*, 1981) and during excessive catecholamine stimulation (Schrader *et al.*, 1977). Adenosine receptors on the external surface of myocardial, smooth muscle and endothelial cells mediate antiadrenergic effects (Schrader *et al.*, 1977; Böhm *et al.*, 1984; 1985) and produce vasodilator actions (Berne, 1963) thereby limiting oxygen demand and increasing blood flow to the critically challenged myocardium. These effects led investigators to suggest that endogenously formed adenosine is an endogenous feed back inhibitor against catecholamine overstimulation and a retaliatory metabolite for the temporal and local control of cellular functions (Newby, 1984; Bellardinelli *et al.*, 1989). Conceivably, a cardioprotective effect has been observed (for review see Forman *et al.*, 1993; van Belle, 1993). Adenosine is rapidly taken up by the nucleoside transporter into endothelial cells and into erythrocytes (Klabunde & Althouse, 1981; Plagemann *et al.*, 1988). As a consequence, the plasma half-life of adenosine is about 10 s when given intravenously in man (Klabunde, 1983). From these data it appeared worthwhile to inhibit the nucleoside transporter so as to enhance the adenosine concentration at its extracellular receptor sites, thereby increasing its cardioprotective effects. Nucleoside

transporter inhibitors have been used in cardioplegic solutions to improve myocardial viability (Ledingham *et al.*, 1990). Draflazine is a compound which selectively inhibits the purine nucleoside transporter (Van Belle & Janssen, 1991) and does not possess other effects as does dipyridamole (Blass *et al.*, 1980; Ahn *et al.*, 1989). Draflazine has also been reported to prevent catecholamine-induced myocardial damage (van Belle *et al.*, 1992). The pharmacological profile of draflazine appears to be advantageous as a compound for cardiac protection, and could be useful as an additional compound in cardioplegic solutions (Flameng *et al.*, 1991). In cardiac transplantation, the availability of donor hearts is markedly limited by the time for which the heart can be kept in cardioplegia for transportation. Thus, the availability of pharmacological agents which preserve cardiac function is of great clinical relevance. Perfusion of the hearts during explantation with cardioprotective agents would allow high myocardial drug concentrations to be achieved. However, this practical approach could be hampered by potential cardiodepressant side effects. In order to evaluate this potentially important question, the effects of draflazine on myocardial force of contraction were investigated on human isolated, electrically driven atrial and ventricular cardiac preparations and were compared to the affinity of the agent at the nucleoside transporter in human myocardial and human erythrocyte membranes. The use of isolated, human myocardial preparations is important because inotropic effects can markedly differ between human and animal tissue

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(Böhm *et al.*, 1989a) and because effects on force of contraction can be studied without interference from pre- or after-load changes or chronotropic effects.

Methods

Myocardial tissue

Experiments were performed on human isolated, electrically stimulated, ventricular papillary muscle strips and right auricular trabeculae or on membrane preparations from human left ventricular myocardium. Tissue was obtained during aortocoronary bypass operations (without heart failure) ($n = 7$, 4 female, 3 male; age: mean 61.4 years, range 41–71) or cardiac transplantation ($n = 3$, 2 female, 1 male; dilated cardiomyopathy). All patients gave written informed consent before surgery. Medical therapy consisted of diuretics, nitrates, ACE-inhibitors and cardiac glycosides. Patients receiving catecholamines, β -adrenoceptor- or Ca^{2+} -antagonists were withdrawn from the study. Drugs used for general anaesthesia were flunitrazepam and pancuronium bromide with isoflurane. Cardiac surgery was performed on cardiopulmonary bypass with cardioplegic arrest during hypothermia. The cardioplegic solution (a modified Bretschneider solution) contained (in mmol l^{-1}): NaCl 15, KCl 10, MgCl_2 4, histidine 180, tryptophan 2, mannitol 30 and potassium dihydrogen oxoglutarate 1.

Contraction experiments

Immediately after excision, the papillary muscles and atrial trabeculae were placed in ice-cold preaerated modified Tyrode solution (composition see below) and delivered to the laboratory within 10 min. The experiments were performed on isolated, electrically driven (1 Hz) muscle preparations. Muscle strips of uniform size with muscle fibres running parallel to the length of the strips (diameter < 1.0 mm, length 5–9 mm) were dissected in aerated bathing solution (composition see below) at room temperature. Connective tissue, if visibly present, was carefully trimmed away. The preparations were attached to a bipolar platinum stimulating electrode and suspended individually in 75 ml glass tissue chambers for recording of isometric contractions. The bathing solution used was a modified Tyrode solution containing in mmol l^{-1} : NaCl 119.8, KCl 5.4, CaCl_2 1.8, MgCl_2 1.05, NaH_2PO_4 0.42, NaHCO_3 22.6, Na_2EDTA 0.05, ascorbic acid 0.28 and glucose 5.0. It was continuously gassed with 95% O_2 and 5% CO_2 and maintained at 37°C; its pH was 7.4. Muscle strip preparations were attached to two stainless metal pins, one of which was connected to a force transducer. Isometric force of contraction was measured with an inductive force transducer (W. Fleck, Mainz, Germany) attached to a Hellige Helco Scriptor (Hellige, Freiburg, Germany) or Gould recorder (Gould Inc., Cleveland, Ohio, U.S.A.). Each muscle was stretched to the length at which developed force was maximal and the resting load was kept constant throughout the experiments. The preparations were electrically paced at 1 Hz with rectangular pulses of 5 ms duration (Grass stimulator SD 9), the voltage was 20% above threshold. All preparations were allowed to equilibrate at least 90 min in a drug-free bathing solution until complete mechanical stabilization. After 45 min, the solution was changed. Concentration-dependent mechanical effects of drafazine and other drugs were obtained. To test mechanical performance, after each experiment the positive inotropic effect mediated by elevation of the extracellular Ca^{2+} -concentration (15 mmol l^{-1}) was measured. All preparations in this experimental series developed an increase in force of contraction after increase in the Ca^{2+} -ion-concentration. Control strips in Tyrode solution revealed a maximally 20% reduction of baseline isometric developed tension over the period necessary to complete pharmacological testing. Agents were

applied cumulatively to the organ bath. Each muscle was used only once to record a concentration-response curve.

Human myocardial membrane preparations

Left ventricular myocardium was chilled in 30 ml ice-cold homogenization buffer (20 mmol l^{-1} Tris/HCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol, pH 8.0). Connective tissue was trimmed away and myocardial tissue was minced with scissors, disrupted with an Ultraturrax (Janke and Kunkel, Staufenbreisgau, Germany) and homogenized with a motor-driven glass teflon Elvehjem-Potter for 1 min. The homogenate was spun at 480 g for 10 min (JA 20, Beckman, Palo Alto, U.S.A.). The supernatant was retained and the pellet discarded. This homogenate was diluted with an equal volume of ice-cold 1 mol l^{-1} KCl and stored on ice for 10 min. The supernatant was centrifuged at $100,000 g$ for 45 min. The pellet was resuspended in 50 volumes of homogenization buffer and recentrifuged at $100,000 g$ for 45 min. The final pellet was resuspended in incubation buffer (50 mmol l^{-1} Tris/HCl, 10 mmol l^{-1} MgCl_2 , pH 7.4). Assays were performed in a total volume of 250 μl incubation buffer.

Human erythrocyte membrane preparation

Heparinized blood (15 ml) was taken from healthy volunteers and centrifuged at 150 g for 20 min at room temperature (Beckman J21B, rotor JA 20). The supernatant was discarded and the sediment was washed twice in 30 ml buffer (50 mmol l^{-1} Tris/HCl, 10 mmol l^{-1} MgCl_2 , 1 mmol l^{-1} dithiothreitol) and again centrifuged at 150 g. The sediment was resuspended in 10 ml buffer and diluted with an equal volume of 1 mol l^{-1} KCl and treated with five strokes of a motor-driven glass teflon Elvehjem-Potter homogenizer. Following an incubation period for 15 min at 40°C, the suspension was centrifuged for 30 min at $100,000 g$. The sediment was resuspended in hypotonic medium (25 mmol l^{-1} MgCl_2 , 10 mmol l^{-1} KHCO_3 , 20 mmol l^{-1} Tris/HCl) and again centrifuged at $100,000 g$ for 30 min. This final pellet was potted and suspended in buffer without dithiothreitol, frozen in liquid nitrogen and stored at -80°C .

Radioligand binding experiment

The nucleoside transporter in human erythrocytes and myocardial membranes was investigated with [^3H]-nitrobenzylthioinosine (^3H -NBTI) as radiolabelled ligand. The incubation was carried out for 60 min at 37°C in a buffer containing 50 mmol l^{-1} Tris/HCl, 10 mmol l^{-1} MgCl_2 and 1 mmol l^{-1} dithiothreitol at a final volume of 250 μl . The assay was started with membrane suspension (20 μg for erythrocytes, 150 μl for myocardial membranes). Nonspecific binding was determined with 30 $\mu\text{mol l}^{-1}$ dipyrindamole. The conditions used allowed complete equilibration with the nucleoside transporter. The reaction was terminated by rapid vacuum filtration through Whatman GB/C filters. The filters were washed three times immediately with 6 ml incubation buffer. Filters were dried and placed in 10 ml of scintillation fluid (Quickszint 501, Zinsser Analytics, Frankfurt, Germany) and radioactivity determined with a LKB- β -liquid scintillation counter. Each determination was performed in triplicate.

Miscellaneous

Protein was determined according to Lowry *et al.* (1951). Binding data (B_{max} and K_D -values) were determined according to Scatchard (1949). K_i values were determined according to Cheng & Prusoff (1973) from the IC_{50} -values which were graphically determined in each individual experiment.

Materials

Draflazine CR 75231, 2-(aminocarbonyl)-N-(4 amino-2,6-dichlorophenyl)-4-(55-bis-(4-fluorophenyl)pentyl)-1-piperazine acetamide) was from Janssen Co, Beerse, Belgium. Dipyridamole, R-N'-phenyl-isopropyladenosine (R-PIA) and carbachol were purchased from Boehringer-Mannheim (Germany). All other chemicals were of analytical grade or the best grade commercially available. For studies with isolated cardiac preparations, stock solutions were prepared and applied to the organ bath. Applied compounds did not change the pH in the bathing solution.

Results

[³H]-NBTI-binding to the nucleoside transporter

Human erythrocyte membranes were prepared from three samples obtained from human volunteers. A typical experiment to characterize binding of [³H]-NBTI to human erythrocyte membranes is shown in Figure 1. Binding of [³H]-NBTI was monophasic and saturable. Transformation of the data according to Scatchard (1949) revealed a linear plot with a density of 23 pmol mg⁻¹ protein [³H]-NBTI bound and an apparent K_D -value of 0.8 nmol l⁻¹. Nonspecific binding was low amounting to about 1% of total binding at K_D and about 5% at 30 nmol l⁻¹ [³H]-NBTI. In order to evaluate the affinity of the nucleoside transporter inhibitors, draflazine and dipyridamole, competition experiments were performed. Figure 2 illustrates the concentration-dependent antagonism of [³H]-NBTI-binding by dipyridamole and draflazine. Both compounds completely antagonized binding of [³H]-NBTI to the erythrocyte nucleoside transporter. Analysis of binding data revealed K_i values of 4.5 nmol l⁻¹ and 48 nmol l⁻¹ for draflazine and dipyridamole, respectively. Figure 3 demonstrates a representative radioligand binding experiment with [³H]-NBTI on membranes from the human left ventricle. As in erythrocyte membranes, [³H]-NBTI concentration-dependently bound to the membranes with a K_D value of 0.8 nmol l⁻¹ and a B_{max} value of 1.18 nmol [³H]-NBTI bound mg⁻¹ protein. Figure 4 shows concentration-dependent inhibition of [³H]-NBTI-binding by draflazine and dipyridamole in human ventricular membranes. As in erythrocyte membranes, the potency of draflazine in inhibiting binding of [³H]-NBTI to membranes was about 10 times higher than that of dipyridamole. Taken together, in radioligand binding studies with [³H]-NBTI, the density of the nucleoside trans-

porter in erythrocytes was determined to be about 19 times higher than in human ventricular membranes. In both systems, the potency of draflazine was about 10 times greater than that of dipyridamole in binding to the nucleoside transporter.

Figure 2 Inhibition of [³H]-nitrobenzylthioinosine ([³H]-NBTI) (0.8 nmol l⁻¹) binding to the nucleoside transporter of human erythrocyte membranes by the nucleoside transporter inhibitors, draflazine (▲) and dipyridamole (●). Ordinate scale: specific binding as a percentage of maximal bound [³H]-NBTI. Abscissa scale: concentration of competitors.

Figure 3 Binding of [³H]-nitrobenzylthioinosine ([³H]-NBTI) (0.1–30 nmol l⁻¹) to the nucleoside transporter of human ventricular membranes. The inset shows the linear transformation of the data obtained in the saturation experiment. Bound [³H]-NBTI (pmol mg⁻¹ protein) is plotted as a function of the ratio of bound to free [³H]-NBTI ($B/F \times 10^3$). The intercept with the abscissa scale is the number of binding sites (B_{max}); the slope is the apparent affinity. Each point represents the mean of triplicate observations: (○) total binding; (▲) specific binding; (◆) nonspecific binding.

Figure 1 Binding of [³H]-nitrobenzylthioinosine ([³H]-NBTI) (0.1–30 nmol l⁻¹) to the nucleoside transporter of human erythrocyte membranes. The inset shows the linear transformation of the data obtained in the saturation experiment. Bound [³H]-NBTI (pmol mg⁻¹ protein) is plotted as a function of the ratio of bound to free [³H]-NBTI ($B/F \times 10^3$). The intercept with the abscissa scale is the number of binding sites (B_{max}); the slope is the apparent affinity. Each point represents the mean of triplicate observations: (○) total binding; (▲) specific binding; (◆) nonspecific binding.

Figure 4 Inhibition of [³H]-nitrobenzylthioinosine ([³H]-NBTI) (0.8 nmol l⁻¹) binding to the nucleoside transporter of human ventricular membranes by the nucleoside inhibitors, draflazine (▲) and dipyridamole (●). Ordinate scale: specific binding in percentage of maximal bound [³H]-NBTI. Abscissa scale: concentration of competitors.

Figure 5 Original recordings illustrating the effects on force of contraction in isolated cardiac preparations from human atrial (left tracings) and human ventricular (right tracings) myocardium of drug-free bathing solution (a,b), draflazine (c,d), the adenosine receptor agonist, R-PIA (e,f) and carbachol (g,h).

Figure 6 Concentration-response curves for the effects on contraction in human isolated, electrically driven atrial trabeculae of draflazine (□), R-PIA (▲) and carbachol (○) ($0.0001-100 \mu\text{mol l}^{-1}$). Basal force of contraction was $11.6 \pm 0.6 \text{ mN}$ ($n = 20$). Ordinate scale: force of contraction in % of predrug values. Abscissa scale: concentration of studied drugs in $\mu\text{mol l}^{-1}$.

Figure 7 Concentration-response curves for the effects on force of contraction in human isolated, electrically driven papillary muscle strips of draflazine (□), R-PIA (▲) and carbachol (○) ($0.0001-100 \mu\text{mol l}^{-1}$). Basal force of contraction was $3.0 \pm 0.5 \text{ mN}$ ($n = 31$). Ordinate scale: force of contraction in % of predrug values. Abscissa scale: concentration of studied drugs in $\mu\text{mol l}^{-1}$.

Effects of draflazine on force of contraction

Original recordings in Figure 5 demonstrate the effect of draflazine on isometric force of contraction in isolated, electrically driven right atrial and left ventricular cardiac preparations. The effects of the A_1 -adenosine receptor agonist, R-PIA and the muscarinic cholinergic agonist, carbachol, as well as the time course of force of contraction in drug-free bathing solution are shown for comparison. Similar to force generation in drug-free bathing solution, draflazine did not produce negative inotropic responses up to $100 \mu\text{mol l}^{-1}$ in atrial or in ventricular cardiac preparations. In contrast, the A_1 -adenosine receptor agonist, R-PIA, and carbachol produced concentration-dependent negative inotropic effects in atrial heart muscle without affecting force development in ventricular heart muscle. Figure 6 shows the concentration-response curves for effects of draflazine, R-PIA and carbachol in atrial preparations. Draflazine had no effects on developed tension over the concentration range 0.001 – $100 \mu\text{mol l}^{-1}$. R-PIA and carbachol concentration-dependently reduced force of contraction. Carbachol was about ten times more potent than R-PIA. In ventricular preparations, R-PIA and carbachol did not reduce developed force of contraction (Figure 7).

Discussion

Adenosine is an endogenous nucleoside which is released from the myocardium in situations where an increased ATP-breakdown occurs. In addition, adenosine acts as an antiplatelet aggregating agent (Cronstein *et al.*, 1985) and potently inhibits leucocyte activation (van Belle, 1985). Therapeutic applications of adenosine include the treatment of supraventricular tachycardias (Di Marco *et al.*, 1983) and controlled hypothermia in neurosurgery (Sollevi *et al.*, 1984). From this profile of actions it has been suggested that adenosine is a local endogenous regulator of cellular metabolism and function (Newby, 1984). Consistently, adenosine has been reported to protect the heart from ischaemia and stunning (Lasley *et al.*, 1990; Bunch *et al.*, 1992). On the contrary, uncoupling of adenosine A_1 -receptors by pertussis toxin treatment was observed to produce myocardial lesions comparable to those observed following catecholamine-treatment (Böhm *et al.*, 1988). One possible physiological intervention to increase the concentration of the nucleoside at its extracellular receptor, is the application of nucleoside transport inhibitors like dipyridamole or lidoflazine which could result in an improved cardiac function following situations like catecholamine stress, ischaemia or long term storage in cardioplegic solutions (Schubert *et al.*, 1989).

The limited time for conservation of ischaemic donor organs is still one important problem in heart transplantation. Cardiac function following cardiac transplantation is inversely related to the time of ischaemic storage of the organ and in general limited to 4–6 h post explantation (Heck *et al.*, 1989; Pflugfelder *et al.*, 1989). Thus, nucleoside transporter inhibition appears to be an attractive mechanism for increasing the adenosine concentration at its extracellular receptor sites thereby preserving mechanical function following storage in cardioplegic solutions. Indeed lidoflazine was observed to improve cardiac function during intermittent aortic cross clamping in dogs (Chang-Chun *et al.*, 1992). The most pronounced effects of the nucleoside transporter inhibitor would be expected at a 100% receptor occupation. In this study, we

determined the concentration by radioligand binding studies using competition experiments with dipyridamole or draflazine. Complete antagonism by draflazine or dipyridamole to [^3H]-NBTI binding was observed at $30 \mu\text{mol l}^{-1}$ and $300 \mu\text{mol l}^{-1}$ draflazine and dipyridamole, respectively, in human myocardial membranes under the used assay conditions. In the erythrocyte membranes, these concentrations were even higher. Thus, it would be advantageous to achieve these rather high concentrations in the myocardium to obtain an efficient nucleoside uptake inhibition. These concentrations could be reached during cardiac transplantation by perfusing the hearts directly following explantation with cardioplegic solution and the nucleoside uptake inhibitor. However, the effects of these compounds are not specific for the inhibition of nucleoside transporter. Dilazep, for instance, possesses Ca^{2+} antagonistic properties (Wainwright *et al.*, 1993) and thus, might elicit cardiodepressant effects. Therefore, these unwanted cardiodepressant effects were studied with draflazine in human isolated cardiac preparations. The use of human myocardium is important because response to inotropic agents can differ between human and laboratory animal myocardium (Böhm *et al.*, 1989a). Draflazine, at concentrations up to $100 \mu\text{mol l}^{-1}$ did not produce negative inotropic effects. Thus, direct negative inotropic effects do not have to be expected when the explanted heart is directly perfused with draflazine. However, one might argue that adenosine could directly produce antiadrenergic effects, when its uptake into cells is maximally inhibited. As shown by the metabolically stable A_1 -adenosine receptor agonist, R-PIA, stimulation of the receptor does not produce any inhibitory direct effect on force of contraction in human ventricular myocardium as previously also reported in rodent myocardium (Böhm *et al.*, 1984; Dobson *et al.*, 1986). Only in atria is adenosine known to produce direct negative inotropic effects (Böhm *et al.*, 1989b). In preparations with oxygenated Tyrode solution, draflazine did not reduce atrial force of contraction. This observation is in agreement with the suggestion that the preparations are well oxygenated and do not release adenosine in amounts able to exert negative inotropic effects even in human atrial myocardium.

Draflazine is a lidoflazine analogue which has been described as a specific inhibitor of nucleoside uptake (van Belle & Janssen, 1991). Its affinity for the nucleoside transporter of membranes from human erythrocytes and human myocardium was about ten times higher than that of dipyridamole as judged from the K_i values for inhibition of [^3H]-NBTI-binding (this study). In addition, the compound elicited antiarrhythmic effects in anaesthetized pigs (Wainwright *et al.*, 1993). From this pharmacological profile it appears that it might be a useful drug for cardiopreservation and long term storage of explanted donor hearts. The first evidence in favour of this suggestion was provided by experiments in dog hearts which were kept in hyperkalaemic cardioplegic solution without or with draflazine. The hearts with the nucleoside inhibitor exhibited almost complete mechanical activity after 24 h, whereas no mechanical activity was observed in the control group (Masuda *et al.*, 1992).

From the present findings and from observations reported in the literature, it is suggested that the nucleoside transporter inhibitor, draflazine, may be a suitable agent for cardiopreservation. Its lack of cardiopressant effects in human myocardium, even at high concentrations could offer the opportunity to perfuse the explanted heart to obtain a complete or at least high degree of nucleoside transporter inhibition.

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