Characterization of the inhibition by stilbene disulphonates and phloretin of lactate and pyruvate transport into rat and guinea-pig cardiac myocytes suggests the presence of two kinetically distinct carriers in heart cells

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1. The kinetics of transport of pyruvate (K_m 0.20 mM), L-lactate (K_m 2.2 mM) and D-lactate (K_i 10.2 mM) into rat cardiac myocytes were studied and compared with those for guinea-pig heart cells [Poole, Halestrap, Price and Levi (1989) *Biochem. J.* **264**, 409–418] whose equivalent values were 0.07, 2.3 and 6.6 mM respectively. Maximal rates of transport were about 5-fold higher in the rat heart cells. 2. 4,4'-Dibenzamidostilbene-2,2'-disulphonate (DBDS), a powerful inhibitor of monocarboxylate transport into erythrocytes [Poole & Halestrap (1991) *Biochem. J.* **275**, 307–312], was found to be a potent but apparently partial inhibitor of lactate and pyruvate transport, with an apparent K_i value at 0.5 mM L-lactate of about 16 μ M in both species. Maximal inhibition was 50% and 80% in rat and guinea-pig cells respectively. 3. The maximal extent of inhibition and apparent K_i values were dependent on both the substrate transported and its concentration. Maximum inhibition was less and the K_i was greater at higher substrate concentrations. 4. A variety of other stilbene disulphonates were stud-

ied which showed different K_i values and maximal extents of inhibition. 5. Phloretin was a significantly less potent inhibitor of transport into both rat (K_i 25 μ M) and guinea-pig (K_i 16 μ M) heart cells than into rat erythrocytes (K_i 1.4 μ M). In the rat but not the guinea-pig heart cells, inhibition appeared partial (maximal inhibition 84%). 6. We demonstrate that our results can be explained by the presence of two monocarboxylate carriers in heart cells, both with K_m values for L-lactate of about 2 mM and inhibited by α -cyano-4-hydroxycinnamate, but with different affinities for other substrates and inhibitors. One carrier is sensitive to inhibition by stilbene disulphonates and has lower K_m values for pyruvate (0.05–0.10 mM) and D-lactate (5 mM), whereas the other has higher K_m values for pyruvate (0.30 mM) and D-lactate (25 mM), and is relatively insensitive to stilbene disulphonates. Rat heart cells possess more of the latter carrier and guinea-pig heart cells more of the former. 7. The significance of these results for the study of lactate transport in the perfused heart is discussed.

INTRODUCTION

The transport of lactic acid across the plasma membrane of heart cells is important in two situations: under aerobic conditions, when lactic acid enters the cell to be used as a respiratory fuel for the heart, and under ischaemic or hypoxic conditions, when lactic acid is produced by glycolysis and must be transported out of the myocytes. We have shown previously that both lactate and pyruvate can be transported across the plasma membrane of guinea-pig cardiac myocytes with an accompanying proton by means of a specific carrier (Poole et al., 1989). This carrier has kinetic properties that are distinct from the well-characterized transporter of the erythrocyte. Most notably, it has a higher affinity for a variety of monocarboxylate substrates that can act as respiratory fuels for the heart, such as pyruvate, acetate, acetoacetate and β -hydroxybutyrate, and its stereoselectivity for L-lactate over D-lactate is much less (Poole et al., 1989, 1990). The monocarboxylate/proton co-transporter is the major route for lactic acid transport across the heart cell membrane under normal physiological conditions. However, the possibility remains that under hypoxic or ischaemic conditions, when intracellular lactate concentrations are very high, there may be a minor role for free diffusion of lactic acid or lactate efflux by an anion transporter coupled to proton movement by means of the Na^+/H^+ transporter (Mainwood and Worsley-Brown, 1975; Juel,

1988; Mason and Thomas, 1988; Vaughan-Jones et al., 1989; Poole et al., 1989). In order to establish the relative importance of these different pathways, a specific inhibitor of the monocarboxylate/proton co-transporter is required that can be used in intact tissue preparations such as the perfused heart. One such inhibitor which has been used extensively is α -cyano-4-hydroxycinnamate (Halestrap and Denton, 1974, 1975; Eisner et al., 1987, 1988; Poole et al., 1989, 1990). However, this compound enters heart cells, where it acts as a potent inhibitor of mitochondrial pyruvate transport and hence of pyruvate oxidation (Halestrap and Denton, 1974, 1975; Halestrap, 1975). This undesirable perturbation of metabolism causes a large stimulation of lactic acid production by the heart perfused with glucose, even under normoxic conditions (Halestrap and Denton, 1975).

We have recently demonstrated that a series of stilbene disulphonates act as potent impermeant inhibitors of lactate transport into rat and human erythrocytes (Poole and Halestrap, 1991; Poole et al., 1991). In the present paper we study the effects of these inhibitors on lactate and pyruvate transport into cardiac myocytes from both the guinea pig and rat. 4,4'-Dibenzamidostilbene-2,2'-disulphonate (DBDS) was found to be a potent, but apparently partial, inhibitor of transport into the cardiac myocytes of both species. It was also found that the kinetics of substrate transport into cardiac myocytes from the

Abbreviations used: DBDS, 4,4'-dibenzamidostilbene-2,2'-disulphonate; NO₂-DBDS, 4,4'-di-(4-nitrobenzamido)stilbene-2,2'-disulphonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulphonate; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate; TBenzDS, NNN'-N'-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate.

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two species are significantly different. It is demonstrated that these results are best explained by the presence of two monocarboxylate carriers in heart cells, with different kinetic properties. The significance of these results for the study of lactate transport in the perfused heart is discussed.

EXPERIMENTAL

Materials

Stilbene disulphonate derivatives were prepared as described previously (Poole and Halestrap, 1991; Poole et al., 1991) with the following exceptions. The preparation of DBDS was modified so as to avoid the need for purification of the product by chromatography on silica gel. Briefly, 4,4'-diaminostilbene-2,2'-disulphonate (1.5 g; 0.004 mol) was dissolved in 60 ml of 20% (v/v) dimethyl sulphoxide containing 2 g of NaHCO₃. Benzoyl chloride (1.57 ml; 0.012 mol) was added drop-wise with stirring. After 2 h the mixture was cooled, and the product collected by filtration and washed with ice-cold water and diethyl ether. The solid was dissolved in the minimum volume of hot methanol, and the final product precipitated by the addition of ice-cold ether. 4,4'-Di(4-nitrobenzamido)stilbene-2,2'-disulphonate (NO₂-DBDS) was prepared by a similar method, except for the substitution of 4-nitrobenzoyl chloride (2.2 g; 0.012 mol, dissolved in 5 ml of anhydrous acetone) for benzoyl chloride.

4-Nitrobenzoyl chloride was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. The sources of all other chemicals, biochemicals and radiochemicals were as given in Poole et al. (1989, 1990, 1991) and Poole and Halestrap (1991).

Preparation of cardiac myocytes from guinea pig and rat

Cardiac myocytes were isolated from rat and guinea-pig hearts by a combination of enzymic and mechanical dispersion as described previously (Lee and Levi, 1991), but with the modifications described below. For both rat and guinea-pig, the perfusion flow rate was kept constant at 9 ml/min per g of heart tissue. The rat heart was perfused with the collagenase/protease buffer for 16 min in the presence of 180 μ M Ca²⁺, whereas for the guinea pig the relevant values were 240 μ M Ca²⁺ and 12 min. The cells were stored at room temperature for 30 min in 'KB' solution (Isenberg and Klockner, 1982) before preparing them for transport measurements as described by Poole et al. (1989).

Measurement of substrate transport, intracellular volume and pH gradients

Initial rates of L- and D-lactate transport into cardiac myocytes were measured at 11 °C by a centrifuge-stop silicone-oil filtration technique as described by Poole et al. (1989, 1990). These experiments were performed in a citrate buffer (80 mM sodium citrate/10 mM Hepes, neutralized to pH 7.4 with NaOH) in order to optimize the uptake of monocarboxylates (Poole et al., 1989, 1990). Correction for extracellular [¹⁴C]substrate was performed by including ³H₂O in the incubations and in parallel experiments measuring the intracellular and extracellular volumes with [¹⁴C]sucrose and ³H₂O. Intracellular [¹⁴C]substrate was calculated as ([¹⁴C]substrate - ³H₂O) space - ([¹⁴C]-sucrose - ³H₂O) space, as described previously (Poole et al., 1989). A 'dead-time' of about 10 s was observed using this technique (see Figure 1); this represents the time taken for the cells to sediment through the oil into the HClO₄ during centrifugation. For pyruvate transport an inhibitor-stop technique was used,

since even at 0 °C transport was too fast to resolve accurately by centrifuge-stop. Essentially the method was the same as for centrifuge-stop, but at the required time 5 mM α -cyano-4-hydroxycinnamate was added to inhibit transport before rapidly transferring samples to centrifuge tubes containing the HClO₄ and oil. No 'dead-time' was observed with this technique, confirming the effectiveness of the inhibitor in stopping transport. With both techniques, uptake after 30 s was used to estimate initial rates of transport for L- and D-lactate, and 10 s for pyruvate; time courses were close to linear over these time periods (see, e.g., Figure 1). Initial rates of L-lactate transport into rat erythrocytes at 7 °C were measured after 15 s by a centrifuge stop technique (without the use of silicone oil) as described previously (Poole and Halestrap, 1991).

Inhibitors were routinely dissolved in dimethyl sulphoxide/methanol (4:1, v/v) since DBDS, NNN'-N'-tetrabenzyl-4,4'-diaminostilbene-2,2'-disulphonate (TBenzDS) and phloretin were relatively insoluble in other suitable solvents. The concentration of stock solutions was such that after addition to the incubation the solvent was never present at more than 1% (v/v). An equivalent volume of the solvent was included in the control incubations. The inhibitors were introduced to the cell suspension and preincubated with cells for 2-5 min before addition of [¹⁴C]substrates to initiate the assay. Any additions or modifications to the methods described above are given in the text or Figure legends.

Presentation of results

All experiments were performed in duplicate for each cell preparation, and carrier-mediated uptake of substrate was corrected for non-carrier-mediated uptake (free diffusion) by subtraction of the substrate transported in the presence of 5 mM α -cyano-4-hydroxycinnamate, which totally inhibits the transporter (Poole et al., 1989, 1990). Data are usually presented as means $\pm$ S.E.M. (error bars) of the number of experiments shown; each experiment was performed on a separate preparation of cardiac myocytes. The data from each experiment were fitted to the relevant equation by non-linear least-squares regression, and then the derived parameter values were combined to give the mean values $\pm$ S.E.M. reported in the Tables and text. It should be noted that K_i values for inhibitors are usually quoted as apparent K_i values, since they are values for half-maximal inhibition at a constant substrate concentration, and do not take account of whether or not inhibition is competitive. However, K_i values for competing substrates have been corrected to account for the competitive nature of the inhibition, as described in the text and Figure legends. All data fitting was performed with FigP and PFit Software (Biosoft, Cambridge, U.K.).

RESULTS

Kinetics of monocarboxylate transport into rat cardiac myocytes

The data of Figure 2 show the concentration-dependence of the rate of uptake of L-lactate and pyruvate in the presence and absence of 5 mM α -cyano-4-hydroxycinnamate, which inhibits carrier-dependent transport. A summary of the kinetic properties of the carrier determined from data such as those of Figure 2 is given in Table 1, where a comparison is made with the data for guinea-pig cardiac myocytes (Poole et al., 1989). Two important points should be noted about the experimental protocol. Firstly, pyruvate uptake was measured by an inhibitor-stop technique at 0 °C, whereas for L- and D-lactate uptake was measured at 11 °C and by centrifuge-stop. The difference in temperature accounts

Figure 1 Time courses for the uptake of L- and D-lactate and pyruvate by isolated cardiac myocytes in the presence and absence of inhibitors

Time courses of the uptake of 0.5 mM L-lactate (I), 0.5 mM D-lactate (II) or 0.2 mM pyruvate (III) by rat (a) and guinea-pig (b) heart cells were determined at 11 °C (L- and D-lactate) or 0 °C (pyruvate) in the absence (●) or presence of 0.2 mM DBDS (■) or 5 mM α -cyano-4-hydroxycinnamate (CNCN; ▲). Data are presented as means $\pm$ S.E.M. (error bars) of the numbers of experiments shown. Further details are given in the Experimental section.

for the difference in $V_{\max}$ between pyruvate and lactate transport reported in Table 1. Secondly, carrier-dependent uptake was calculated by subtracting the uptake of substrate in the presence of 5 mM α -cyano-4-hydroxycinnamate from that in its absence. This also overcomes a problem associated with apparent negative uptake sometimes observed at early time points in the presence of inhibitors (see Figure 1), and especially with D-lactate as substrate. Such observations imply that in the presence of α -cyano-4-hydroxycinnamate [14 C]lactate is excluded from a larger volume of the extracellular medium than is [14 C]sucrose. Whether or not this is a real effect or an artefact of the technique, it represents only a small discrepancy and is circumvented by the procedure outlined above.

It is clear that the properties of the transporter of rat heart cells are similar to, but not identical with, those of the guinea pig. Both carriers have a measured K_m for L-lactate of about 2 mM and a measured K_i for D-lactate of 6–10 mM. The value of the K_m for D-lactate is probably the same as that for its K_i , since, for the

guinea pig, both values appear to represent the dissociation constant of the carrier–substrate complex (Poole et al., 1989). The rate of carrier-mediated transport of 0.5 mM D-lactate by rat heart cells (0.12 nmol/min per μ l of intracellular space, derived from Figure 1a ii) was slightly less than that for guinea-pig heart cells (0.20 nmol/min per μ l; Figure 1b ii), although rates were too low to measure accurately enough for detailed kinetic studies. However, approximate values for the $V_{\max}$ of D-lactate transport into rat and guinea-pig heart cells of 2.6 and 2.8 nmol/min per μ l respectively can be estimated from the rates of transport at 0.5 mM D-lactate and the measured K_i values for D-lactate. These calculations assume that K_i and K_m values are identical, as argued above. In contrast, the rat cells showed a 4–5-fold greater $V_{\max}$ for transport of both pyruvate and L-lactate than did the guinea-pig cells (10.3 compared with 2.3 nmol/min per μ l), whereas both their K_m for pyruvate transport and K_i for pyruvate inhibition of L-lactate transport were 2–3-fold higher (about 0.2 and 0.08 mM for rat and guinea pig respectively). After 5 min

Figure 2 Concentration-dependence of the rates of pyruvate and L-lactate transport into rat heart cells in the presence ($\blacktriangle$) and absence ($\bullet$) of 5 mM α -cyano-4-hydroxycinnamate (CNCN)

Initial rates of uptake of L-lactate and pyruvate were measured by centrifuge-stop (11 °C) and inhibitor-stop (0 °C) techniques respectively. Details are given in the Experimental section. Values are shown as means $\pm$ S.E.M. (error bars) of 4 or 5 experiments, and derived K_m and V_{max} values for the inhibitor-sensitive uptake are given in Table 1.

Table 1 Comparison of kinetic properties of the cardiac myocyte lactate carriers from rat and guinea pig

All data were obtained for cells incubated in citrate buffer as described in the Experimental section. The K_i values given were derived from measurements of the inhibition of the transport of 0.5 mM L-lactate by increasing concentrations of the competing substrate. Data were then fitted by regression analysis to the equation for competitive inhibition $v = V_{max}/(1 + ([I]/K_i)(1 + K_m/[S]))$, with $[S]$ set at 0.5 mM and the K_m for L-lactate set at the relevant value shown in the Table. Results are given as means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. * Values taken from Poole et al. (1989).

Kinematic parameters and substrates	Rat	Guinea pig
K_m (mM)		
L-Lactate (11 °C)	2.15 $\pm$ 0.09 (4)	2.27 $\pm$ 0.19 (5)*
Pyruvate (0 °C)	0.20 $\pm$ 0.02 (5)	0.07 $\pm$ 0.01 (5)*
K_i (mM)		
D-Lactate (11 °C)	10.2 $\pm$ 1.69 (4)	6.60 $\pm$ 1.20 (4)*
Pyruvate (11 °C)	0.19 $\pm$ 0.02 (4)	0.08 $\pm$ 0.01 (4)*
V_{max} (nmol/min per μ l of intracellular space)		
L-Lactate (11 °C)	10.3 $\pm$ 0.60 (4)	2.27 $\pm$ 0.24 (5)*
Pyruvate (0 °C)	2.09 $\pm$ 0.23 (5)	0.41 $\pm$ 0.06 (5)*
pH gradient (at 0.5 mM L-lactate)	0.64 $\pm$ 0.02 (4)	0.47 $\pm$ 0.03 (4)*
Equilibrium uptake (nmol/ μ l of intracellular space)		
L-Lactate (0.5 mM)	2.10 $\pm$ 0.11 (7)	1.99 $\pm$ 0.05 (2)
D-Lactate (0.5 mM)	0.69 $\pm$ 0.03 (5)	0.76 $\pm$ 0.05 (3)
Pyruvate (0.2 mM)	0.55 $\pm$ 0.02 (2)	0.46 $\pm$ 0.05 (2)

uptake, when a steady state had been reached, the intracellular concentrations of pyruvate, L- and D-lactate, although different from each other, were similar in both cell types. As we have discussed previously (Poole et al., 1989), this equilibrium may represent the balance between uptake on the H^+ /lactate co-transporter and efflux via a pathway which transports monocarboxylates in their anionic form and without stereoselectivity (Bashford and Pasternak, 1984; Mason and Thomas, 1988; Lynch and Best, 1990). The rates of L-lactate and pyruvate transport into guinea-pig heart cells measured in this study are very similar to those reported previously (Poole et al., 1989)

Figure 3 Inhibition of L-lactate transport by DBDS into rat and guinea-pig heart cells at 11 °C

Isolated rat ($\blacktriangle$) and guinea-pig ($\bullet$) heart cells were preincubated with the required concentration of DBDS for 2–5 min. Initial rates of transport of 0.5 mM L-lactate were then measured after 30 s as described in the Experimental section. Results are expressed as a percentage of carrier-mediated transport (α -cyano-4-hydroxycinnamate-inhibitable) in the absence of DBDS, as means $\pm$ S.E.M. (error bars) of 5 or 6 experiments. The continuous lines drawn through the data points were by non-linear least-squares regression analysis of the data to the equation $V = V_0/(1 + [I]/K_i) + V_n$, as described in the text. The derived parameter values are given in Table 2. The dashed lines and open symbols represent rates of L-lactate transport calculated theoretically, assuming the presence of two carriers as described in Table 4, and then fitted to the same equation as the experimental data. The theoretical parameter values are given in Table 4.

Inhibition of transport by DBDS and other stilbene disulphonates

The data of Figure 1 show the effects of 0.2 mM DBDS and 5 mM α -cyano-4-hydroxycinnamate on the time courses of pyruvate, L- and D-lactate uptake into rat and guinea-pig cardiac myocytes. It is clear that the inhibition by 0.2 mM DBDS is substantially less than that by 5 mM α -cyano-4-hydroxycinnamate, especially in the rat cardiac myocytes. Indeed, in these cells inhibition by DBDS of D-lactate transport was too small to determine with accuracy. This was partly due to the relatively low rate of carrier-specific uptake of D-lactate in these cells relative to that of L-lactate. The concentrations of both inhibitors were chosen to give the maximum practical inhibition. DBDS could not be used at concentrations higher than 0.2 mM, because of its relative insolubility in the buffer used for transport studies. However, inspection of Figures 3 and 4 suggests that higher concentrations of inhibitor would not give greater in-

Figure 4 Inhibition by DBDS of pyruvate and L-lactate transport into rat and guinea-pig heart cells at different substrate concentrations

Guinea-pig (a,b) and rat (c) cardiac myocytes were preincubated with the required concentration of DBDS for 2–5 min. In (b) initial rates of transport at 0.2 mM (●) and 5 mM (▲) L-lactate were measured as described in the legend to Figure 3. In (a) and (c), uptake of 0.04 mM (●) and 1.0 mM (▲) pyruvate at 0 °C (a,c) was measured after 10 s and 30 s respectively by using an inhibitor-stop technique described in the Experimental section. These conditions were chosen so that uptake of pyruvate was linear with respect to time over the period chosen (see Figure 1). Data are shown as means $\pm$ S.E.M. (error bars) of the numbers of experiments indicated below and were analysed as described in the legend to Figure 3. The derived K_i values ($\pm$ S.E.M.) for the guinea-pig heart cells in the presence of 0.04 mM and 1.0 mM pyruvate and for 0.2 mM and 5 mM L-lactate were $10.0 \pm 1.5 \mu\text{M}$ ($n = 3$), $50.7 \pm 3.0 \mu\text{M}$ ($n = 2$), $8.1 \pm 0.4 \mu\text{M}$ ($n = 4$) and $20.1 \pm 2.2 \mu\text{M}$ ($n = 4$) respectively. The corresponding maximal inhibitable rates (V_i) were $90.9 \pm 2.5\%$, $65.5 \pm 2.7\%$, $79.9 \pm 2.8\%$ and $79.7 \pm 7.1\%$. For rat heart cells in the presence of 0.04 mM and 1 mM pyruvate the derived values were $16.1 \pm 1.0 \mu\text{M}$ ($n = 5$), $25.8 \pm 0.5 \mu\text{M}$ ($n = 2$), $66.9 \pm 3.1\%$ and $52.1 \pm 0.5\%$. The dashed lines and open symbols represent rates of transport calculated theoretically, assuming the presence of two carriers as described in Table 4, and then fitted to the same equation as the experimental data.

inhibition. For the transport of both pyruvate and L-lactate, DBDS inhibition was apparently only partial and could best be fitted to the equation:

$$V_i = V_0 / (1 + [I]K_i) + V_n$$

where V_i is the rate of transport in the presence of inhibitor as a percentage of the rate in its absence, and V_0 and V_n represent that fractional percentage of the carrier-mediated transport that can and cannot be inhibited by DBDS respectively. The data of Figure 4 show that the value of V_0 is smaller at higher pyruvate concentrations, falling from $88 \pm 8\%$ ($n = 3$) to $63 \pm 5\%$ ($n = 2$) as pyruvate was increased from 0.04 to 1.0 mM in guinea-pig cardiac myocytes (Figure 4a). The equivalent values for rat cardiac myocytes (Figure 4c) were $67 \pm 2\%$ ($n = 5$) and $50 \pm 2\%$ ($n = 3$). The decreases in maximal extent of inhibition were accompanied by an increase in the calculated apparent K_i values from $10.0 \pm 1.5 \mu\text{M}$ to $50.7 \pm 3.0 \mu\text{M}$ in guinea-pig heart cells and from $16.1 \pm 1.0 \mu\text{M}$ to $25.8 \pm 0.5 \mu\text{M}$ in rat heart cells. The effects of increasing the L-lactate concentration from 0.2 to 5 mM on inhibition by DBDS were also studied in guinea-pig heart cells (Figure 4b). The calculated apparent K_i value increased from $8.1 \pm 0.4 \mu\text{M}$ to $20.1 \pm 2.2 \mu\text{M}$ ($n = 4$), but there was no change in the calculated maximal inhibition observed, which remained at about 80% ($79.9 \pm 2.8\%$ and $79.7 \pm 7.1\%$ respectively). Detailed kinetic experiments to determine the nature of inhibition by DBDS were not attempted, in view of the apparent partial inhibition observed. However, the increase in the calculated apparent K_i values as the substrate concentration was raised suggest that the inhibition has some competitive element. The inhibition of L-lactate transport into rat erythrocytes by DBDS is also thought to be competitive (Poole and Halestrap, 1991). The presence of DBDS had no effect on the ability of α -cyano-4-hydroxycinnamate to inhibit transport. Thus in four separate experiments, 5 mM α -cyano-4-hydroxycinnamate inhibited L-lactate transport by $86.3 \pm 1.0\%$ and $83.2 \pm 3.3\%$ in the absence and presence of 0.2 mM DBDS respectively.

Table 2 summarizes the derived values for the maximum inhibition of L-lactate (0.5 mM) transport caused by DBDS and other stilbene disulphonates, and their apparent K_i values. Data are presented for both rat and guinea-pig heart cells and also for rat erythrocytes to allow comparisons to be made. Three features emerge clearly. Firstly the heart cells from guinea pigs exhibit more DBDS-sensitive L-lactate and pyruvate transport than do those from rats, although the derived apparent K_i values are similar in both species. Secondly, the maximum observed inhibition is not the same for all the inhibitors used. Thirdly, the potency of the various stilbene disulphonates as inhibitors of L-lactate transport into rat heart cells is different from their potency as inhibitors of L-lactate transport into rat erythrocytes. Most notably, 4,4'-dinitrostilbene-2,2'-disulphonate (DNDS) has a substantially lower apparent K_i value in the heart cells ($17 \mu\text{M}$) than in erythrocytes ($1120 \mu\text{M}$), although the maximal extent of inhibition is only 40%.

Kinetic properties of the DBDS-insensitive transport of L-lactate

In two experiments we investigated the kinetics of pyruvate transport into rat heart cells on the DBDS-insensitive carrier directly by measuring initial rates of transport at 0 °C in the presence and absence of 0.2 mM DBDS. Six different concentrations of pyruvate between 0.04 mM and 1 mM were used, each in duplicate. In the presence of DBDS the K_m values ($\pm$ S.E.M.) derived by least-squares regression to the Michaelis-Menten equation were 0.47 ± 0.09 mM and 0.35 ± 0.05 mM. V_{max} values of 1.09 ± 0.11 and 1.20 ± 0.07 nmol/min per μl of intracellular space were obtained. In the absence of DBDS the K_m values were 0.25 ± 0.05 and 0.20 ± 0.01 mM and the V_{max} values 1.47 ± 0.11 and 2.09 ± 0.05 nmol/min per μl of intracellular space. However, it is not possible to derive reliable results from such experiments since increasing pyruvate concentrations may con-

Table 2 Comparison of the inhibition of L-lactate transport into cardiac myocytes from rat and guinea pig by stilbenedisulphonate derivatives and phloretin

Initial rates of 0.5 mM L-lactate transport into cardiac myocytes were measured at different concentrations of inhibitors as described in the text, and are given as percentages $\pm$ S.E.M. of the control (α -cyano-4-hydroxycinnamate-sensitive) rates of transport, for the numbers of experiments shown in parentheses. Apparent K_i values were calculated as described in the text and legend to Figure 3 and represent the inhibitor concentration giving 50% of the maximum obtainable inhibition in the presence of 0.5 mM L-lactate. * Values for rat erythrocytes are taken from Poole and Halestrap (1991) and Poole et al. (1991).

Inhibitor	Apparent K_i (μ M) (at 0.5 mM L-lactate)			Maximal extent of inhibition (% of control)	
	Rat erythrocytes	Rat myocytes	Guinea-pig myocytes	Rat myocytes	Guinea-pig myocytes
DIDS	53*	—	31.1 $\pm$ 1.1 (3)	—	70.9 $\pm$ 2.8
DBDS	36*	16.9 $\pm$ 3.9 (5)	16.0 $\pm$ 2.3 (6)	49.6 $\pm$ 2.5	79.9 $\pm$ 2.3
TBenzDS	1.5*	2.6 $\pm$ 1.3 (5)	—	50.6 $\pm$ 5.5	—
SITS	132*	93.3 $\pm$ 18 (3)	—	63.1 $\pm$ 6.2	—
DNDS	1120*	16.9 $\pm$ 1.7 (4)	60.2 $\pm$ 9.0 (3)	39.6 $\pm$ 2.8	60.9 $\pm$ 2.8
NO ₂ -DBDS	9.8	15.6 $\pm$ 0.5 (2)	—	38.7 $\pm$ 4.3	—
Phloretin	1.4	25.7 $\pm$ 2.4 (5)	16.1 $\pm$ 2.3 (5)	83.9 $\pm$ 3.2	97.6 $\pm$ 3.0

Figure 5 Pyruvate and D-lactate inhibition of L-lactate transport into isolated rat cardiac myocytes in the presence and absence of DBDS

The cell suspension was preincubated at 11 °C with (▲) or without (●) 0.2 mM DBDS for 2–5 min, before transport was initiated by addition of 0.5 mM L-[¹⁴C]lactate and pyruvate (b) or D-lactate (a) at the final concentrations indicated. Transport was terminated after 30 s by centrifugation as described in the Experimental section. The data are expressed in terms of the rate of uptake in the presence of pyruvate or D-lactate as a percentage of that in its absence. The curves drawn show the fit by non-linear least-squares regression analysis to the equation for competitive inhibition, $V = V_{\max} / \{1 + (K_m/[S]) + ([I]/K_i)\}$, by using the K_m value derived for L-lactate transport given in Table 1. The derived competitive K_i values for pyruvate and D-lactate in the absence and presence of DBDS are given in the text.

comitantly decrease the extent of inhibition by DBDS of the DBDS-sensitive component. This problem cannot be circumvented by the use of higher concentrations of DBDS, because its maximum solubility in the buffers used was about 0.2 mM. However, we have established previously that, for the lactate transporter from guinea-pig heart cells, measured K_m values for pyruvate and L-lactate are the same as their measured K_i values as competitive inhibitors (Poole et al., 1989). Thus we have studied the ability of pyruvate and D-lactate to inhibit L-lactate transport in the presence of 0.2 mM DBDS. The data for rat heart cells are shown in Figure 5. Both pyruvate and D-lactate appeared to be capable of fully inhibiting the DBDS-insensitive transport of L-lactate with measured K_i values (means $\pm$ S.E.M. of four separate experiments) of 0.26 ± 0.03 and 22.3 ± 1.7 mM respectively. In similar experiments the measured K_i values in the absence of DBDS were 0.18 ± 0.02 and 9.8 ± 0.5 mM respectively. These data imply that the transport of both pyruvate and D-lactate in the presence of DBDS may have a slightly lower substrate affinity than in its absence. Similar experiments were attempted with guinea-pig heart cells, but the amount of DBDS-insensitive α -cyano-4-hydroxycinnamate-insensitive L-lactate

uptake at 30 s was too small to yield reliable results (see Figure 1).

Inhibition of L-lactate transport into heart cells and erythrocytes by phloretin

The apparent partial nature of the inhibition of lactate and pyruvate transport into heart cells by stilbene disulphonates is unusual. It was therefore of interest to establish whether another class of potent inhibitors exhibits the same property. Phloretin has been reported to inhibit many transport processes such as sugar transport (LeFevre and Marshall, 1959) and the red-blood-cell anion transporter (Deuticke, 1977). Deuticke et al. (1978) have also reported that 300 μ M phloretin inhibited lactate transport into erythrocytes by 95%, but no concentration-dependence of the inhibition was given. The data of Figure 6 show the concentration-dependence of phloretin inhibition of lactate transport into rat erythrocytes and guinea-pig and rat cardiac myocytes. Three important points emerge. Firstly, erythrocytes were more sensitive to phloretin than were the cardiac myocytes. The apparent K_i values for phloretin in the

Figure 6 Inhibition by phloretin of L-lactate transport into cardiac myocytes from rat ($\blacktriangle$) and guinea-pig ($\bullet$) and from rat erythrocytes ($\blacksquare$)

Initial rates of uptake of 0.5 mM L-lactate were measured at 11 °C in the presence of the concentrations of phloretin indicated. For the erythrocytes, data are presented as means of 2 experiments in which the K_i value, derived by least-squares regression assuming 100% maximal inhibition, agreed within 5%. For cardiac myocytes, data are presented as means $\pm$ S.E.M. (error bars) of 5 separate experiments, and the curves drawn represent the fit to an equation for partial inhibition as described in the legend to Figure 3. The derived parameter values are given in Table 2.

presence of 0.5 mM L-lactate were 1.43 and 1.39 μ M in two separate experiments with rat erythrocytes, and $25.7 \pm 2.4 \mu$ M and $16.1 \pm 2.3 \mu$ M ($n = 5$) for rat and guinea-pig cardiac myocytes respectively. Secondly, for rat cardiac myocytes the inhibition clearly appeared to be partial, reaching a maximum value of $83.9 \pm 3.2\%$, whereas in heart cells from the guinea pig inhibition appeared to reach completion ($97.6 \pm 3.0\%$), as was also the case for rat erythrocytes. Thirdly, for both rat and guinea-pig heart cells the first addition of phloretin gave a variable degree of activation of transport; thereafter inhibition followed a conventional pattern. We have no explanation of this result, but it is known that phloretin can interact with the phospholipid bilayer and influence the activity of many membrane proteins (see Deuticke et al., 1991). For the regression analysis used to calculate K_i values and maximal extents of inhibition, data obtained in the absence of phloretin were not included.

DISCUSSION

Apparent partial inhibition of lactate and pyruvate transport by DBDS and phloretin can be explained by the presence of two carriers

The data presented in this paper demonstrate that DBDS and other stilbene disulphonates are only partial inhibitors of lactate and pyruvate transport into both rat and guinea-pig cardiac myocytes. The maximal extent of inhibition was dependent on both the inhibitor used and the species from which the heart cells were derived. This is shown most clearly in Table 2 by a comparison of SITS, DBDS and DNDS. These differences could be observed when the various inhibitors were used on the same cell preparations, and are therefore not a consequence of day-to-day variations in the proportion of sensitive and insensitive cells in the preparation. Phloretin also appears to be only a partial inhibitor in rat heart cells, whereas inhibition in cells from the guinea pig approached 100%. It may be significant that maximum percentage inhibition of transport by the stilbene disulphonates was always greater in the guinea-pig heart cells than in those from the rat.

Two explanation for partial inhibition by DBDS can be expounded: either a single carrier species whose interaction with

the inhibitor does not totally inhibit transport, or the presence of two carriers, only one of which is strongly inhibited by DBDS. However, the single-carrier model does not easily explain how the maximal extent of inhibition by stilbene disulphonates or phloretin varies with both the particular inhibitor used and the species from which the heart cells were derived (Table 2) and why it is also affected by the substrate concentration (Figure 4). In contrast, the two-carrier model can account for these observations and provide an explanation for the differences in kinetic behaviour seen in heart cells from rat and guinea pig, as described below.

The two-carrier model assumes that each carrier has different K_i and K_m values for substrates and inhibitors and is present at different concentrations in guinea-pig and rat heart cells. An initial consideration of our data suggested that one of the carriers might have a low K_m for pyruvate, be inhibited by DBDS and phloretin and be able to transport D-lactate, whereas the other carrier might be insensitive to these inhibitors, have a higher K_m for pyruvate and be relatively poor at transporting D-lactate. Both carriers would have similar K_m values for L-lactate of about 2 mM. Since, in comparison with guinea-pig heart cells, rat heart cells demonstrate less inhibition by stilbene disulphonates and phloretin (see Figure 3; Table 2), a higher overall K_m for pyruvate (see Table 1), and relatively lower rates of D-lactate transport (Figure 1), it seems probable that they contain less of the DBDS-sensitive carrier. Further support for the two-carrier model comes from the data of Figure 5, where it is shown that D-lactate has a lower affinity for the DBDS-insensitive carrier than for the DBDS-sensitive carrier. Thus it inhibited L-lactate transport in the presence of DBDS with a measured competitive K_i of 22.3 ± 1.7 mM, significantly less than the value of 9.8 ± 0.5 mM measured in the absence of DBDS.

In theory, it is possible to fit all our data by regression analysis to an equation representing such a two-carrier model. However, in practice, with the limited quantity of heart cells available for each experiment, it was not possible to generate the necessary quantity of data with sufficient accuracy to allow a reliable fit to such a complex equation containing many closely related variables. In an attempt to overcome this problem, we have estimated, by trial and error, values for the K_m , K_i and V_{max} of the two carriers that would produce the observed kinetic behaviour and pattern of inhibition. In order to fit all the experimental data to the same two-carrier model with a common set of parameter values, it was found necessary for the first carrier ('DBDS-sensitive') to be inhibited in mixed fashion by the inhibitors and the second carrier to be competitively inhibited. It should also be noted that, for the purposes of these calculations, it is assumed that K_m and K_i values for competing substrates such as D-lactate are equivalent, as discussed above. The process adopted to select the optimal parameter values for the two carriers was as follows. Initial guesses of the parameter values were made, and from these were calculated predicted rates of transport at different substrate and inhibitor concentrations by using the relevant equations described in the legends to Tables 3 and 4. These predicted data values were then fitted to the same equations as the experimental data, which assumes the presence of a single carrier. After comparison of the parameter values derived from the predicted and measured data, guesses for the initial parameter values were refined and the procedure was repeated. This was continued until there was a reasonable agreement between the theoretical fits and the fits using the experimental data of Tables 1 and 2 and Figures 3 and 4.

The final values derived for K_m and V_{max} of the two carriers are summarized in Table 3, whereas K_i values for the different inhibitors are given in Table 4. The calculated values ($\pm$ S.E.M.)

Table 3 Kinetic parameters predicted for the two carriers of rat and guinea-pig cardiac myocytes

The K_m and V_{max} values for the two putative carriers were set at the values shown, since these were found to give the best fit to the experimental data as described in the text. The theoretical rates of transport were then calculated at 10 different substrate concentrations similar to those used in the kinetic experiments. These theoretical data were then fitted by non-linear least-squares regression to the normal Michaelis-Menten equation for a single carrier. The derived K_m and V_{max} values ($\pm$ S.E.M.) are shown and compared with the experimentally derived values.

Substrate	Species	Carrier 1		Carrier 2	
		Set K_m^1 (mM)	Set V_{max}^1 (nmol/min per μ l)	Set K_m^2 (mM)	Set V_{max}^2 (nmol/min per μ l)
Pyruvate, 0 °C	Guinea pig	0.05	0.30	0.30	0.15
	Rat	0.10	0.80	0.30	1.40
L-Lactate, 11 °C	Guinea pig	2.50	1.50	2.00	0.75
	Rat	2.50	4.00	2.00	7.00
D-Lactate, 11 °C	Guinea pig	5.00	1.50	25.00	0.75
	Rat	5.00	4.00	25.00	7.00
Single carrier assumed					
Substrate	Species	Predicted K_m (mM)	Measured K_m (mM)	Predicted V_{max} (nmol/min per μ l)	Measured V_{max} (nmol/min per μ l)
Pyruvate, 0 °C	Guinea pig	0.076 ± 0.003	0.07 ± 0.01	0.42 ± 0.01	0.41 ± 0.06
	Rat	0.184 ± 0.003	0.20 ± 0.02	2.12 ± 0.02	2.09 ± 0.23
L-Lactate, 11 °C	Guinea pig	2.310 ± 0.001	2.27 ± 0.19	2.25 ± 0.01	2.27 ± 0.24
	Rat	2.160 ± 0.001	2.15 ± 0.09	11.0 ± 0.01	10.30 ± 0.60
D-Lactate, 11 °C	Guinea pig	6.420 ± 0.100	6.60 ± 1.20	2.01 ± 0.02	ND
	Rat	9.370 ± 0.120	10.20 ± 1.70	9.20 ± 0.12	ND

Table 4 Predicted inhibition constants for phloretin and various stilbene disulphonates as inhibitors of L-lactate transport into rat and guinea-pig cardiac myocytes, assuming the presence of two carriers

The rates of transport of 0.5 mM L-lactate at increasing concentrations of inhibitor were calculated by using the equation that assumes carrier 1 to be inhibited in mixed fashion, with K_1^1 and K_2^2 representing the competitive and uncompetitive K_i values respectively, and carrier 2 to be inhibited competitively (K_3^3), i.e.:

$$v = \frac{V_{max}^1 \{ (K_m^1/[S]) (1 + [I]/K_1^1) + (1 + [I]/K_2^2) \} + V_{max}^2 \{ 1 + (K_m^2/[S]) (1 + [I]/K_3^3) \}}{1 + [I]/K_1^1 + (1 + [I]/K_2^2) + 1 + (K_m^2/[S]) (1 + [I]/K_3^3)}$$

The K_m and V_{max} values for the two putative carriers were set at the values shown for L-lactate in Table 3, whereas K_i values for the inhibitors were set at the values shown in the present Table. Twelve calculated rates of transport at inhibitor concentrations in the same region as used to determine the experimentally derived K_i values shown in Table 2 were fitted by non-linear least-squares regression to the equation for inhibition of a single carrier as described in Table 2. The derived K_i values and maximal inhibitable rate (V_0) are shown as means $\pm$ S.E.M., and can be compared with the experimentally derived values in Table 2.

Inhibitor	Species	Carrier 1		Carrier 2 Set K_3^3 (μ M)	Single carrier assumed	
		Set K_1^1 (μ M)	Set K_2^2 (μ M)		Predicted K_i (μ M)	Predicted V_0 (%)
DBDS	Guinea pig	9	60	250	15.9 ± 1.3	75.8 ± 1.7
	Rat	5	30	400	17.7 ± 3.8	48.3 ± 2.8
DNDS	Guinea pig	50	500	5000	61.1 ± 0.3	64.4 ± 1.0
	Rat	11	80	2000	17.2 ± 1.2	36.6 ± 0.7
TBenzDS	Rat	0.5	1	75	2.6 ± 0.8	46.0 ± 3.7
SITS	Rat	40	200	500	92.5 ± 6.0	60.2 ± 1.4
DIDS	Guinea pig	20	100	500	29.8 ± 1.4	72.7 ± 0.8
Phloretin	Guinea pig	10	80	25	16.4 ± 0.3	98.0 ± 0.5
	Rat	4	40	60	24.8 ± 3.3	83.9 ± 2.9

for the K_m , V_{max} , K_i and maximal extent of inhibition assuming the combined action of both carriers are also given. These can be compared with the experimentally derived values in Tables 1 and 2, and it will be seen that the two sets of values agree very well. For DBDS inhibition of L-lactate transport into both rat and guinea-pig heart cells this is demonstrated graphically by the open symbols and dashed lines in Figure 3. The dashed lines in

Figure 4 show how well the fit is maintained at different concentrations of pyruvate and L-lactate. Although the agreement between experimental and theoretical curves is not as good as at 0.5 mM L-lactate, the similarities between the experimental and theoretical curves are very pronounced. It must be stressed that the constraints imposed by having to fit the data from many experiments performed with different inhibitors and at different

substrate concentrations to the same set of parameter values for the two carriers are considerable. Thus, if the agreement between theoretical and measured data is reasonable, the model is likely to be a good one, much as is the case for a normal non-linear least-squares regression analysis. Some independent support for the model comes from the K_m values for pyruvate transport into the rat heart cell carrier determined in the presence of 0.2 mM DBDS. Under such conditions only the DBDS-insensitive carrier would operate. In two such experiments the values obtained ($\pm$ S.E.M.) were 0.47 ± 0.09 mM and 0.35 ± 0.05 mM. These values are slightly higher than the K_m proposed for the DBDS-insensitive carrier (0.3 mM), but this would be predicted by the model, since the DBDS-insensitive carrier is also inhibited competitively by DBDS, with a K_i value of 400 μ M. Thus at 200 μ M DBDS the predicted apparent K_m for pyruvate would be approx. 450 μ M.

One exception to the agreement between experimentally and theoretically derived parameter values is the predicted V_{max} for D-lactate transport, especially in rat heart cells. This is substantially higher than extrapolation of the rates of 0.5 mM D-lactate would suggest. However, this discrepancy is easily accounted for if the V_{max} of either or both carriers for the transport of D-lactate is different from that for the transport of L-lactate and pyruvate. Tables 3 and 4 also demonstrate that some minor species differences in K_m and K_i values of the two carriers are required in order to fit the data for rat and guinea-pig heart cells. Such variations in K_m and K_i values are known to occur in the kinetics of erythrocyte monocarboxylate and phosphate transport between different species such as human and rat (Deuticke, 1982; Halestrap et al., 1990; Gruber and Deuticke, 1973) and might merely reflect differences in phospholipid composition or protein glycosylation.

The presence of two isoforms of the monocarboxylate carrier in heart cells with distinct kinetic properties and distinguishable by their inhibitor sensitivity is not without precedent. Thus the existence of more than one type of the Na^+/H^+ antiporter in some cells has been demonstrated by their different sensitivities to amiloride derivatives and other inhibitors such as cimetidine and clonidine (Haggerty et al., 1988; Ramamoorthy et al., 1991). Different isoforms of the glucose transporter within the same cell have also been described (Gould and Bell, 1990; Yang et al., 1992). In order to confirm this conclusion, purification and characterization of the protein(s) involved is required. Our recent identification and partial purification of the erythrocyte lactate carrier (Poole and Halestrap, 1992) should assist the attainment of this goal.

Potential use of stilbene disulphonates as inhibitors of lactate transport in the heart

The ability to inhibit lactate transport across the plasma membrane with a specific impermeant inhibitor could have many uses. In particular, it would aid the study of the importance of lactic acid transport for the regulation of intracellular pH during hypoxia and ischaemia followed by reperfusion. The build-up of lactic acid during hypoxia or ischaemia (Opie et al., 1973; Richards et al., 1987) causes intracellular acidification, and its subsequent removal from the cell during normoxia or reperfusion restores the intracellular pH (Garlick et al., 1979; Brooks and Willis, 1983; Allen et al., 1985; Allen and Orchard, 1987). There is considerable evidence that low intracellular pH impairs cardiac muscle contraction on the one hand (Fabiato and Fabiato, 1978; Eisner et al., 1987; Vaughan-Jones et al., 1987), but may protect hearts from the damage that can occur following reperfusion

after ischaemia (see Poole-Wilson, 1989; Bond et al., 1991; Halestrap, 1991). Some preliminary studies on the importance of lactate transport have been performed with α -cyanocinnamate analogues (Halestrap and Denton, 1975; Eisner et al., 1987, 1988), but these inhibitors have the distinct disadvantage of entering the cell and inhibiting mitochondrial pyruvate transport and oxidation (Halestrap and Denton, 1974, 1975; Halestrap, 1975). In contrast, DBDS neither enters the cell nor inhibits ADP-stimulated pyruvate oxidation by heart mitochondria at concentrations up to 0.5 mM (results not shown). Thus the stilbene disulphonates may have a useful part to play in studies on the metabolic and physiological significance of lactate transport in the heart, although the partial nature of their inhibition seen in the isolated-cell experiments suggest limitations to their use. The extent to which this limitation applies may well be species-dependent in the light of the difference in behaviour of rat and guinea-pig heart cells described here. However, partial inhibition of lactate transport could be an advantage for the heart when it is reperfused following a period of ischaemia. The return to normal intracellular pH might be slower, and so protect the heart from reperfusion damage as outlined above.

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