The kinetics of transport of lactate and pyruvate into isolated cardiac myocytes from guinea pig

Kinetic evidence for the presence of a carrier distinct from that in erythrocytes and hepatocytes

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1. Time courses for the uptake of L-lactate, D-lactate and pyruvate into isolated cardiac ventricular myocytes from guinea pig were determined at 11 °C or 0 °C (for pyruvate) in a citrate-based buffer by using a silicone-oil-filtration technique. These conditions enabled initial rates of transport to be measured without interference from metabolism of the substrates. 2. At a concentration of 0.5 mM, transport of all these substrates was inhibited by approx. 90 % by 5 mM- α -cyano-4-hydroxycinnamate; at 10 mM-L-lactate a considerable portion of transport could not be inhibited. 3. Initial rates of L-lactate and pyruvate uptake in the presence of 5 mM- α -cyano-4-hydroxycinnamate were linearly related to the concentration of the monocarboxylate and probably represented diffusion of the free acid. The inhibitor-sensitive component of uptake obeyed Michaelis–Menten kinetics, with K_m values for L-lactate and pyruvate of 2.3 and 0.066 mM respectively. 4. Pyruvate and D-lactate inhibited the transport of L-lactate, with K_i values (competitive) of 0.077 and 6.6 mM respectively; the K_i for pyruvate was very similar to its K_m for transport. The K_i for α -cyano-4-hydroxycinnamate as a non-competitive inhibitor was 0.042 mM. 5. These results indicate that L-lactate, D-lactate and pyruvate share a common carrier in guinea-pig cardiac myocytes; the low stereoselectivity for L-lactate over D-lactate and the high affinity for pyruvate distinguish it from the carrier in erythrocytes and hepatocytes. The metabolic roles for this novel carrier in heart are discussed.

INTRODUCTION

Transport of L-lactate and pyruvate across the plasma membrane has been studied most extensively in erythrocytes, where a specific monocarboxylate carrier is present (Halestrap, 1976; Deuticke *et al.*, 1978; Dubinsky & Racker, 1978; Deuticke, 1982). At their physiological concentrations, no more than 10 % of total transport occurs by the diffusion of the free acid or on the inorganic-anion exchanger, band 3 (Deuticke *et al.*, 1982). The lactate carrier of erythrocytes catalyses the electroneutral transport of a proton with a monocarboxylate anion (Dubinsky & Racker, 1978; Leeks & Halestrap, 1979). It is stereoselective for L- over D-lactate (Deuticke *et al.*, 1978) and inhibited by a variety of compounds, including α -cyanocinnamate derivatives, organomercurials, amino reagents, quercetin, and high concentrations of the stilbenedisulphonate DIDS (Halestrap & Denton, 1974; Halestrap, 1976; Deuticke *et al.*, 1978; Belt *et al.*, 1979; Jennings & Adams-Lackey, 1982). This carrier has recently been solubilized and functionally reconstituted into lipid vesicles (Poole & Halestrap, 1988). A carrier very similar to that in erythrocytes has been shown to be present in hepatocytes (Monson *et al.*, 1982; Fafournoux *et al.*, 1985; Edlund & Halestrap, 1988; Quintana *et al.*, 1988). Less extensive studies have indicated the presence of a broadly similar plasma-membrane carrier in many other tissues [see Poole & Halestrap (1988) for references].

The transport of lactate and pyruvate across the cardiac sarcolemmal membrane is of considerable

physiological importance. These metabolites are readily oxidized by the heart [see, e.g., Illingworth *et al.* (1975) and Taegtmeyer *et al.* (1980)]; indeed, under aerobic conditions L-lactate may be a major fuel (Lassers *et al.*, 1971; Mowbray & Ottaway, 1973). In contrast, under conditions of hypoxia or ischaemia, net efflux of lactic acid from the heart occurs, as cytosolic [NADH]/[NAD⁺] rises and pyruvate is diverted from oxidation by the citric acid cycle to lactate formation (Opie, 1976; Katz & Sahlin, 1988). Some studies on lactate and pyruvate transport across the cardiac sarcolemmal membrane have been carried out with the perfused heart. On the basis of measurements of intra- and extra-cellular L-lactate and pyruvate, early results indicated that a carrier-mediated transport mechanism must exist (Watts & Randle, 1967; Mowbray & Ottaway, 1973). It was noted in such studies that the ratio of intracellular to perfusate [pyruvate] approached unity (Opie & Mansford, 1971), whereas the same ratio for L-lactate was often considerably greater than 1. These data would suggest that pyruvate equilibrates more readily across the sarcolemmal membrane than does L-lactate. Indeed, Henderson *et al.* (1969) found the relative efflux rate for pyruvate from perfused rat heart to be 15 times that of L-lactate. More recently, more direct attempts to measure lactate and pyruvate transport in the perfused heart have been made, using the paired tracer-dilution technique (Dennis *et al.*, 1985; Mann *et al.*, 1985). Such studies are only able to reveal gross kinetic parameters for lactate clearance by the heart, and do not discriminate between transport

Abbreviations used: pCMBS, *p*-chloromercuribenzenesulphonate; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; DMO, 5,5'-dimethylloxazolidine-2,4-dione.

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from the perfusion medium into the interstitial space and transport across the plasma membrane. Nevertheless, these investigations have shown saturation kinetics for L-lactate uptake, and inhibition of L-lactate uptake by pyruvate. Apparent K_m values for L-lactate uptake were 6 and 19 mM for rat and rabbit hearts respectively (Dennis *et al.*, 1985; Mann *et al.*, 1985). Mann *et al.* (1985) also presented evidence that the carrier was stereoselective for L- over D-lactate and sensitive to inhibition by α -cyano-4-hydroxycinnamate.

More suitable systems for obtaining accurate kinetic parameters of the transport are cardiac sarcolemmal membrane vesicles or isolated ventricular myocytes. Trosper & Philipson (1987) have presented data on L-lactate transport in rabbit cardiac sarcolemmal membrane vesicles, and shown that lactate uptake could be stimulated by a proton gradient, but not by a Na^+ gradient. α -Cyano-4-hydroxycinnamate and pCMBS were found to be inhibitors of this proton-linked uptake, and a K_m for L-lactate in the range 16–40 mM was obtained. However, in their analysis Trosper & Philipson (1987) did not determine the kinetic parameters for carrier-mediated transport in isolation from diffusion of the free acid; the latter can become the major contribution to uptake at L-lactate concentrations of 10 mM and above (see Edlund & Halestrap, 1988). An account of L-lactate and pyruvate uptake by isolated cardiac myocytes from rat was presented by Kammermeier *et al.* (1985). They claimed saturation at a concentration of approx. 12 mM for L-lactate, but again did not differentiate between carrier-mediated and diffusion components of uptake. We have therefore carried out a more detailed analysis of carrier-mediated L-lactate and pyruvate transport in isolated guinea-pig cardiac myocytes under the same conditions as we have used to study transport in erythrocytes and hepatocytes. This has enabled direct comparisons to be drawn between these systems.

EXPERIMENTAL

Materials

All chemicals and biochemicals were obtained from the sources given previously (Edlund & Halestrap, 1988; Poole & Halestrap, 1988). Collagenase (type 1) was obtained from Worthington, Irvine, Scotland, U.K., and protease (type XXV) from Sigma Chemical Co., Poole, Dorset, U.K. Dunkin Hartley guinea pigs were supplied by Bantin and Kingman, Hull, U.K. Radiochemicals were from Amersham International, Amersham, Bucks., U.K.

Isolation of cardiac ventricular myocytes

Myocytes were isolated from guinea-pig hearts by a combination of enzymic and mechanical dispersion (Lee, 1987). The hearts were retrogradely perfused via the aorta at a constant flow rate of 9 ml/min. The perfusion buffer contained 130 mM-NaCl, 5 mM-Hepes, 10 mM-glucose, 20 mM-aurine, 10 mM-creatine, 5.4 mM-KCl, 3.5 mM-MgCl₂ and 0.4 mM-NaH₂PO₄ in ultra-pure water (Milli Q instrument from Millipore Corp.), neutralized to pH 7.25 with NaOH and gassed with 100% O₂ (buffer A). The heart was perfused at 37 °C with buffer A + 750 μM -CaCl₂ for 4 min, and then for 4 min with buffer A + 100 μM -EGTA. Finally the heart was perfused with buffer A + 80 μM -CaCl₂, 1 mg of

collagenase (Worthington type 1)/ml and 0.1 mg of protease (Sigma type XXV)/ml for 10 min. The digested heart was removed, chopped coarsely and shaken for four 5 min periods in a plastic flask containing the enzyme solution (above) with the addition of 1% (w/v) bovine serum albumin. After each 5 min incubation the tissue was filtered through a nylon gauze (pore size 200 μm), and undigested material shaken for a further period. Isolated cells which filtered through the gauze were collected by centrifugation at 40 *g* for 45 s. The supernatant was then changed to a storage solution containing 10 mM-Hepes, 100 mM-glutamate, 30 mM-KCl, 1 mM-EGTA, 5 mM-pyruvate, 20 mM-aurine, 20 mM-glucose, 5 mM-MgCl₂, 5 mM-succinate, 5 mM-creatine, 5 mM-ATP and 5 mM- β -hydroxybutyrate, pH 7.2 (KB solution: Isenberg & Klockner, 1982). This preparation routinely yielded (2–4) $\times 10^6$ Ca²⁺-tolerant cardiac myocytes with rod-shaped morphology. To prepare the cells for transport measurements, the supernatant was removed and the pellet suspended in 30 vol. of Ca²⁺-free bicarbonate-buffered saline (Krebs & Henseleit, 1932) + 0.5 mM-EGTA to wash the myocytes free of glucose, lactate and pyruvate. The cells were then allowed to settle by gravity, and the supernatant was removed and replaced with 30 vol. of 84 mM-sodium citrate, adjusted to pH 7.4 with NaH₂PO₄, at the temperature used for transport measurements. The cells were allowed to sediment once more, and then suspended to the final incubation volume in the same buffer. When pyruvate was assayed enzymically in the cell supernatant, it was found to be present at a concentration of less than 2 μM . The percentage of rod-shaped cells in the final incubation was 65–90%; most of those cells which had rounded up were leaky, as judged by Trypan Blue exclusion. Since we have expressed our results as uptake of substrate/ μl of intracellular space, small variations in the percentage of viable rod-shaped cells should not affect measurements of transport; this was found to be the case. In some experiments uptake of L-lactate was measured in the physiological saline medium of Krebs & Henseleit (1932) containing 0.5 mM-EGTA, in which 25 mM-Hepes replaced bicarbonate as buffer. Albumin was not present in the incubation buffers, since it is known to bind α -cyano-4-hydroxycinnamate (Thomas & Halestrap, 1981). In order to prevent damage to the large ventricular myocytes by passage through a narrow-aperture 200 μl automatic pipette, tips were modified by removal of 5 mm of the tip with a sharp blade.

Cell protein was measured by a modified biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard.

Measurement of transport, intracellular volumes and pH gradients

Initial rates of transport were measured by an oil filtration technique, essentially as described by McGivan *et al.* (1977) and Edlund & Halestrap (1988), but with a ratio of silicone oil MS550 (sp.gr. 1.07) to dinonyl phthalate (sp.gr. 0.97) of 13:7 (v/v). Cardiac ventricular myocytes at approx. 2–4 mg of protein/ml were equilibrated with constant shaking at 11 °C or 0 °C as indicated. Transport was initiated by mixing 10 vol. of cells with 1 vol. of citrate buffer, the latter containing ¹⁴C-labelled substrate and ³H₂O at approx. 15 $\mu\text{Ci/ml}$ and 100 $\mu\text{Ci/ml}$ respectively. Samples (110 μl) of cell suspension were layered on top of the oil, and uptake

was terminated at the appropriate time by centrifugation for 25 s. Thereafter the methodology was the same as reported by Edlund & Halestrap (1988). Corrections were made for the contribution by radiolabelled substrate to the total substrate concentration. Since the specific radioactivity of the [^{14}C]pyruvate was only 30 mCi/mmol, for pyruvate concentrations of 0.04 mM or less no additional unlabelled pyruvate was added and the amount of ^{14}C was decreased accordingly. The pH gradients and intracellular volumes were measured by using $^3\text{H}_2\text{O}$ (10 $\mu\text{Ci}/\text{ml}$ final sp. radioactivity) and either 0.5 mM-5,5'-dimethyl[2- ^{14}C]oxazolidine-2,4-dione (DMO) (0.5 mM, 1.5 $\mu\text{Ci}/\text{ml}$ final sp. radioactivity) or 0.5 mM-[U- ^{14}C]sucrose (1.5 $\mu\text{Ci}/\text{ml}$ final sp. radioactivity) as described previously (Addanki *et al.*, 1968; Edlund & Halestrap, 1988). Volumes were measured in quadruplicate for each cell preparation. A mean ($\pm$ S.E.M.) value of $2.76 \pm 0.06 \mu\text{l}/\text{mg}$ of protein at 11 $^{\circ}\text{C}$ was determined for 28 separate preparations of cardiac myocytes. It was noted previously that direct centrifugation of hepatocytes yielded a greater value for the cell volume than did centrifugation through oil, probably as a result of loss of water during the passage of the cells through the oil (Edlund & Halestrap, 1988; Halestrap, 1988). Measurement of myocyte volumes by direct centrifugation yielded values that were $114 \pm 7\%$ (mean $\pm$ S.E.M. for 15 cell preparations) of those obtained by oil filtration. Since it is technically difficult to measure small volumes by direct centrifugation, and the difference in values between the two techniques is small, oil filtration was used for all subsequent experiments.

Any modifications to the above protocols are noted in the text or Figure legends.

RESULTS AND DISCUSSION

Time courses of lactate and pyruvate uptake into isolated cardiac myocytes at 11 $^{\circ}\text{C}$

Time courses of lactate and pyruvate uptake were measured at 11 $^{\circ}\text{C}$ in order to minimize metabolism of these compounds and enable measurement of true initial rates of transport into the cells. Experiments were performed in citrate medium, since both initial rates of transport and uptake at equilibrium were found to be about twice the values of those obtained in Hepes-buffered Krebs-Henseleit medium. This was also observed for lactate transport into liver cells (Edlund & Halestrap, 1988) and reflects a greater pH gradient (alkaline inside) across the plasma membrane in citrate buffer. In the present experiments, the values for the pH gradient in citrate buffer and Hepes-buffered Krebs-Henseleit medium were 0.47 ± 0.03 ($n = 4$) and 0.18 ± 0.02 ($n = 3$) respectively. The use of citrate buffer also enables the results to be compared directly with those we have previously obtained for erythrocytes and hepatocytes. Time courses of L-[^{14}C]lactate uptake into myocytes incubated in citrate buffer are shown in Fig. 1. At 0.5 mM-L-lactate uptake was substantially inhibited in the presence of 5 mM- α -cyano-4-hydroxycinnamate; this concentration of the compound gave maximal inhibition of transport. The values of the pH gradient for control and α -cyano-4-hydroxycinnamate incubations were 0.47 ± 0.03 and 0.47 ± 0.03 (means $\pm$ S.E.M., $n = 4$) respectively. Thus it is clear that the inhibition of lactate transport was not through an indirect effect on the pH

gradient. At an L-lactate concentration of 10 mM, uptake was decreased in the presence of α -cyano-4-hydroxycinnamate, but not to the extent observed at the lower concentration of substrate.

The time courses of uptake were fitted by non-linear least-squares regression analysis to the first-order rate equation as described previously (Edlund & Halestrap, 1988):

$$L_t = L_{\text{tot}}(1 - e^{-k(t+x)})$$

where L_t is the lactate uptake at time t , L_{tot} is the total uptake of lactate at equilibrium, k is the rate constant and x is the 'dead-time' of centrifugation (the time taken to sediment the cells through the oil layer into HClO_4). Such analysis of time courses of substrate uptake into cardiac myocytes revealed a dead-time of approx. 7 s; this is similar to values of approx. 5 s reported for hepatocytes (McGivan *et al.*, 1977; Edlund & Halestrap, 1988). This is shown as the negative intercept on the abscissa. The data in Fig. 1 show that at both concentrations of L-lactate uptake was approximately a linear function of time for about the first 30 s, and this time point was used as an estimate of initial rates of transport in the determination of the kinetic parameters of the transport process.

From the combined data of Fig. 1, L-lactate uptake at equilibrium could be determined by least-squares regression analysis, which gave values of 1.03 and 12.2 nmol/ μl of intracellular space for 0.5 mM- and 10 mM-L-lactate respectively; the corresponding values for the pH gradient calculated from [^{14}C]DMO distribution were 0.47 ± 0.03 ($n = 4$) and 0.33 ± 0.02 ($n = 4$). These data suggest that lactate enters the cell with a proton, as in the erythrocyte (Dubinsky & Racker, 1978; Leeks & Halestrap, 1979; Jennings & Adams-Lackey, 1982), leading to a dissipation of the pH gradient. However, if distribution of lactate were determined solely by the pH gradient, equilibrium uptake would be 1.47 and 21.4 nmol/ μl of intracellular space at 0.5 mM- and 10 mM-L-lactate respectively. These discrepancies, which are greater at high lactate concentrations, have been noted previously for the transport of lactate into hepatocytes (Edlund & Halestrap, 1988), and may reflect the presence of an anionic pathway capable of driving lactate out of the cell under the influence of the membrane potential, particularly when it is present at high concentrations. Such a pathway is thought to exist in striated muscle (Mason, 1986) and Lettré cells (Bashford & Pasternak, 1984).

In Fig. 2 we show time courses for the uptake of 0.5 mM-D-lactate and -pyruvate into cardiac myocytes at 11 $^{\circ}\text{C}$ in the absence and presence of α -cyano-4-hydroxycinnamate. In both cases uptake was substantially inhibited by α -cyano-4-hydroxycinnamate, and pyruvate uptake was very fast by comparison with either D- or L-lactate. The observation of inhibitor-sensitive uptake of D-lactate is surprising, since in erythrocytes and hepatocytes this stereoisomer is a very poor substrate for the lactate carrier, and is believed to cross the plasma membrane largely by diffusion of the free acid (Deuticke *et al.*, 1978; Monson *et al.*, 1982; Edlund & Halestrap, 1988; Poole & Halestrap, 1988).

Kinetics of L-lactate transport at 11 $^{\circ}\text{C}$

Initial rates of L-lactate transport were measured at lactate concentrations in the range 0.25–10 mM, and data from these experiments are shown in Fig. 3. It is clear

Fig. 1. Time courses of L-lactate uptake by isolated cardiac myocytes in the presence and absence of α -cyano-4-hydroxycinnamate

The time courses of the uptake of (a) 0.5 mM- and (b) 10 mM-L-lactate by isolated guinea-pig cardiac myocytes were determined at 11 °C, in the absence (●) or presence (○) of 5 mM- α -cyano-4-hydroxycinnamate as described in the Experimental section. Results are shown as means $\pm$ S.E.M. for four experiments in each case. In each experiment individual values were the result of duplicate determinations. The lines drawn through the points were those obtained by fitting the data to a first-order rate equation [$L_t = L_{\text{tot}}(1 - e^{-k(t+x)})$], where L_t is the lactate uptake at time t , L_{tot} is the lactate uptake at equilibrium, k is the rate constant and x is the 'dead-time' of centrifugation (the intercept on the abscissa, which represents the time taken to sediment the cells through the oil layer into HClO_4).

Fig. 2. Time courses for the uptake of D-lactate and pyruvate in the absence and presence of α -cyano-4-hydroxycinnamate

Time courses are shown for the uptake of 0.5 mM-D-lactate (a) and -pyruvate (b) by isolated cardiac myocytes at 11 °C in the absence (●) and presence (○) of 5 mM- α -cyano-4-hydroxycinnamate. Results are shown as mean values for two experiments, which agreed within 10%.

that in the presence of 5 mM- α -cyano-4-hydroxycinnamate uptake was a linear function of substrate concentration; these data suggest that the α -cyano-4-hydroxycinnamate-insensitive portion of uptake is due to non-ionic diffusion of the free acid. In order to calculate the carrier-mediated component of uptake, this inhibitor-insensitive component was subtracted from the control value, to leave the carrier-mediated component. This carrier-mediated L-lactate transport obeyed Michaelis-Menten kinetics (Fig. 3b). For five separate experiments, the K_m and V_{max} ($\pm$ S.E.M.) were found to be 2.27 ± 0.19 mM and 2.72 ± 0.24 nmol/min per μl of in-

tracellular space. This K_m value is very similar to that found for L-lactate transport into rat erythrocytes and hepatocytes (Edlund & Halestrap, 1988).

We have attempted to measure pyruvate transport kinetics under the same conditions as for L-lactate, but using a 15 s time point to determine initial rates. At low concentrations of pyruvate, uptake reached equilibrium too rapidly to allow measurement of initial rates of transport at 11 °C. This prevented determination of the K_m for pyruvate at this temperature. However, we were able to estimate the V_{max} , and in two such experiments a mean value of 1.9 nmol/min per μl of intracellular

Fig. 3. Kinetics of L-lactate transport into isolated cardiac myocytes at 11 °C

Initial rates of L-lactate transport at increasing concentrations were calculated from uptake at 30 s, by assuming a dead-time of 7 s. In (a) initial rates in the absence (●) and presence (○) of 5 mM α -cyano-4-hydroxycinnamate are shown. In (b) a Lineweaver-Burk plot is shown for the carrier-mediated (α -cyano-4-hydroxycinnamate-sensitive) component of transport. Data are shown as means $\pm$ S.E.M. of duplicate determinations from five separate experiments. The line drawn through the data on the Lineweaver-Burk plot was that obtained by fitting the data by non-linear least-squares regression analysis to the Michaelis-Menten equation. Derived parameter values from these experiments are given in the text and Table 3.

Fig. 4. Time courses of pyruvate uptake into isolated cardiac myocytes at 0 °C in the presence and absence of α -cyano-4-hydroxycinnamate

The experimental protocol was the same as for Fig. 1, except that the temperature was 0 °C, and the pyruvate concentrations were 0.02 mM (a) and 0.5 mM (b). Results are shown as mean values $\pm$ S.E.M. for four (a) or five (b) experiments, and were fitted to a first-order rate equation modified to account for metabolism of the label:

$$L_t = L_{\text{tot}}(1 - e^{-k(t+x)}) + k_{\text{met}} t$$

where L_t , L_{tot} and x are defined as in Fig. 1, and k_{met} is a rate constant for the metabolism of pyruvate. The symbols are used in the same way as in Figs. 1 and 2.

space was obtained. This is slightly lower than the value determined for L-lactate (see above), but is within the limits of variation between experiments.

Kinetics of pyruvate transport measured at 0 °C

In order to determine the K_m for pyruvate, the kinetics of pyruvate uptake into isolated cardiac myocytes were studied at 0 °C. In Fig. 4 we show time courses of

pyruvate uptake at this temperature for two concentrations of the substrate. Even at this temperature uptake of 0.02 mM-pyruvate was very rapid, and approx. 10-fold accumulation was achieved after 5 min incubation, considerably greater than would be predicted by the pH gradient. This would suggest that metabolism of the label is occurring under these conditions. Indeed, the data for pyruvate time courses at such low concentrations

Fig. 5. Kinetics of pyruvate transport into isolated cardiac myocytes at 0 °C

The experimental protocol and treatment of the data were exactly the same as in Fig. 3, except that the data were obtained at 0 °C and a 15 s time point was used for the calculation of initial rates. Data are given from five experiments; the derived parameter values for the individual experiments are given in the text and Table 3.

Fig. 6. Pyruvate and D-lactate inhibition of L-lactate transport into isolated cardiac myocytes

Uptake of 0.5 mM-L-lactate was determined at 11 °C as described in the Experimental section. Pyruvate (a) or D-lactate (b) was added to the cell suspension with the ^{14}C -labelled substrate to give the final concentrations indicated in the Figure. Results are shown as percentages of the control (no added pyruvate or D-lactate) carrier-mediated (α -cyano-4-hydroxycinnamate-sensitive) transport. The data are given as means $\pm$ S.E.M. for four experiments with either pyruvate or D-lactate, and were fitted by non-linear least-squares regression analysis to the equation for competitive inhibition, by using the K_m value derived for L-lactate transport given in the text and Table 3. The K_i values derived were 0.077 ± 0.012 mM and 6.6 ± 1.2 mM for pyruvate and D-lactate respectively.

could not be fitted satisfactorily to the first-order equation used for the L-lactate time courses at 11 °C, unless the equation was modified to include an additional parameter which allows for a constant rate of metabolism, as indicated in the legend to Fig. 4. The data indicate that a 15 s time point gives a reasonable estimate of the initial rate of pyruvate transport, even at very low concentrations of the substrate.

In Fig. 5 we show kinetic data from experiments where initial rates of pyruvate uptake into cardiac myocytes were measured at 0 °C over a range of concentrations up to 0.5 mM. As with L-lactate, uptake in the presence of α -cyano-4-hydroxycinnamate appeared to be a linear func-

tion of [pyruvate], suggesting that this represents diffusion of the free acid. The inhibitor-sensitive component of uptake was found to obey Michaelis-Menten kinetics, and the K_m (0.066 ± 0.007 mM) and $V_{\max}$ (0.412 ± 0.06 nmol/min per μ l of intracellular space at 0 °C) were determined from five experiments. This K_m value for pyruvate shows that it is a very good substrate for the carrier. In other cells pyruvate is also a better substrate for the monocarboxylate carrier than is L-lactate (Halestrap, 1976; Leeks & Halestrap, 1979; Edlund & Halestrap, 1988), but the K_m values are at least 10 times those reported here for cardiac myocytes. This very low K_m value for pyruvate suggests that the monocarboxylate

Table 1. Kinetic parameters for α -cyano-4-hydroxycinnamate inhibition of pyruvate transport into cardiac myocytes at 0 °C

Cells were preincubated in the absence or presence of α -cyano-4-hydroxycinnamate at concentrations of 0.025, 0.05, 0.1, 0.2 and 0.5 mM. Initial rates for the uptake of 0.02 mM- and 0.1 mM-pyruvate at 0 °C were calculated by using a 15 s time point. Initial rates were corrected for any uptake that occurred in the presence of 5 mM- α -cyano-4-hydroxycinnamate. The combined data from five separate experiments (60 data points in all) were fitted by non-linear least-squares regression analysis to the equations for mixed, competitive and non-competitive inhibition. Derived parameter values are expressed as means, with 95 % confidence limits shown in parentheses.

Inhibition model	Pyruvate K_m (μ M)	K_{i1} (μ M)	K_{i2} (μ M)	Residual sum of squares
Mixed	58 (36–80)	31 (11–51)	54 (16–92)	2173
Competitive	52 (32–72)	16 (12–20)	–	2448
Non-competitive	65 (54–75)	41 (35–47)	–	2203

carrier in cardiac myocytes may be different from that present in other tissues, although the difference may reflect only a species variation in the kinetic properties of a common carrier. For example, it has been found that the K_m value for L-lactate in human erythrocytes (Halestrap, 1976) is approx. 5 times that for rat erythrocytes (Edlund & Halestrap, 1988). However, experiments with guinea-pig erythrocytes have shown these cells to have a K_m value for pyruvate of approx. 0.7 mM (R. C. Poole, unpublished work); this is very similar to that found for rat erythrocytes and hepatocytes (Edlund & Halestrap, 1988).

Kinetics of inhibition by pyruvate and D-lactate of L-lactate transport into cardiac myocytes at 11 °C

If pyruvate and both L-lactate and D-lactate are transported on the same carrier in cardiac myocytes, they would be expected to be competitive inhibitors of transport with respect to each other. In many cases K_i values (competitive) have been determined which are equivalent to the K_m value for transport of the compound involved. This is the case for pyruvate and L-lactate in human erythrocytes (Halestrap, 1976). Data are shown in Fig. 6 for the inhibition of transport of 0.5 mM-L-lactate into cardiac myocytes at 11 °C by both pyruvate and D-lactate. Fitting the data from individual experiments to the equation for competitive inhibition, K_i values of 0.077 ± 0.012 mM and 6.6 ± 1.2 mM (means $\pm$ S.E.M., $n = 4$ in each case) could be derived. These data show pyruvate to be a potent inhibitor of L-lactate transport into cardiac myocytes; this has been noted previously (Kammermeier *et al.*, 1985). The K_i is very similar to the K_m value of 0.066 mM determined for pyruvate transport; this implies that the K_m value represents the dissociation constant of the substrate–transporter complex. The same conclusion was reached for the lactate carrier of human erythrocytes (Halestrap, 1976), and implies that transport rather than binding is the rate-limiting process. Although no detailed studies of the kinetics of D-lactate transport have been made, we have compared transport of 0.5 mM-L- and D-lactate at 11 °C in the same experiments. The initial rate of D-lactate uptake was 60 ± 6 % ($n = 4$) of that for L-lactate. If the K_m value for L-lactate is taken as 2.27 mM (see above), then a K_m of 4.2 mM could be estimated for the D-isomer, assuming the same V_{max} value for both

isomers. This is quite similar to the K_i value of 6.6 mM derived from inhibition experiments.

Inhibitors of myocyte lactate transport

α -Cyanocinnamate derivatives have been shown to inhibit monocarboxylate transport into many other cells, including erythrocytes (Halestrap & Denton, 1974; Halestrap, 1976), Ehrlich ascites cells (Spencer & Lehninger, 1976) and hepatocytes (Monson *et al.*, 1982; Edlund & Halestrap, 1988). We have studied the kinetics of α -cyano-4-hydroxycinnamate inhibition of pyruvate transport into isolated cardiac myocytes at 0 °C. The combined data were fitted by non-linear least-squares regression analysis to the equations for competitive, non-competitive and mixed inhibition, and the derived parameter values are shown in Table 1. As with the kinetic data from rat hepatocytes, it was possible to fit the data satisfactorily to each of these models. However, statistically the best fit was obtained by using either the equation for mixed inhibition, values ($\pm$ S.E.M.) for K_{i1} and K_{i2} being 0.031 ± 0.01 mM and 0.054 ± 0.019 mM respectively, or for non-competitive inhibition with a K_i of 0.041 ± 0.003 mM. These K_i values, whatever the model, are similar to the values of 50–100 μ M determined for the erythrocyte and hepatocyte monocarboxylate carriers in citrate medium (Halestrap, 1976; Edlund & Halestrap, 1988). For erythrocytes the K_i is higher (0.45 mM) in saline medium, where a much smaller pH gradient exists (Leeks & Halestrap, 1979). Since α -cyanocinnamate derivatives are weak acids and should distribute according to the transmembrane pH gradient, these observations have been taken as evidence that they inhibit from the cytoplasmic side of the plasma membrane. Furthermore, if inhibition occurs as a result of the α -cyano-4-hydroxycinnamate binding to the inner face of the transporter, non-competitive kinetics would be predicted after preincubation with inhibitor. Under these conditions the substrate is present initially on the opposite side of the carrier to that at which the inhibitor is binding, and is therefore unable to compete. We have previously shown this to be the situation with mitochondrial pyruvate transport. For this carrier inhibition is competitive with respect to internal substrates of the carrier, but not with those added externally that cannot diffuse across the inner mitochondrial membrane (see Halestrap *et al.*, 1980).

Table 2. Effects of pCMBS, quercetin and DIDS on L-lactate transport and the pH gradient across the plasma membrane of isolated cardiac myocytes

Myocytes were preincubated at 11 °C for 20 min with the inhibitors at the concentrations indicated before measurement of the uptake of 0.5 mM-L-lactate at 30 s. Values are expressed as the percentage inhibition of carrier-mediated (α -cyano-4-hydroxycinnamate-sensitive) transport and are given as means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. Values for Δ pH measured with [14 C]DMO (see the Experimental section) were obtained from parallel incubations.

Condition	Inhibition of carrier-mediated uptake	Δ pH (alkaline inside)
Control (no additions)	0	0.47 $\pm$ 0.03 (4)
5 mM- α -Cyano-4-hydroxycinnamate	100	0.47 $\pm$ 0.03 (4)
0.2 mM-pCMBS	55.5 $\pm$ 2.9 (5)	0.46 $\pm$ 0.02 (4)
0.5 mM-Quercetin	77.5 $\pm$ 0.7 (4)	0.35 $\pm$ 0.02 (4)
0.5 mM-DIDS	70.0 $\pm$ 1.0 (4)	0.30 $\pm$ 0.03 (4)

Table 3. Kinetic properties of the cardiac myocyte, hepatocyte and erythrocyte monocarboxylate carriers

Values for rat erythrocytes and hepatocytes are taken from Edlund & Halestrap (1988), and those for human erythrocytes from Halestrap (1976). All data were obtained for cells incubated in citrate buffer; K_i values for α -cyano-4-hydroxycinnamate are given for the models of inhibition indicated.

Cell type and temperature	K_m value (mM)			$\frac{K_m(\text{L-lactate})}{K_m(\text{pyruvate})}$	K_i for α -cyano-4-hydroxycinnamate (mM)
	L-Lactate	Pyruvate	D-Lactate		
Guinea-pig cardiac myocytes (the present work), 11 °C	2.27	0.066	6.6*	34	0.041 (non-competitive)
Rat hepatocytes, 0 °C	2.42	0.63	> 25	3.8	0.109 (competitive)
Rat erythrocytes, 7 °C	2.99	0.96	> 25	3.1	—
Human erythrocytes, 10 °C	9.14	1.89	> 25	4.8	0.049 (competitive)

* The K_m for D-lactate in cardiac myocytes is taken as the K_i value determined for inhibition of L-lactate transport.

In addition to α -cyanocinnamate derivatives, the lactate carrier in other tissues has been characterized by its sensitivity to a variety of other inhibitors. In erythrocytes lactate transport is highly sensitive to inhibition by the organomercurial thiol reagent pCMBS (Deuticke *et al.*, 1978), unlike the transport of inorganic anions in these cells. Other inhibitors include the bioflavonoid quercetin (Belt *et al.*, 1979) and the stilbene-disulphonate DIDS and its analogue, dihydro-DIDS (Jennings & Adams-Lackey, 1982; Donovan & Jennings, 1985; Edlund & Halestrap, 1988). DIDS and related compounds are very potent inhibitors of chloride exchange across the erythrocyte membrane, maximal inhibition being achieved at concentrations of approx. 5–10 μ M (see Halestrap, 1976); inhibition of lactate transport in erythrocytes (Jennings & Adams-Lackey, 1982; R. C. Poole & A. P. Halestrap, unpublished work) and hepatocytes (Edlund & Halestrap, 1988) requires 10–100 times this concentration. In Table 2 we show the effects of these inhibitors on the transport of 0.5 mM-L-lactate into cardiac myocytes. DIDS, pCMBS and quercetin were all found to be inhibitors of lactate transport; however, DIDS and quercetin had a small effect on the pH gradient. It is of note that pCMBS gave approx. 55% inhibition of transport at a concentration which would result in >90% inhibition in erythrocytes and

hepatocytes (Deuticke *et al.*, 1978; Edlund & Halestrap, 1988).

General conclusion

The results presented here show that lactate and pyruvate transport across the cardiac sarcolemmal membrane is fast and predominantly carrier-mediated at low, physiological, concentrations of these metabolites. At high concentrations an increasingly large proportion of transport is by non-ionic diffusion, as was previously found for hepatocytes (Monson *et al.*, 1982; Fafournoux *et al.*, 1985; Edlund & Halestrap, 1988). In Table 3 we summarize the kinetic data obtained for the guinea-pig cardiac myocyte lactate carrier and compare these with the parameters previously determined in this laboratory for rat erythrocytes and hepatocytes. On the basis of very similar K_m values for both L-lactate and pyruvate, and a high degree of stereoselectivity, we have suggested that the erythrocyte and hepatocyte lactate carriers may be the same protein (Edlund & Halestrap, 1988). The identification of a patient with an apparent deficiency in both skeletal-muscle and erythrocyte transporters (Fishbein, 1986) supports the common identity of these carriers also. In contrast, the kinetic parameters reported here for isolated cardiac myocytes from guinea pig

indicate that a different protein may be involved in sarcolemmal L-lactate/pyruvate transport. Although the K_m for L-lactate is very similar to that in rat erythrocytes and hepatocytes, the value for pyruvate (0.066 mM) is approx. 10-fold lower. In addition, the myocyte lactate carrier exhibits a low degree of stereoselectivity by comparison with erythrocytes and hepatocytes. We have shown that these differences are not due to a species difference in a common carrier, since guinea-pig erythrocytes resemble those of rat very closely in terms of both pyruvate affinity and stereoselectivity (R. C. Poole & A. P. Halestrap, unpublished work). It is of note that cardiac myocytes are not the only cell type to have a low degree of stereoselectivity for L- over D-lactate transport; the proton-linked carrier in Ehrlich ascites cells has identical K_m values for the two stereoisomers (Spencer & Lehninger, 1976).

The most significant finding of the present studies was the very low K_m for pyruvate transport in cardiac myocytes, lower than that for pyruvate transport into mitochondria (see Halestrap *et al.*, 1980). These observations, along with the high transport activity, imply that pyruvate should readily equilibrate across the sarcolemmal membrane, and this is actually observed in the perfused heart (Henderson *et al.*, 1969; Opie & Mansford, 1971). However, as outlined in the Introduction, this does not appear to be the case for L-lactate. Such an observation may be readily explained by the much higher K_m of the carrier for this substrate. The observation that L-lactate/pyruvate ratios are lower in the perfusate than in the cytosol (Opie & Mansford, 1971; Mowbray & Ottaway, 1973) could also be explained in this manner, and suggest that caution should be applied in using the perfusate [L-lactate]/[pyruvate] ratio to estimate cytoplasmic [NADH]/[NAD⁺].

Taegtmeyer *et al.* (1980) determined the utilization of 5 mM-L-lactate by the perfused working rat heart at 37 °C, and obtained a value of 1400 $\mu\text{mol/h}$ per g dry wt. From a value of 360 μl of cell water/g wet wt. (Opie & Mansford, 1971) and a wet-weight/dry-weight ratio of 5.5 (Taegtmeyer *et al.*, 1980), this would represent a value of 12 nmol/min per μl of intracellular space. Extrapolating the initial rate of L-lactate uptake at 5 mM concentration from 11 °C to 37 °C by using the activation energy for the human erythrocyte lactate carrier (Halestrap, 1976) yields a value of 28 nmol/min per μl of intracellular space. Since initial rates of monocarboxylate uptake in citrate medium, where a large pH gradient exists across the plasma membrane, are likely to be an overestimation of those occurring *in vivo*, it is possible that rates of lactate transport in the heart are quite similar to maximal rates of utilization. Although these extrapolations can only be approximate, they do raise the possibility that lactate metabolism by the heart may be constrained to some extent by plasma-membrane transport. Since heart metabolism is aerobic under all but pathological situations, it is possible that the monocarboxylate carrier may function to exchange L-lactate entering the cell with pyruvate leaving. The pyruvate could be formed from the lactate by lactate dehydrogenase, which would lead to the production of NADH for oxidation by the mitochondria. In this context it is of interest that a specific isoenzyme of L-lactate dehydrogenase with lower K_m for pyruvate and greater sensitivity to inhibition by pyruvate predominates in heart muscle (Holbrook *et al.*, 1975).

This work was supported by a Medical Research Council Research Studentship to R.C.P., and by research grants from the Wellcome Trust and the British Heart Foundation.

REFERENCES

- Addanki, S., Cahill, F. D. & Soto, J. F. (1968) *J. Biol. Chem.* **243**, 2337–2348
- Bashford, C. L. & Pasternak, C. A. (1984) *J. Membr. Biol.* **79**, 275–284
- Belt, J. A., Thomas, J. A., Buchsbaum, R. N. & Racker, E. (1979) *Biochemistry* **19**, 3506–3511
- Dennis S., Kohn, M. C., Anderson, G. J. & Garfinkel, D. (1985) *J. Mol. Cell. Cardiol.* **17**, 987–995
- Deuticke, B. (1982) *J. Membr. Biol.* **70**, 89–103
- Deuticke, B., Rickert, I. & Beyer, E. (1978) *Biochim. Biophys. Acta* **507**, 137–155
- Deuticke, B., Beyer, E. & Forst, B. (1982) *Biochim. Biophys. Acta* **684**, 96–110
- Donovan, J. A. & Jennings, M. L. (1985) *Biochemistry* **24**, 561–564
- Dubinsky, W. P. & Racker, E. (1978) *J. Membr. Biol.* **44**, 25–36
- Edlund, G. L. & Halestrap, A. P. (1988) *Biochem. J.* **249**, 117–126
- Fafournoux, P., Demigne, C. & Remesy, C. (1985) *J. Biol. Chem.* **260**, 292–299
- Fishbein, W. N. (1986) *Science* **234**, 1254–1256
- Gornall, H. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756
- Halestrap, A. P. (1976) *Biochem. J.* **156**, 193–207
- Halestrap, A. P. (1988) *Biochem. J.* **253**, 622–623
- Halestrap, A. P. & Denton, R. M. (1974) *Biochem. J.* **138**, 313–316
- Halestrap, A. P., Scott, R. D. & Thomas, A. P. (1980) *Int. J. Biochem.* **11**, 97–105
- Henderson, A. H., Craig, R. J., Gorlin, R. & Sonnenblick, E. H. (1969) *Am. J. Physiol.* **217**, 1752–1756
- Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossman, M. G. (1975) *Enzymes* 3rd Ed. **11**, 191–292
- Illingworth, J. A., Ford, W. C. L., Kobayashi, K. & Williamson, J. R. (1975) in *Recent Advances in Cardiac Structure and Metabolism*, vol. 8 (Roy, P. E. & Harris, P., eds.), pp. 271–290, University Park Press, Baltimore
- Isenberg, G. & Klockner, U. (1982) *Pflügers Arch.* **395**, 6–18
- Jennings, M. L. & Adams-Lackey, M. (1982) *J. Biol. Chem.* **257**, 12866–12871
- Kammermeier, H., Wein, B. & Graf, W. (1985) *Basic Res. Cardiol.* **80**, suppl. 1, 57–60
- Katz, A. & Sahlin, K. (1988) *J. Appl. Physiol.* **65**, 509–518
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–36
- Lassars, R. W., Kaijser, M. L., Wahlquist, M. L. & Carson, L. A. (1971) *Lancet* **ii**, 448–451
- Lee, K. S. (1987) in *Electrophysiology of Single Cardiac Cells* (Noble, D. & Powell, T., eds.), pp. 69–103, Academic Press, London
- Leeks, D. R. & Halestrap, A. P. (1979) in *Function and Molecular Aspects of Biomembrane Transport* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C. & Siliprandi, N., eds.), pp. 427–430, Elsevier/North-Holland, Amsterdam
- Mann, G. E., Zlokovic, B. V. & Yudilevich, D. L. (1985) *Biochim. Biophys. Acta* **819**, 241–248
- Mason, M. J. (1986) Ph.D. Thesis, University of Bristol

- McGivan, J. D., Bradford, N. M. & Beavis, A. D. (1977) *Biochem. J.* **162**, 147–156
- Monson, J. P., Smith, J. A., Cohen, R. D. & Iles, R. A. (1982) *Clin. Sci.* **62**, 411–420
- Mowbray, J. & Ottaway, J. H. (1973) *Eur. J. Biochem.* **36**, 362–368
- Opie, L. H. (1976) *Circ. Res.* **38**, suppl. 1, 52–74
- Opie, L. H. & Mansford, K. R. L. (1971) *Eur. J. Clin. Invest.* **1**, 295–306
- Poole, R. C. & Halestrap, A. P. (1988) *Biochem. J.* **254**, 385–390
- Quintana, I., Felipe, A., Remesar, X. & Pastor-Anglada, M. (1988) *FEBS Lett.* **235**, 224–228
- Spencer, T. L. & Lehninger, A. L. (1976) *Biochem. J.* **154**, 404–414
- Taegtmeyer, H., Hems, R. & Krebs, H. A. (1980) *Biochem. J.* **186**, 701–711
- Thomas, A. P. & Halestrap, A. P. (1981) *Biochem. J.* **196**, 471–479
- Trosper, T. L. & Philipson, K. D. (1987) *Am. J. Physiol.* **252**, C483–C489
- Watts, D. J. & Randle, P. J. (1967) *Biochem. J.* **104**, 51P

Received 2 May 1989/7 August 1989; accepted 9 August 1989