J. Membrane Biol. 112, 15–23 (1989)

The Journal of
Membrane Biology

Functional Characteristics of the Cardiac Sarcolemmal Monocarboxylate Transporter

Terry L. Trosper and Kenneth D. Philipson
Departments of Medicine and Physiology and the Cardiovascular Research Laboratory, University of California, Los Angeles, School of Medicine, Los Angeles, California 90024-1760

Summary. We have previously shown that a mechanism for transporting L-lactate is located in cardiac sarcolemmal membranes (*Am. J. Physiol.* **252:**C483–C489, 1987). This mechanism has now been shown to transport pyruvate also. The transporter recognizes a wide range of monocarboxylic acids with chain lengths of three to six carbons, as evidenced by their ability to inhibit L-lactate uptake into sarcolemmal vesicles. The ability of the monocarboxylate analogues to inhibit depends strongly on the nature of substituents, particularly at the second carbon. L-lactate and pyruvate transport are not affected by dicarboxylates other than oxaloacetate. The transporter is inhibited by the protein modifiers diethylpyrocarbonate, dinitrofluorobenzene, and phenylisothiocyanate. Diethylpyrocarbonate inhibition is not reversed by hydroxylamine, nor is dinitrofluorobenzene inhibition reversed by thiol reagents, suggesting that the target residues are not histidine, or tyrosine or cysteine, respectively. Several monocarboxylates effectively protect the transporter from inhibition by the modifying reagents, suggesting that the modified residue(s) may be at or near the binding site. Alternatively, the target amino acid(s) in the transport protein may become inaccessible due to a conformation change triggered by the substrate analogues. Overall, the results suggest that a sensitive free amino group, associated with substrate binding, is attacked by the protein-modifying reagents.

Key Words monocarboxylate transport · cardiac sarcolemma · amino acid modification · lysyl and histidyl residues

Introduction

A mechanism that facilitates the transport of L-lactate across cardiac sarcolemmal membranes has recently been reported [33]. This transport system has many kinetic properties and inhibitor sensitivities similar to those of the monocarboxylate transporters in erythrocytes [4, 8, 10], Ehrlich ascites cells [31] and hepatocytes [11, 12], although some significant differences exist. A component of L-lactate accumulation by skeletal muscle may be due to a similar system [35]. Uptake of L-lactate by sarcolemmal vesicles appears to proceed as L-lactate$^-$/H^+ cotransport; also, uptake is inhibited by an outwardly directed proton gradient [33]. These characteristics have recently been reported for a lactate transport system in placental brush border membranes [1], and monocarboxylate$^-$/H^+ cotransport has previously been suggested to be the mode of transport in erythrocytes [4, 8, 10], Ehrlich ascites cells [31], and hepatocytes [12].

It is of interest to determine whether, as suggested by preliminary data on sarcolemmal vesicles [33] and by earlier studies on acidification of Purkinje fibers [5] and on perfused hearts [20], the cardiac sarcolemmal L-lactate transporter is also a general monocarboxylate-transporting system, and to identify molecular characteristics required for substrate recognition. We report below on pyruvate transport by cardiac sarcolemma. The effect of a wide array of other monocarboxylates and of dicarboxylates on L-lactate or pyruvate transport was also investigated. The cardiac sarcolemmal system appears to respond to some substrate analogues differently from monocarboxylate-transporting mechanisms in other cells.

No plasma membrane monocarboxylate/proton cotransporter has yet been isolated or characterized molecularly. However, the observed inhibitory effects of the sulfhydryl group reagents pCMBS[1] and NEM contributed to the conclusion that the systems are proteinaceous [8, 33]. In an attempt to identify amino acid residues essential for substrate binding or transport, several other protein-modifying reagents have now been tested for their ability to inhibit monocarboxylate transport in cardiac sarco-

[1] Abbreviations used are: DEPC = diethyl pyrocarbonate (ethoxyformic anhydride); DMSO = dimethyl sulfoxide; DNFB = 2,4-dinitrofluorobenzene; DTT = dithiothreitol; FCCP = carbonylcyanide 4-trifluoromethoxyphenyl hydrazone; FITC = fluoroscein isothiocyanate; MOPS = 4-morpholine propanesulfonic acid; NEM = N′-ethylmaleimide; PITC = phenylisothiocyanate; pCMBS = 4-(chloromercuri)benzenesulfonate.

lemmal vesicles. The results of these experiments all suggest that an amino group in a hydrophobic environment at or near the binding site is essential for transport function.

Interpretation of protein-modification results was aided by the fact that some substrate analogues were able to provide significant protection from inhibition by certain group-modifying reagents. This information also contributes to a description of the nature of the interaction of substrates with the transport proteins. Additionally, it should be useful in studies leading to the isolation of the cardiac sarcolemmal monocarboxylate$^-$/H^+ cotransporter.

Materials and Methods

Sarcolemmal Vesicles

Sarcolemma was isolated from trimmed left ventricles of canine hearts as previously described [14, 26]. The sarcolemmal band from the sucrose gradient was diluted about eightfold with 140 mM sucrose, 5 mM MOPS, pH adjusted to 7.4 with either Tris base or NaOH, and centrifuged at 155,000 × *g* for 75 min. The resulting pellet was resuspended to a protein concentration of 5 to 10 mg/ml in 280 mM sucrose containing 10 mM of the same buffer, and stored in liquid N_2 until use. These preparations were typically enriched over 50-fold in the sarcolemmal markers *p*-nitrophenyl phosphatase and Na^+/K^+-dependent ATPase [14] and contained negligible amounts of the mitochondrial membrane marker lipid, cardiolipin (R. Vermuri, this laboratory, *unpublished results*). The preparation is a mixture of right-side-out and inside-out vesicles which are >80% sealed, as judged by the effects of ionophores on Na^+/K^+-dependent ATPase activity [14].

Monocarboxylate Transport

Transport of monocarboxylate was measured as uptake of ^{14}C-labeled L-lactate or pyruvate by sarcolemmal vesicles using a procedure described previously [33]. Briefly, a 10-μl aliquot of vesicle suspension, typically 1.5–2.0 mg protein/ml in 280 mM sucrose buffered as described above, was mixed rapidly with 40 μl of uptake medium containing 0.2 to 0.25 μCi of ^{14}C-monocarboxylic acid substrate plus cold Na^+ or Tris salt of the monocarboxylate to the desired concentration, 140 mM NaCl, 10 mM MOPS/Tris or MOPS/NaOH buffer at the same pH as the vesicles, and 5 μM monensin. Monensin was added from a freshly prepared stock solution of 5% ethanol in DMSO, such that the final concentrations of ethanol and DMSO were 0.05 and 1%, respectively. These amounts of the solvents had no effect on uptake. Measurements were made at room temperature unless otherwise indicated.

Uptake was stopped after the desired time by manual addition of 1 ml ice-cold, buffered, isotonic KCl solution to the suspension. One ml of this mixture was immediately filtered through a 0.45 μm nitrocellulose filter (Sartorius No. 11336) and washed twice with 3 ml of the same solution. Radioactivity remaining on the filter was determined by scintillation counting after addition of 6 ml of a mixture of 60% Betafluor (National Diagnostics) and 40% ethanol, which rendered the wet filters essentially transparent without significantly quenching counts. Measurements of uptake of pyruvate or L-lactate as a function of time indicated that uptake was approximately linear for 10 or 20 sec, respectively (Fig. 1 and ref. 33). These uptake times were used in subsequent experiments for determination of initial rates of uptake.

Carboxylic acid inhibitors were added to the uptake media as Na^+ or Tris salts, adjusted to the same pH as the medium. The ionic strength was maintained by adding the Na^+ or Tris salt of the nontransportable anion, gluconic acid, to control samples, and/or by decreasing the concentration of NaCl in the uptake solutions. Specific details are mentioned in the results where appropriate.

Protein Modification

Sarcolemmal vesicles were preincubated with protein modifying reagents in buffered sucrose solutions in the absence or presence of monocarboxylates which might protect from modification. Osmotic strength was maintained at 280 mOsmolar by adjusting the amount of sucrose added to the solutions, after ionic strength was adjusted with sodium gluconate. If present, monocarboxylate salts were added to vesicle suspensions 5 to 7 min before addition of the protein modifier. Typical reaction mixture volumes of 40 to 50 μl contained 100 μg membrane protein. Modification reactions were terminated by dilution of the suspension into 8 to 10 volumes of ice-cold 280 mM sucrose, 10 mM MOPS/Tris, pH 7.4, and immediate centrifugation in a Beckman airfuge at 175,000 × *g* for 20 min. The resulting sarcolemmal membrane pellet was resuspended in the dilution medium and incubated ~30 min at room temperature prior to uptake measurements.

Particular conditions used for individual modifiers were as follows: Phenylglyoxal was recrystallized from hot water and stored desiccated at room temperature. Stock solutions were freshly prepared in 100 mM lutidine (2,6-dimethylpyridine) buffer at pH 8.4. The incubation suspension contained 50 mM lutidine buffer at pH of 8.1. Stock solutions of DEPC in ethanol were prepared daily. Intermediate dilutions of this reagent into buffered sucrose were made immediately prior to addition to the sarcolemmal vesicle suspension, which was subsequently incubated for 15–20 min at room temperature. DNFB solutions were freshly prepared in methanol each day. An intermediate dilution into buffered sucrose was made just prior to addition to the sarcolemmal suspension. All solutions or suspensions containing DNFB were kept in the dark. PITC solutions were prepared in DMSO. Intermediate dilutions into buffered sucrose were opalescent; solutions of PITC at the final concentrations used in incubation media were clear. No Tris was present in any solution used for these modifications. Final concentrations of organic solvents were 0.2% for ethanol, and 0.5% for methanol and DMSO. These concentrations had no effect on uptake of L-lactate by control sarcolemma.

When reversal of a protein modification reaction was investigated, a 40 or 50 μl aliquot of incubated vesicle suspension was diluted into 385 μl of 280 mM sucrose, 10 mM MOPS/Tris, pH 7.4, containing 20 mM NH_2OH (for DEPC) or 5 mM DTT (in the dark, for DNFB), and incubated for the given times at room temperature before being centrifuged as described above.

Materials

DEPC, DNFB, FITC, PITC, D- and L-lactic acids, pyruvic acid, 3-hydroxybutyrate sodium salt, D,L-2-hydroxyvalerate sodium salt, gluconic acid sodium salt, succinic acid, oxaloacetic acid, L-

alanine, NEM, DTT and monensin were from Sigma; 2-methyl,2-hydroxy propionic acid sodium salt, 2-hydroxybutyric acid sodium salt, L-malic acid, 2-oxobutyric acid sodium salt, oxaloacetic acid, 2-oxovaleric acid, 2-oxo,4-methylvaleric acid, and 2-oxoglutaric acid were from Fluka; 2-hydroxy,2-methylbutyric acid, D,L-2-hydroxycaproic acid, L-norvaline, L-2-hydroxy,4-methylvaleric acid, 4-hydroxybutyric acid sodium salt, 2-oxocaproic acid, L-isoleucine, S(−)2-chloropropionic acid, *n*-butyric acid, *n*-valeric acid, and 2,6-dimethylpyridine were from Aldrich. Other chemicals were of reagent grade. Deionized water of resistivity 18 MΩ was used to prepare solutions for modification and uptake procedures. U-^{14}C-L-lactic acid sodium salt and 1-^{14}C-pyruvic acid were from Amersham.

Results

Pyruvate Transport by Sarcolemmal Vesicles

The time course of pyruvate uptake by sarcolemmal vesicles is shown in Fig. 1. As can be seen from the inset, uptake remains linear for approximately 10 sec at room temperature. At pH 7.4 the initial uptake rate is a saturable function of pyruvate concentration (Fig. 2). From Eadie-Hofstee plots of such data the apparent affinity of the sarcolemmal transporter for pyruvate was calculated to be 5.0 ± 1.6 mM (n = 4). An example is shown in the inset of Fig. 2. As suggested by preliminary work [33], L-lactate is an effective inhibitor of pyruvate uptake by sarcolemmal vesicles; a 100-fold excess of L-lactate inhibits pyruvate uptake by 77 ± 7%.

Pyruvate and L-lactate transport share many characteristics. We previously presented evidence that L-lactic acid crosses the sarcolemma as an L-lactate$^-$/H$^+$ cotransport. It was shown that in the presence of an inwardly directed Na$^+$ gradient, monensin enhances L-lactate uptake about sixfold by preventing buildup of protons inside the vesicles [33]. The effect is not specific for Na$^+$; K$^+$ + nigericin will serve as well. Pyruvate uptake by cardiac sarcolemmal vesicles has now been observed to increase 4- to 10-fold under the same conditions. Furthermore, a K$^+$ gradient in the presence of FCCP + valinomycin stimulates the rate of uptake to the same extent that the Na$^+$ gradient + monensin does, whether the monocarboxylate transported is L-lactate or pyruvate. Both L-lactate and pyruvate transport display a very large temperature dependence between 10 and 37°C; initial uptake rates for both substrates are increased 10-fold over this temperature range. The sensitivity of pyruvate and L-lactate transport to inhibition by other monocarboxylates and by dicarboxylates is similar (*see below*).

As is the case for L-lactate transport [33], pyruvate transport is sensitive to sulfhydryl group reagents. Preincubation of sarcolemmal vesicles with

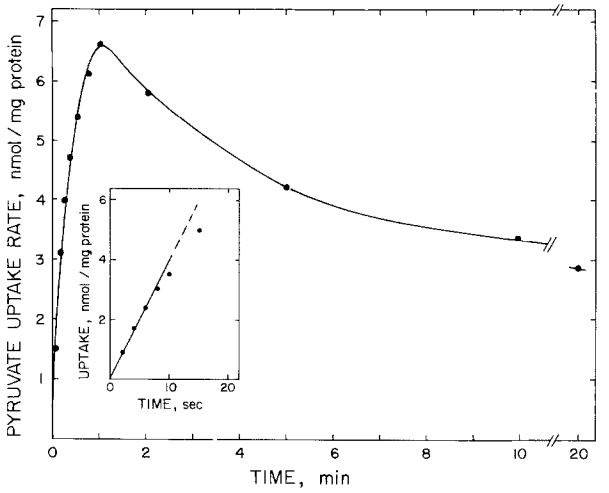

Fig. 1. Uptake of pyruvate by cardiac sarcolemmal vesicles loaded with 280 mM sucrose, 10 mM MOPS/Tris, pH 7.4, as a function of time at room temperature. External medium contained 1 mM pyruvate, 112 mM NaCl, 56 mM sucrose, 10 mM MOPS, pH 7.4, and 6.5 μM monensin. *Inset:* Uptake determined over short time periods using another vesicle preparation and the same conditions

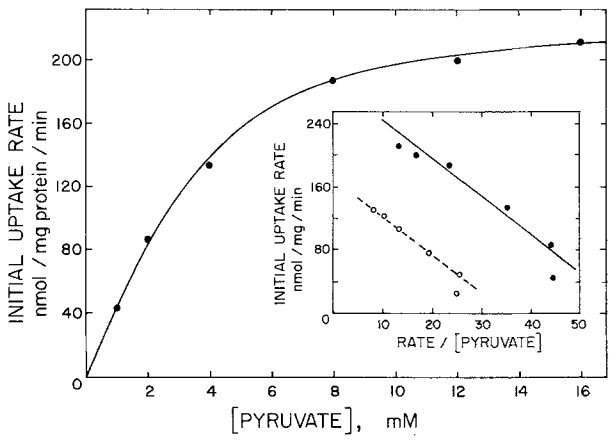

Fig. 2. Dependence of pyruvate uptake by cardiac sarcolemmal vesicles on external pyruvate concentration; uptake conditions as in Fig. 1. *Inset:* Eadie-Hofstee plot of data in Fig. 2, (-●-), and of data from uptake measurements using sarcolemmal vesicles that had been treated with 7 μM DEPC, (-○-). Slope of solid line, which is least squares fit of data, gives apparent K_m for pyruvate of 4.8 mM; treated vesicles have K_m of 4.7 mM

10 mM NEM for 30 min at 37°C and pH 7.4 inhibited over 90% of pyruvate uptake. Lower concentrations of reagent or shorter incubation times resulted in less complete inhibition.

Inhibition of L-Lactate Uptake by Other Monocarboxylates

A wide variety of substituted and unsubstituted monocarboxylic acids is able to inhibit L-lactate up-

Table 1. Inhibitory effectiveness of various monocarboxylates[a]

Substituent	C3 compounds	% inhib.	C4 compounds	% inhib.	C5 compounds	% inhib.	C6 compounds	% inhib.
None	Propionate	42 ± 8	Butyrate	65 ± 7	Valerate	79 ± 3	(Hexanoate)	
Hydroxy	L-2-OH (=L-lactate)	56 ± 6	2-OH	49 ± 1	D,L-2-OH	62 ± 3	D,L-2-OH	67 ± 9
	D-2-OH (=D-lactate)	13 ± 7						
	2-OH,2CH_3	10 ± 6	2-OH,2-CH_3	13 ± 4				
			2-OH,3-CH_3	34 ± 2	L-2-OH,4CH_3	84 ± 1		
			3-OH	23 ± 1				
			4-OH	23 ± 2				
Oxo	2-oxo (=pyruvate)	58 ± 3	2-oxo	75 ± 4	2-oxo	84 ± 4	2-oxo	87 ± 5
					2-oxo,4-CH_3	90 ± 1		
Methyl	2-CH_3 (=isobutyrate)	39 ± 1						
Chloro	S(−)-2-Cl	56 ± 2						
Amino	L-2NH_3	9[b]			L-2NH_3	13[b]		
					D-2NH_3	0[b]		

[a] Percent inhibition of the initial rate of uptake of 1 mM external L-lactate by 10 mM inhibitor added as Na salt. Controls contained 10 mM Na gluconate. Data are averages of three or more experiments, ± SE unless otherwise indicated.
[b] Average of two experiments.

take by sarcolemmal vesicles, as measured by their effect on initial rate of L-lactate uptake from 112 mM Na^+, 56 mM sucrose buffered at pH 7.4. For comparative purposes the percent inhibition caused by a 10-fold excess of the different monocarboxylates tested is listed in Table 1. Sodium gluconate was used to balance ionic strength in controls. Vesicles were loaded with 280 mM sucrose, 10 mM MOPS, pH 7.4. All uptake media contained 5 μM monensin, to prevent acidification of the vesicle interior.

Increasing inhibitor chain length from three to five carbons progressively increases inhibitory potency for all classes of substituents (Table 1). The addition of one more methylene group to the chain to produce a molecule six carbons long may not have any further effect. Also, the nature and location of substituent groups can markedly alter the extent of inhibition for compounds of the same chain length.

A hydrogen bonding group is not required at the *2* position for effective inhibition; equal inhibitions are caused by S(−)2-chloropropionate and L-lactate. The transporter may recognize L isomers of compounds substituted at the *2* position preferentially; however, unavailability of both pure isomeric forms of many of the compounds prevented a thorough investigation of this point. When the inhibitor used was a mixture of stereoisomers, the extent of inhibition probably was less than that which would be observed for the pure L isomer, due to stereoselectivity of the transporter. Addition of a second substituent at the C2 position reduces effectiveness of the inhibitor, as does the presence of amino groups at this position.

Monocarboxylates with hydrogen bonding substituents in the *3* or *4* position, rather than at the second carbon, are not effective inhibitors of the transporter (e.g., *compare* 2- and 3- or 4-hydroxybutyrates). However, addition of a methyl group at the *4* position, such as in 2-oxo,4-methylvalerate, may be advantageous.

The extent of inhibition of the initial rate of 1 mM L-lactate uptake depended strongly on inhibitor concentration. Essentially complete inhibition was caused by 100 mM L-2-hydroxy,4-methyl- or 2-oxovalerates. The sigmoidal dependence of percent inhibition on the log of inhibitor concentration is shown for D,L-2-hydroxyvalerate, pyruvate, and cold L-lactate in Fig. 3. Dixon plots of data for the competitive inhibitors L-lactate and pyruvate suggested that approximate K_I's were 15–20 mM for both (*data not shown*). These concentrations lie in the range of substrate affinities of the transporter. Also, the effectiveness of the 2-hydroxyvalerate may have been decreased because it was a racemic mixture. The pure L-isomer is not available commercially.

Inhibition of 1 mM pyruvate uptake by various monocarboxylates was similar to inhibition of L-lactate uptake. For example, 10 mM L-lactate or 2-oxovalerate reduced the initial rate of 1 mM pyruvate uptake by 62 and 86%, respectively, compared with 56 ± 6% and 84 ± 4% for 1 mM L-lactate uptake.

Table 2. Effect of amino acid modifiers on L-lactate transport

Reagent	Target residue(s)	Preinc. conditions	% inhibition[a]	Reversibility
DEPC	histidine [2, 3, 19, 23] amino groups [19] tyrosine [19, 21]	>40 μM, pH 7.4, 15 min	>95%	
		10 μM, pH 7.4, 15 min	69 ± 12%	0[b]
		10 μM, pH 6.6, 15 min	49 ± 10%	5[b]
DNFB	amino groups [2, 19] tyrosine [24] cysteine [24] histidine [24]	0.4 mM, pH 7.4, 30 min, dark	82 ± 10%	16 ± 11%[c]
FITC	amino groups [2, 19]	0.5 mM, pH 7.4, 30 min, dark	−1 ± 2%	
PITC	amino groups [21, 29]	0.4 mM, pH 7.4, 30 min, dark	78 ± 6%	
Phenylglyoxal	arginine [19, 32]	5–10 mM, pH 8.1, 75 min	47 ± 4%	

[a] Average of three or more experiments, ± SE, except for FITC, for which $n = 2$.
[b] *See* Materials and Methods for procedural details.
[c] 30 min incubation; *see* Materials and Methods for additional details.

Effect of Dicarboxylates on L-Lactate Uptake

Tenfold excess succinate, L-malate or 2-oxoglutarate in the external medium at pH 7.4 had negligible effect on L-lactate uptake by cardiac sarcolemmal vesicles. Also, inhibition of less than 20% of initial uptake rates were repeatedly observed when 100-fold excesses of these dicarboxylates were present. However, 10- and 100-fold excess oxaloacetate reduced the monocarboxylate transport by an average of 24 and 53%, respectively, in several experiments. Oxaloacetate had a similar effect on pyruvate uptake.

Effect of Protein Modifiers on L-Lactate or Pyruvate Transport

Several amino acid modifiers, other than the sulfhydryl group reagents previously used [33], were tested for their effect on monocarboxylate transport. The modifiers were selected for their ability to react with amino acid residues that can be positively charged at pH 7.4, and thus act as monocarboxylate anion binding sites on transporter peptides. After modification, the sarcolemmal vesicles were removed from reagent-containing solution by centrifugation, as described in Materials and Methods, resuspended in 280 mM sucrose, 10 mM MOPS/Tris, pH 7.4, and diluted into uptake media containing Na^+ and monensin, for the usual initial uptake rate measurements. Control vesicles were always incubated under modification conditions, but in the absence of the modifying reagent, and transport activities were normalized to resuspended membrane protein.

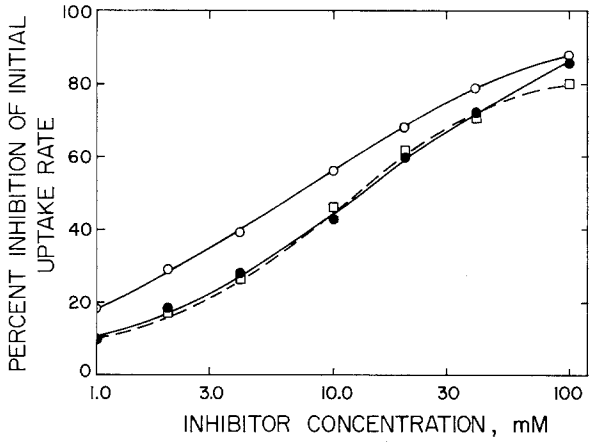

Fig. 3. Inhibition of 1 mM ^{14}C-L-lactate uptake by cardiac sarcolemmal vesicles by L-lactate (-●-), pyruvate (-□-), or D,L-2-hydroxyvalerate (-○-) as a function of inhibitor concentration. Note log scale on abscissa. Vesicles were loaded with 280 mM sucrose, 10 mM MOPS/Tris, pH 7.4. External medium contained 56 mM sucrose, 140 mM Na^+, 10 mM NaMOPS, pH 7.4, 6.5 μM monensin, the indicated concentration of monocarboxylate, and gluconate to balance ionic strength at 140 mM

Table 2 shows the results obtained with the modifying agents used. DEPC was the most powerful inhibitor investigated. The concentrations of DEPC used correspond to approximately 5 to 20 nmol reagent/mg membrane protein, several-fold less than usual [23], and among the lowest DEPC concentrations reported effective [30].

Histidine and tyrosine modifications by DEPC can be reversed by hydroxylamine, provided a large excess of modifying reagent has not caused a second carboxyethylation to occur at each histidine residue [23]. Despite the use of low concentrations

Table 3. Protection of L-lactate transport from DEPC inhibition[a]

Monocarboxylate	% protection
L-2-OH propionate (L-lactate)	5 ± 5
2-oxo propionate (pyruvate)	36 ± 8
2-CH_3 propionate	25 ± 2
L-2-Cl propionate	31 ± 6
n-butyrate	69 ± 8
2-OH butyrate	68 ± 8
n-valerate	77 ± 5
D,L-2-OH valerate	62 ± 8
2-OH,4-CH_3 valerate	89 ± 5
2-oxo,4-CH_3 valerate 1 mM	38[b]
25 mM	73 ± 1
50 mM	89[c]

[a] Vesicles were preincubated for 5–7 min with 50 mM Na monocarboxylate (unless otherwise noted), 180 mM sucrose, 10 mM NaMOPS, pH 7.4, before 12 μM DEPC was added. Controls contained 50 mM Na gluconate, ± DEPC. After 20 min, vesicles were diluted, centrifuged, and resuspended for uptake measurements as described in Materials and Methods. Data, which are means ± SE of results of three or more experiments, are presented as percent of DEPC inhibition prevented by presence of the monocarboxylate.

[b] $n = 1$.

[c] $n = 2$.

of DEPC in the present study, investigation of reversal was hampered by significant inhibition of L-lactate uptake by high concentrations of NH_2OH at pH 7.4. Incubations with 20 mM NH_2OH for 2 to 4 hr (Table 2) were not adequate to cause measurable reversal of inhibitions ranging from 23 to 75%.

DEPC appears to be a noncompetitive inhibitor of the monocarboxylate transporter, as expected. Modification conditions that reduced V_{max} for pyruvate uptake by up to 70% did not significantly affect the affinity for pyruvate. In one experiment (Fig. 2, inset) the apparent K_m of a sarcolemmal vesicle preparation was 4.8 mM before and 4.7 mM after DEPC modification. Another experiment gave similar results.

In contrast to the results in Table 2, the initial rate of L-lactate uptake by DEPC-treated vesicles was inhibited only 10% when the uptake medium was at pH 5.9 ($n = 2$). This should be compared to an average 78% inhibition of uptake from pH 7.4 medium, measured for the same modified vesicle preparations. This result is consistent with the previous observation that passive diffusion contributes significantly to L-lactate sequestration at the lower pH [33]. Furthermore, the ability of the DEPC-treated vesicles to accumulate L-lactate indicates that the modification treatment did not render the vesicles leaky.

Despite the fact that the amino group modifiers DNFB, PITC, and FITC were preincubated with sarcolemmal vesicles at pH 7.4, a pH lower than that at which these reagents are usually effective, DNFB and PITC caused major inhibition of the initial uptake rate of L-lactate (Table 2). The extent of this inhibition was dependent on inhibitor concentration (*data not shown*). However, 0.5 mM FITC had no inhibitory effect whatsoever.

Because DNFB may attack residues other than amino groups in a reversible reaction at neutral pH [28], reversal of DNFB inhibition by dithiothreitol was investigated under conditions where the sulfhydryl reagent did not itself affect monocarboxylate uptake. The results (Table 2) suggest that modification of substituents other than amino groups makes at most a minor contribution to the inhibitory effect of DNFB.

Table 2 reports the maximum effect that was obtained with phenylglyoxal modification. The conditions used are harsher than those reported to cause >80% inhibition of the mitochondrial pyruvate transporter [25] or the erythrocyte inorganic anion exchanger [18].

Protection of the Monocarboxylate Transporter from Inhibition by Protein Modifiers

It was possible to protect the transporter from inhibition by DEPC, DNFB and PITC. If ≥50 mM of any one of several substrate analogues, which are themselves good inhibitors of L-lactate uptake, is present during the incubation of sarcolemma with the protein modifiers, a significant fraction of the inhibition is prevented (Table 3). Incubation of control vesicles with the protecting monocarboxylate alone, followed by removal of that monocarboxylate by dilution and centrifugation as described in Materials and Methods, did not significantly affect subsequent uptake of L-lactate or pyruvate. Such a control was performed for every set of experiments.

The effectiveness of the 4- and 5-carbon monocarboxylates as protectors from DEPC inhibition is similar to their effectiveness as inhibitors of L-lactate uptake (*compare* Tables 1 and 3). The 3-carbon monocarboxylates are less effective as protectors than as inhibitors. Surprisingly, the substrate L-lactate does not protect the transporter from DEPC inhibition. Substrate analogues could not prevent protein modification by inactivating the protein reagents directly, because none of the monocarboxylates used contain targeted substituent groups. The lack of protection provided by L-lactate, and the complete ineffectiveness of sodium gluconate as a protector, support the correctness of this assumption.

L-lactate did not behave anomalously in protecting from uptake inhibition by DNFB (Table 4). In fact, pyruvate and L-lactate are equally effective against DNFB, which is consistent with their equal inhibitory potencies. Also, L-lactate is a good protector from the hydrophobic amino group reagent PITC, being as effective as one of the best substrate analogue inhibitors, L-2-hydroxy,4-methylvalerate, in preventing inhibition.

Sulfhydryl group modification also inhibits monocarboxylate uptake by sarcolemmal vesicles [33]. However, 50 mM 2-hydroxyvalerate was not capable of protecting the transporter from inhibition by the sulfhydryl group reagent NEM.

Table 4. Protection of L-lactate uptake from DNFB or PITC inhibition[a]

Compound	% Protection from	
	DNFB	PITC
L-2-OH propionate (L-lactate)	27 ± 4	56 ± 8
2-oxo propionate (pyruvate)	33 ± 4	—
2-OH valerate	65 ± 4	—
L-2-OH,4CH_3 valerate	80 ± 2	47 ± 12

[a] Vesicles were preincubated with 50 mM Na^+ monocarboxylate; protein modifier was subsequently added to 0.8 mM. Protocol and controls were as described in Table 3 and text.

Discussion

The results presented above indicate that cardiac sarcolemmal vesicles contain a pyruvate-transporting mechanism with kinetic properties and inhibitor sensitivities very similar to those of the L-lactate transporter previously identified in these membranes [33]. As is the case for hepatocyte monocarboxylate transport [11], the affinity of the mechanism for pyruvate is about four times that for L-lactate. The present results confirm the earlier suggestion [33] that the cardiac sarcolemmal L-lactate transporter is in fact a monocarboxylate transporter. The effects of ionophores support the hypothesis that protons are transported with the monocarboxylate.

The transporting mechanism is inhibited by many short chain (3 to 6 carbon) monocarboxylic acids with a wide variety of substituents at the 2-carbon position. Inhibitions cannot be due to acidification of the vesicle interior by transport or diffusion of nonionized monocarboxylic acids, because all uptake experiments were performed in the presence of monensin and external Na^+, which would prevent buildup of an externally directed proton gradient. Diffusion of the analogues into the membrane could possibly result in inhibition of L-lactate or pyruvate uptake. However, if this were a significant mechanism of inhibition, the same effectiveness would not be expected for compounds of widely varying hydrophobicities (*cf.* 2-chloropriopionate, pyruvate and D,L-2-hydroxyvalerate), because they would be expected to equilibrate rapidly in the membrane to different extents [5, 34]. Nor should compounds with similar hydrophobicities cause significantly different inhibitions (*cf.* 2-hydroxy and 3-hydroxybutyrates, or 2-methylpropionate and *n*-butyrate, or 2-hydroxy,4-methylvalerate and 2-hydroxyhexanoate).

Alternatively, it is more likely that the monocarboxylate analogues interact directly with the transporting mechanism. For example, significant inhibition by 2-chloropropionate is consistent with its ability to acidify Purkinje fiber intracellular space in a manner sensitive to α-cyano,4-hydroxycinnamic acid [5], a potent inhibitor of the L-lactate transporter [11, 33].

The results presented in Table 1 suggest several possible requirements for recognition of the monocarboxylates by the transporter. Positively charged groups hinder interaction, as does the presence of two substituents on the second carbon. A strong hydrogen bonding substituent on the second carbon is not essential, although it does enhance recognition. 2-L compounds are preferred. Polar groups toward the end of the molecule opposite the carboxylic acid group decrease interaction, whereas additional hydrophobic groups in this region enhance it. Some of these conclusions have previously been drawn from results of studies on the effects of monocarboxylates on L-lactate efflux from erythrocytes [7, 8].

The experiments with the dicarboxylates are consistent with these suggestions, with the notable exception of oxaloacetate. Oxaloacetic acid from three different suppliers was used to eliminate the possibility that the compound was contaminated with a potent inhibitor. A satisfactory explanation of the ability of this dicarboxylate to inhibit monocarboxylate transport has not been obtained. The possible existence of an oxaloacetate-specific transporter, with very fast kinetics, which cotransports two protons and would cause rapid acidification of the vesicle interior despite the presence of monensin, should be checked.

The response of the sarcolemmal monocarboxylate transporter to 3-hydroxybutyrate distinguishes it from the transporter in other cells. This compound has been reported to be a good inhibitor of L-lactate uptake in Ehrlich ascite cells [31], thymocytes [27], and hepatocytes [22], and, when

present externally, to stimulate L-lactate efflux from erythrocytes [7, 27]. However, the present results suggest that 3-hydroxybutyrate interacts rather poorly with the cardiac sarcolemmal monocarboxylate transporter. These results are consistent with a previous report that a 5.6-fold excess of 3-hydroxybutyrate reduced 1 mM L-lactate uptake by perfused hearts by only 20% [6]. Also, 3-hydroxybutyrate is not as effective an inhibitor of the sarcolemmal transport system as 2-methylpropionate (Table 1). The latter compound, in contrast, has been observed not to interact with the erythrocyte transporter, whereas 3-hydroxybutyrate does [7]. Furthermore, a recently reported L-lactate transport mechanism in skeletal muscle fibers is not inhibited by either 2- or 3-hydroxybutyrate [35]. The present findings suggest that cardiac sarcolemma may have a distinct transporting system for 3-hydroxybutyrate, which is a major metabolic substrate for cardiac cells and is taken up from blood.

Protein modification experiments using group specific reagents do not routinely yield unequivocal evidence for a particular essential amino acid residue [15]. The studies reported above are no exception. However, the results may be interpreted consistently as follows: DEPC has been shown in many cases to be more specific for histidine at pH ~6 than at pH values greater than 7 [19, 23]. Thus its greater effectiveness in experiments at the higher pH, and the nonreversibility of DEPC inhibition with NH_2OH treatment suggest that a lysine, rather than or in addition to a histidine, may be the target residue in sarcolemma [23]. Successful modification by DNFB at physiological pH could be attributed to reaction with histidine, tyrosine or cysteine rather than amino groups, but the failure of thiol reagents to cause significant reversal of the effect argues against this. Instead, the results suggest an unusual lysine residue that is not protonated at pH 7.4 [13, 28, 36]. Effective inhibition by the hydrophobic amino group modifier PITC at this pH, and the failure of the much more polar reagent FITC to inhibit, are consistent with this interpretation, because an amino group in a hydrophobic environment might be expected to be unprotonated at pH 7.4 [29]. It should also be noted that phosphatidylserine is subject to attack by DNFB under these conditions [29, 36], and that alteration of membrane lipids could contribute to the observed inhibition of monocarboxylate uptake by the vesicles.

The ability of substrates and substrate analogues to protect the sarcolemmal monocarboxylate transport mechanism against inhibition by protein-modifying reagents suggests that these reagents react with amino acid residues that are essential for transport activity. Because the substrate analogues are themselves inhibitors of L-lactate or pyruvate uptake, they may interact with the transporting mechanism at or near the substrate binding site. Interestingly, the three carbon monocarboxylates provided less protection than expected on the basis of their inhibitory behavior. Thus the protective capacity of the longer substrate analogues could stem from the nonpolar portion of these molecules interacting with hydrophobic region(s) of the transporter at or near the recognition site for substrates. This region could contain the putative target lysine residue. Alternatively, binding of these compounds may trigger a conformational change in the transporter molecules which buries the reactive residue. The failure of L-lactate to protect against DEPC inhibition could be owing to the short (3-carbon) substrate molecule being unable to interact with, and thus block, a nearby hydrophobic region of the transporter and/or to a slightly different conformation change caused by the physiological substrate, which might leave the important residue exposed. It should also be noted that the poor protection afforded by L-lactate might be caused in part by the slightly lower affinity the transporter displays for this substrate, compared with that for pyruvate, for instance. The physiological substrate might also cause protein conformational changes during its transport, with the result that different residues are exposed to attack by the modifiers. The successful protection afforded by L-lactate against PITC inhibition would then suggest that this modifier may interact with different amino acid residues in the transport polypeptides from those modified by DEPC or DNFB.

Some substrate analogues can completely protect the cardiac sarcolemmal monocarboxylate transporter from the inhibition caused by covalent modification of specific amino acid residues. These analogues should be useful in differential labeling experiments [15] designed to identify membrane polypeptides which participate in monocarboxylate transport. The small size and reversible binding of the substrate analogues, compared with that of the irreversibly bound isobutylcarbonyl lactyl anhydride used for erythrocyte labeling [9, 16, 17], might simplify design and interpretation of experiments to identify the peptide(s) involved in sarcolemmal monocarboxylate transport.

We thank R. Ward and Dr. R. Vemuri for most of the vesicle preparations, Dr. J.C. Schaeffer for DNFB and many helpful discussions, and Drs. M.M. Bersohn, G.N. Pierce and R. Vemuri for their comments on the manuscript. This research was supported by National Science Foundation grant No. PCM 840459.

Reference

1. Balkovetz, D.F., Leibach, F.H., Mahesh, V.B., Ganapathy, V. 1988. A proton gradient is the driving force for uphill transport of lactate in human placental brush border membrane vesicles. *J. Biol. Chem.* **263:**13823–13830
2. Bindslev, N., Wright, E.M. 1984. Histidyl residues at the active site of the Na/succinate cotransporter in rabbit renal brush borders. *J. Membrane Biol.* **81:**159–170
3. Davis, M.H., Jung, D.W., Brierly, G.P. 1987. Inhibition of the Na^+/Ca^{2+} antiport of heart mitochondria by diethyl pyrocarbonate. *J. Bioenerg. Biomembr.* **19:**515–524
4. DeBruijne, A.W., Vreeburg, H., van Stevenick, J. 1983. Kinetic analysis of L-lactate transport in human erythrocytes via the monocarboxylic-specific carrier system. *Biochim. Biophys. Acta* **732:**562–568
5. de Hemptinne, A., Marrannes, R., Vanheel, B. 1983. Influence of organic acids on intracellular pH. *Am. J. Physiol.* **245:**C178–C183
6. Dennis, S.C., Kohn, M.C., Anderson, G.J., Garfinkel, D. 1985. Kinetic analysis of monocarboxylate uptake into perfused rat hearts. *J. Mol. Cell Cardiol.* **17:**987–995
7. Deuticke, B. 1980. Kinetic properties and substrate specificity of the monocarboxylate carrier in the human erythrocyte membrane. *In:* Membrane Transport in Erythrocytes. U.V. Lassen, H.H. Ussing, and J.O. Wieth, editors. pp. 539–551. Munksgaard, Copenhagen
8. Deuticke, B. 1982. Monocarboxylate transport in erythrocytes. *J. Membrane Biol.* **70:**89–103
9. Donovan, J.A., Jennings, M.L. 1985. Membrane polypeptide in rabbit erythrocytes associated with the inhibition of L-lactate transport by a synthetic anhydride of lactic acid. *Biochemistry* **24:**561–564
10. Dubinsky, W.P., Racker, E. 1978. The mechanism of lactate transport in human erythrocytes. *J. Membrane Biol.* **44:**25–36
11. Edlund, G.L., Halestrap, A.P. 1988. The kinetics of transport of lactate and pyruvate into rat hepatocytes. *Biochem. J.* **249:**117–126
12. Fafournoux, P., Demigné, C., Rémésy, C. 1985. Carrier-mediated uptake of lactate in rat hepatocytes. *J. Biol. Chem.* **260:**292–299
13. Ferguson, S.J., Lloyd, W.J., Radda, G.K. 1974. An unusual reversible chemical modification of soluble beef heart mitochondrial ATPase. *FEBS Lett.* **38:**234–236
14. Frank, J.S., Philipson, K.D., Beydler, S. 1984. Ultrastructure of isolated sarcolemma from dog and rabbit myocardium. *Circ. Res.* **54:**414–423
15. Freedman, J. 1978. Determination of essential residues in enzymes by chemical modification. *In:* Techniques in the Life Sciences: Biochemistry, Vol. B1, pt. II. H.L. Kornberg et al, editors. pp. B117/1-B117/23. Elsevier, Amsterdam
16. Jennings, M.L., Adams-Lackey, A.M. 1982. A rabbit erythrocyte membrane protein associated with L-lactate transport. *J. Biol. Chem.* **257:**12866–12871
17. Johnson, J.H., Belt, J.A., Dubinsky, W.P., Zimniak, A., Racker, E. 1980. Inhibition of lactate transport in Ehrlich ascites tumor cells and human erythrocytes by a synthetic anhydride of lactic acid. *Biochemistry* **19:**3836–3840
18. Julien, T., Zaki, L. 1988. Studies on inactivation of anion transport in human red blood cell membrane by reversibly and irreversibly acting arginine-specific reagents. *J. Membrane Biol.* **102:**217–224
19. Lundblad, R.L., Noyes, C.M. 1984. Chemical Reagents for Protein Modification. CRC, Boca Raton, FL
20. Mann, G.E., Zlokovic, B.V., Yudilevich, D.L. 1985. Evidence for a lactate transport system in the sarcolemmal membrane of the perfused rabbit heart. *Biochim. Biophys. Acta* **819:**241–248
21. Means, G.E., Feeney, R.E. 1971. Chemical Modification of Proteins. Holden-Day, San Francisco
22. Metcalfe, H.K., Monson, J.P., Cohen, R.D., Welsh, S.G. 1985. Interrelationships of 3-hydroxybutyrate and lactate transport in isolated rat hepatocytes and the isolated perfused rat liver. *In:* Diabetes, Obesity, and Hyperlipidemias. G. Crepaldi, A. Tiengo, and G. Babbio, editors. pp. 325–330. Elsevier, Amsterdam
23. Miles, E.W. 1977. Modification of histidyl residues in proteins by diethyl pyrocarbonate. *Meth. Enzymol.* **47:**431–442
24. Murphy, C.J., Shepherd, M.G., Sullivan, P.A. 1983. Chemical modification of L-lactate 2-monooxygenase with fluorodinitrobenzene: Evidence for two essential histidine residues. *Biochemistry* **22:**1665–1669
25. Paradies, G. 1988. The effect of phenylglyoxal on the transloction of pyruvate in rat heart mitochondria. *Biochim. Biophys. Acta* **932:**1–7
26. Philipson, K.D., Ward, R. 1986. Modulation of Na^+–Ca^{2+} exchange and Ca^{2+} permeability in cardiac sarcolemmal vesicles by doxylstearic acids. *Biochim. Biophys. Acta* **897:**152–158
27. Regen, D.M., Tarpley, H.L. 1978. Effects of pH on β-hydroxybutyrate transport in rat erythrocytes and thymocytes. *Biochim. Biophys. Acta* **508:**539–550
28. Shaltiel, S. 1967. Thiolysis of some dinitrophenyl derivatives of amino acids. *Biochem. Biophys. Res. Commun.* **29:**178–183
29. Sigrist, H., Zahler, P. 1978. Characterization of phenylisothiocyanate as a hydrophobic membrane label. *FEBS Lett.* **95:**116–120
30. Soon, C.Y., Shepard, M.G., Sullivan, P.A. 1977. Modification of lactate oxidase with diethyl pyrocarbonate. *Biochem. J.* **165:**385–393
31. Spencer, T.L., Lehninger, A.L. 1976. L-lactate transport in Ehrlich ascites tumour cells. *Biochem. J.* **154:**405–414
32. Takahashi, K. 1968. The reaction of phenylglyoxal with arginine residues in proteins. *J. Biol. Chem.* **243:**6171–6179
33. Trosper, T.L., Philipson, K.D. 1987. Lactate transport by cardiac sarcolemmal vesicles. *Am. J. Physiol.* **252:**C483–C489
34. Walter, A., Gutknecht, J. 1984. Monocarboxylic acid permeation through lipid bilayer membranes. *J. Membrane Biol.* **77:**255–264
35. Watt, P.W., MacLennan, P.A., Hundal, H.S., Kuret, C.M., Rennie, M.J. 1988. L(+)-Lactate transport in perfused rat skeletal muscle: Kinetic characteristics and sensitivity to pH and transport inhibitors. *Biochim. Biophys. Acta* **944:**213–222
36. Weber, J., Semenza, G. 1983. Chemical modification of the small intestinal Na^+/D-glucose cotransporter by amino group reagents. Evidence for a role of amino group(s) in the binding of the sugar. *Biochim. Biophys. Acta* **731:**437–447

Received 1 May 1989