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Articles

Membrane Polypeptide in Rabbit Erythrocytes Associated with the Inhibition of L-Lactate Transport by a Synthetic Anhydride of Lactic Acid†

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Received July 30, 1984

ABSTRACT: The synthetic lactyl anhydride isobutylcarbonyl lactyl anhydride (iBCLA), a selective and potent inhibitor of L-(+)-lactate transport in rabbit erythrocytes, reduces the chemical labeling of a 40–50-kdalton polypeptide by tritiated 4,4′-diisothiocyanato-2,2′-dihydrostilbenedisulfonate ([^{3}H]H_2DIDS). iBCLA does so in a dose-dependent manner at concentrations that strongly inhibit lactate exchange but not chloride–phosphate exchange. These labeling experiments and inhibition reversal studies using iBCLA, *p*-(chloromercuri)benzenesulfonic acid (pCMBS), and dithiothreitol (DTT) suggest that iBCLA does not act at sulfhydryl groups but at or near an amino group that is near a disulfide linkage in the polypeptide which catalyzes lactate transport. These experiments support the association between specific monocarboxylate transport and a 40–50-kdalton membrane-bound polypeptide of the rabbit erythrocyte.

Specific monocarboxylate transport appears to be a common feature in a variety of mammalian cells. Similarities in kinetics and susceptibility to such inhibitors as organomercurials (Deuticke et al., 1978; Spencer & Lehninger, 1976), cyanocinnamates (Spencer & Lehninger, 1976; Halestrap, 1976), and anhydrides (Johnson et al., 1980) occur in the lactate transport systems of human, rabbit, and other mammalian erythrocytes (Deuticke et al., 1978; Deuticke, 1982; Halestrap, 1976; Dubinsky & Racker, 1978), Ehrlich ascites tumor cells (Spencer & Lehninger, 1976; Johnson et al., 1980), and thymocytes (Anderson et al., 1978; Regen & Tarpley, 1978). Rabbit erythrocytes have been shown (Deuticke et al., 1978) to have a high capacity for lactate transport, suggesting that these cells have an abundance of this transport protein in the plasma membrane. Therefore, we have chosen these cells for the chemical labeling of the lactate transport protein. Jennings & Adams-Lackey (1982) presented evidence suggesting that a 40–50-kdalton integral membrane polypeptide is associated with lactate transport in rabbit erythrocytes. The evidence

†This work was supported by Research Grant GM-26861 from the National Institutes of Health. J.A.D. received support from Medical Scientist Training Program Grant GM-07337 from the NIH. M.L.J. received support from NIH RCDA AM-01137.

0006-2960/85/0424-0561$01.50/0

was derived from chemical labeling experiments in which tritiated 4,4'-diisothiocyanato-2,2'-dihydrostilbenedisulfonate [(^{3}H]H_2DIDS)[1] was used to label this polypeptide at concentrations where the [^{3}H]H_2DIDS acted as a selective, concentration-dependent inhibitor of specific lactate transport. This evidence, while suggestive of an association between a particular polypeptide and lactate transport, is nonetheless circumstantial. Such evidence would be strengthened by the further association of that polypeptide with a more specific inhibitor. The synthetic lactyl anhydride iBCLA developed by Racker and co-workers (Johnson et al., 1980) is a more potent and selective inhibitor of lactate transport than H_2-DIDS. In this paper, we show that iBCLA can be used to diminish in a dose-dependent manner the labeling of the 40K–50K polypeptide by [^{3}H]H_2DIDS in the rabbit erythrocyte membrane. iBCLA does this at concentrations that strongly inhibit lactate–lactate exchange but not chloride–phosphate exchange. It has been suggested that iBCLA inhibits lactate transport by acting at sulfhydryl groups in the polypeptide chain (Johnson et al., 1980). However, in rabbit erythrocytes, we found that the effects of iBCLA on lactate transport were not reversible by DTT, a disulfide-reducing agent (Cleland, 1964; Konigsberg, 1972), even after a 6-h incubation at high concentrations at 37 °C.

Experimental Procedures

Materials. Red blood cells (EDTA anticoagulant) were collected by ear vein phlebotomy from New Zealand white rabbits. Cells were used after a maximum of 2 days storage at 4 °C. L-(+)-[^{14}C]Lactic acid (90 mCi/mmol) was purchased from ICN Radiochemicals. H^{36}Cl was purchased from ICN and neutralized with NaOH before use. H_2DIDS and [^{3}H]H_2DIDS were prepared in our laboratory (Jennings et al., 1984) according to methods adapted from Lepke et al. (1976) and Levinson et al. (1979). iBCLA was prepared according to the method of Johnson et al. (1980) and stored at −70 °C. Isobutyl chloroformate and tetrahydrofuran used in the synthesis of iBCLA were purchased from Aldrich. pCMBS, BSA, DTT, L-(+)-lactic acid, and phthalic anhydride were obtained from Sigma. Phloretin was from ICN K & K Laboratories. All other chemicals were of at least reagent grade.

[^{14}C]*Lactate Efflux.* The measurement of lactate efflux was performed exactly as previously described (Jennings & Adams-Lackey, 1982).

$^{36}Cl^-$*–Phosphate Exchange.* Band 3 mediated anion transport was assessed by measuring the exchange of intracellular chloride for extracellular phosphate. Cells washed in PBK 7.4 (145 mM KCl and 10 mM sodium phosphate, pH 7.4) were pelleted by centrifugation and resuspended in PBK 7.4 plus $^{36}Cl^-$ (2 μCi/mL) at room temperature. Cells were centrifuged 2 min at 10000*g*, the supernatant was removed, and the cells were equilibrated to 15 °C. Efflux was initiated by resuspending ^{36}Cl-loaded cells (100 μL) into 10 mL of 140 mM sodium phosphate, pH 6.0 at 15 °C. Aliquots of 1 mL were drawn each minute and centrifuged immediately in the Eppendorf microcentrifuge; 0.5 mL of the supernatants was drawn and counted for radioactivity. Infinity time points were measured with 9% trichloroacetic acid, just as for [^{14}C]lactate efflux (Jennings & Adams-Lackey, 1982).

H_2DIDS and [^{3}H]H_2DIDS Treatment. Cells washed in PBK 7.4 were incubated at 5% hematocrit in PBK 7.4 plus 5 μM H_2DIDS for 80 min at 37 °C. The cells were then washed twice in PBK 7.4 plus 0.2% bovine serum albumin (BSA) followed by two washes in PBK 7.4. The cells were then treated with iBCLA (see below) and again washed in PBK 7.4. Cells were then labeled at 10% hematocrit in PBK 7.4 with 30 μM [^{3}H]H_2DIDS (specific activity 54 mCi/mmol) at 37 °C for 60 min. Cells were then washed twice in PBK 7.4 plus 0.2% BSA and then twice in 150 mM $NaHCO_3$.

iBCLA Treatment. Cells washed in PBK 7.4 were incubated at 1% hematocrit with various concentrations of iBCLA (introduced as 1 mg/mL ethanolic solution in 40 mL total volume) for 60 min at 0 °C. Treated cells were then washed 3 times in PBK 7.4.

pCMBS Treatment. Cells washed in PBK 7.4 were incubated at 1% hemotocrit in PBK 7.4 plus 2 mM pCMBS for 5 min at room temperature and then washed 3 times in PBK 7.4.

DTT Treatment. Cells washed in PBK 7.4 were incubated at 5% hematocrit in PBK 7.4 plus 100 mM DTT for 1 h at 37 °C and then washed 3 times in PBK 7.4.

Inhibition Reversal Studies. The ability of the reducing agent DTT to reverse the inhibition of lactate exchange was investigated for cells treated with pCMBS, iBCLA, pCMBS followed by iBCLA, and iBCLA followed by pCMBS, as shown in Figure 2 and according to the above protocols.

Membrane Isolation and Polyacrylamide Gel Electrophoresis. Cells treated with 5 μM H_2DIDS, various concentrations of iBCLA, and 30 μM [^{3}H]H_2DIDS as described above were lysed osmotically in ice-cold 5 mM $NaHCO_3$ and subsequently washed 3 times by centrifugation at 20000 rpm. Aliquots of packed membranes were solubilized in an equal volume of 4% SDS, 80 mM DTT, 20% glycerol, 120 mM Tris-HCl, pH 6.8, and 0.01% bromophenol blue and heated 3 min at 100 °C. The samples were electrophoresed according to the method of Laemmli (Laemmli, 1970) on a 12-cm polyacrylamide gel (6–18% acrylamide, 5% stacking gel). Lanes were excised and cut into 2.5-mm segments, which were subsequently digested in 30% H_2O_2 at 45 °C overnight. Radioactivity in the segments was measured by liquid scintillation counting. Prior to slicing, the gels were stained in Coomassie Blue G and the lanes scanned spectrophotometrically to permit a comparison to be made between peaks of radioactivity and the molecular weight of standards run on the same gel.

Results

Lactate Transport Inhibition. iBCLA causes a dose-dependent inhibition of lactate exchange at a much lower concentration than is required for a similar inhibition of chloride–phosphate exchange, as shown in Figure 1. Treatment with 10 μM iBCLA resulted in 45% inhibition of lactate–lactate exchange but almost no inhibition of chloride–phosphate exchange. Phthalic anhydride inhibits lactate–lactate exchange 50% at 90 μM but similarly inhibits chloride–phosphate exchange at this concentration (data not shown). H_2DIDS, as previously reported (Jennings & Adams-Lackey, 1982), strongly inhibits band 3 mediated anion transport at 4 μM but requires 20 μM to inhibit lactate–lactate exchange 50%. pCMBS, an organomercurial, very strongly inhibits lactate transport (Deuticke et al., 1978) (see Figure 2) and is known to penetrate the diffusion barrier of other mammalian erythrocytes only very slowly (Knauf & Rothstein, 1971; Smith & Ellman, 1973).

Reversal of Inhibition by DTT. Deuticke et al. (1978) have shown that, in human erythrocytes, the inhibition of lactate–

[1] Abbreviations: [^{3}H]H_2DIDS, tritiated 4,4'-diisothiocyanato-2,2'-dihydrostilbenedisulfonate; iBCLA, isobutylcarbonyl lactyl anhydride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; pCMBS, *p*-(chloromercuri)benzenesulfonic acid; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

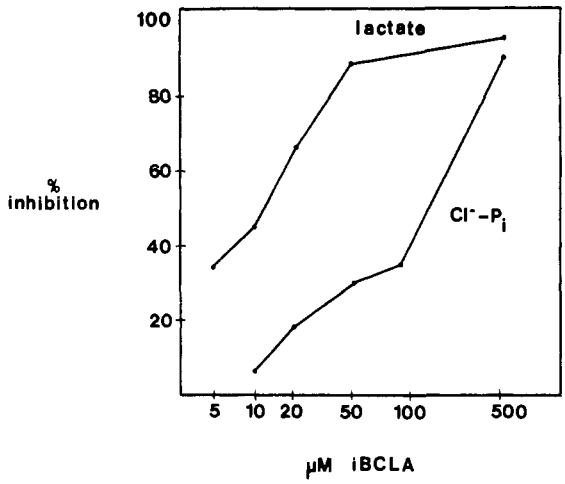

FIGURE 1: Inhibition of lactate–lactate and chloride–phosphate exchange by iBCLA. Rabbit erythrocytes were treated with iBCLA and subjected to the flux assays under Experimental Procedures. Percent inhibition was calculated as previously described (Jennings & Adams-Lackey, 1982).

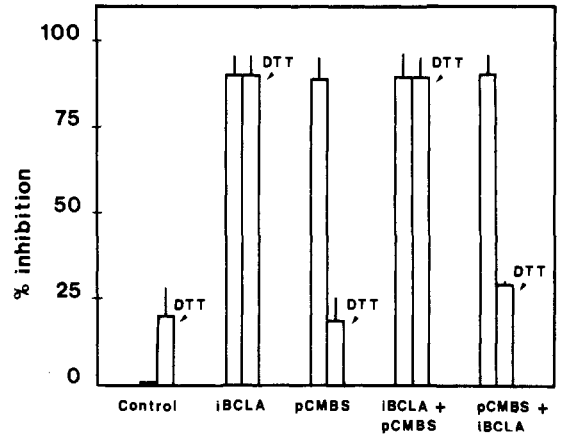

FIGURE 2: Reversal of inhibition of lactate exchange in rabbit erythrocytes. Control cells were treated with 100 mM DTT for 60 min at 37 °C and washed prior to [^{14}C]lactate loading. For iBCLA, cells were treated with 80 μM iBCLA for 60 min at 0 °C, washed, and treated with DTT. For pCMBS, cells were treated with 2 mM pCMBS for 5 min at room temperature, washed and treated with DTT. For iBCLA–pCMBS, cells were first treated with 80 μM iBCLA, then 2 mM pCMBS, and then DTT as above, with washings between each treatment. For pCMBS–iBCLA, cells were treated with 2 mM pCMBS, then 80 μM iBCLA, and then DTT as above. None of these agents were present in the lactate exchange flux medium. Although DTT reversed the inhibitory effects of pCMBS, it had no effect on iBCLA-induced inhibition, even when the incubation was extended to 6 h. Cells treated with pCMBS followed by iBCLA had lactate exchange restored by DTT.

lactate exchange brought about by 3 mM pCMBS is reversible by treatment with 100 mM concentrations of a variety of thiol compounds, including dithioerythritol. We found that the thiol DTT, a known disulfide-reducing agent (Cleland, 1964; Konigsberg, 1972), readily reverses the inhibition of lactate transport in rabbit erythrocytes caused by pCMBS as shown in Figure 2. Cells treated with 2 mM pCMBS and subsequently treated with 100 mM DTT had lactate exchange returned to control (DTT alone) levels following a 1-h incubation at 37 °C. DTT mildly inhibits lactate transport at this concentration but does not increase the mechanical fragility of the cells. Cells treated with iBCLA fully retained the inhibition of lactate transport following subsequent incubation in 100 mM DTT, even when the incubation was extended to 6 h. Johnson et al. (1980) demonstrated a reversal of iBCLA-induced inhibition of lactate exchange in Ehrlich ascites tumor cells using DTT and hydroxylamine. Rabbit erythrocytes were found to be acutely sensitive to hydroxylamine, and attempts to reverse iBCLA-induced inhibition of lactate exchange with this agent resulted in the immediate destruction of the cells. Cell treated with pCMBS and then iBCLA followed by DTT exhibited a return of lactate transport to control levels. However, cells treated with iBCLA, then pCMBS, and then DTT retained the inhibition.

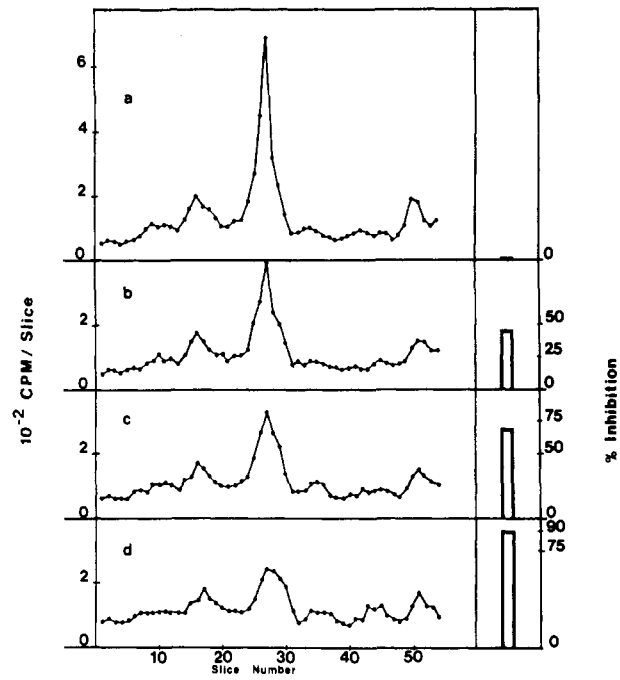

FIGURE 3: Labeling of rabbit erythrocyte membranes by [^{3}H]H_2DIDS following pretreatment with H_2DIDS and iBCLA. Cells were first treated with 5 μM H_2DIDS for 80 min at 37 °C, washed, then treated with 0 (a), 10 (b), 20 (c), and 50 μM (d) iBCLA for 60 min at 0 °C, washed, and then treated with 30 μM [^{3}H]H_2DIDS for 60 min at 37 °C. Electrophoresis of isolated solubilized membranes was performed as described under Experimental Procedures. Gels were sliced and solubilized, and the radioactivity was counted by liquid scintillation counting. Comparisons to molecular weight standards (not shown) electrophoresed on the same gels showed a broad band of radioactivity from 40 to 50 kdaltons, as previously reported (Jennings & Adams-Lackey, 1982). The labeling of this band was diminished in a dose-dependent manner by iBCLA at concentrations corresponding to the dose-dependent inhibition of lactate exchange, shown at right.

Labeling of Rabbit Erythrocyte Membranes with [^{3}H]-H_2DIDS following iBCLA Treatment. Cells were treated successively with 5 μM H_2DIDS, iBCLA at various concentrations, and 30 μM [^{3}H]H_2DIDS. The membranes were isolated, solubilized, and subjected to electrophoresis as described under Experimental Procedures. A dose-dependent decrease in the radioactive labeling of the 40–50-kdalton band was seen with increasing concentrations (Figure 3). These concentrations were in the range where lactate transport was strongly inhibited but chloride–phosphate exchange was not. A decrease in the labeling of this band was also obtained with 90 μM phthalic anhydride instead of iBCLA, as well as in several experiments using higher concentrations of iBCLA (data not shown).

Discussion

The labeling experiment in Figure 3 provides excellent supporting evidence that the 40–50-kdalton band is associated with lactate transport in rabbit erythrocytes. iBCLA is a more selective and potent inhibitor of lactate exchange than H_2-DIDS. Concentrations of iBCLA sufficient to strongly inhibit lactate transport but not chloride–phosphate exchange sig-

nificantly reduce the labeling of this polypeptide by [^{3}H]-H_2DIDS. Thus, evidence for the involvement of the 40–50-kdalton band with specific lactate transport is provided at two levels. First, that iBCLA reduces the labeling of a particular polypeptide by *any* labeling method under the conditions described is evidence in its own right that this polypeptide may be associated with lactate transport. Second, since the labeling method uses a radiolabel which is also a fairly potent and specific inhibitor of lactate transport (Jennings & Adams-Lackey, 1982), the further association of this labeled polypeptide with iBCLA makes the putative connection between the 40–50-kdalton polypeptide and lactate transport all the more plausible. While such evidence is supportive, it remains circumstantial until it can be verified by reconstitution that this protein in fact catalyzes lactate transport.

In Ehrlich ascites tumor cell, 100 mM DTT has been reported to reverse iBCLA-induced inhibition of lactate transport (Johnson et al., 1980), and thiols are known reducing agents of disulfide and organomercurial–sulfide bonds (Deuticke et al., 1978). Thus, it has been suggested that iBCLA may inhibit lactate transport by acting at sulfhydryl groups on the polypeptide chain (Johnson et al., 1980). In rabbit erythrocytes, this is probably not the case, since DTT does not reverse iBCLA-induced inhibition at concentrations and conditions that quickly and completely reverse the effects of the classical sulfhydryl reagent pCMBS (Figure 2). While pCMBS treatment appears to prevent iBCLA from binding near the active site for lactate transport, it is also true that iBCLA prevents [^{3}H]H_2DIDS from binding, and isothiocyanatostilbenedisulfonates are known to react at amino groups in other polypeptides (Ramjeesingh et al., 1981; Jennings, 1982; Legrum et al., 1980). Also, other anhydrides are believed to react at amino groups (Means & Feeney, 1971). iBCLA is structurally similar to ethoxyformic anhydride (EFA), in that both are anhydrides derived from alkoxyformic acids. EFA is a well-known reagent for histidine and amino groups (Muhlrad et al., 1969; Melchior & Fahrney, 1970). The resistance of iBCLA to reversal by DTT, the protection from iBCLA given by pCMBS pretreatment, and the similarity of iBCLA to other anhydrides structurally and functionally in its ability to inhibit lactate exchange and reduce labeling at a probable amino group of a band associated with lactate transport suggest that iBCLA acts at a site separate from but near a sulfhydryl group. This site may be at or very near an ϵ-amino group on the polypeptide chain. This is reasonable, since the iBCLA, pCMBS, and H_2DIDS binding sites are probably all near the functionally active site of the transport protein, which accounts for their action as functional inhibitors. The precise relationships of these sites to structure and function may have to wait for the elucidation of primary and higher order structure in order to be fully understood.

Registry No. L-Lactic acid, 79-33-4.

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