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Characterisation of erythrocyte transmembrane exchange of trifluoroacetate using ^{19}F-NMR: evidence for transport via the monocarboxylate transporter

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The transport of trifluoroacetate (TFA) and difluorophosphate (DFP) into and out of human and sheep erythrocytes was measured using ^{19}F-NMR. The pathways for the transport in human erythrocytes were characterised by differentiating between the transport inhibition caused by different reagents. (1) Pre-treatment of human erythrocytes with *N*-ethylmaleimide (10 mM) caused a decrease of the membrane-permeability coefficients for TFA influx and efflux to 0.74 ± 0.05 and 0.83 ± 0.09-times, respectively, of those determined in the absence of inhibition. Concomitantly there was no apparent effect on the band-3-mediated transport of DFP. Thus, the decrease of the permeability of TFA is consistent with the inhibition being that of the monocarboxylate transporter. (2) Inhibition of TFA and DFP exchange was also seen in human erythrocytes treated with *p*-chloromercuriphenylsulfonate (pCMBS). The extent of inhibition reached a maximum value for the pCMBS concentrations beyond which further inhibition was not achieved and there was substantial residual exchange of the two solutes. (3) Residual flux of TFA was found in the presence of high concentrations of the inhibitors, α-cyano-4-hydroxycinnamate (≥ 4 mM) or 4,4′-dinitrostilbene-2,2′-disulfonate (≥ 1 mM) when each compound was used alone. (4) Complete inhibition of TFA uptake was obtained when human erythrocytes were treated with both α-cyano-4-hydroxycinnamate (4 mM) and a stilbene disulfonate. It was, therefore, concluded that simple diffusion of TFA via the lipid bilayer was negligible in human erythrocytes and that incomplete inhibition of the monocarboxylate transporter occurred when the compounds were used alone.

Introduction

Erythrocyte transmembrane exchange of a number of monocarboxylates, such as pyruvate, lactate and acetate, has been reported to be via several pathways, namely, via band-3, simple diffusion via the lipid bilayer, and via the monocarboxylate transporter [1–6]. Studies by Donovan and Jennings [7], using competitive labelling of membrane protein of rabbit erythrocytes with *N*-hydroxylsulfosuccinimido esters and 4,4′-diisothiocyanato-2,2′-dihydrostilbene disulfonate (H_2DIDS), suggested that a membrane polypeptide, band-R (40–50 kDa) was the monocarboxylate transporter. Poole and Halestrap [8] recently reported the identification of the L-lactate transporter of erythrocytes of rabbit, rat and guinea pig using anti-DIDS antibody. The transporter is a polypeptide of 35–45 kDa in rat erythrocytes and of 40–50 kDa in rabbit and guinea-pig erythrocytes.

TFA, an analogue of acetate, exists almost entirely as an anion at physiological pH. When TFA is added to erythrocyte suspensions, it gives rise to two well-resolved ^{19}F-NMR resonances from its transmembrane populations, with the intracellular peak being shifted to high frequency of the extracellular resonance. The principal physical basis of this phenomenon has been discussed elsewhere [9]. Because of the separate resonances, it is possible to estimate unequivocally the transmembrane distribution of the solute; at equilibrium the ratio of peak areas is dependent on the membrane potential of the erythrocytes [10].

London and Gabel [10] suggested that because the transport was only partially inhibited by 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate (SITS), band-3 *and* simple membrane diffusion were the pathways for TFA exchange. In the present work the inhibition

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of different pathways by various compounds revealed that they were less specific than sometimes thought, and they affect both the monocarboxylate transporter and band-3. Thus, the 0.5 mM SITS which was used, would have simultaneously inhibited the monocarboxylate transporter, thus leaving open the question of whether the inhibited transport had been via band-3 *and* the monocarboxylate transporter. It is this aspect of TFA transport which we investigated in the present work.

Materials and Methods

Chemicals and solutions

TFA (> 99%) was from Merck (Munich, Germany) and was used without further purification. DFP which was a concentrated solution (approx. 97%) with small amounts of monofluorophosphate and fluoride ions, and 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), were from Pfaltz & Bauer (Waterbury, CT, USA). 4,4′-Di-isothiocyanatostilbene-2,2′-disulfonate (DIDS), *N*-ethylmaleimide (NEM, > 98%), and α-cyano-4-hydroxycinnamate (> 97%) were from Aldrich (Milwaukee, WI, USA). pCMBS monosodium salt was from Sigma (St. Louis, MO, USA). Heparin sodium salt was from Fisons Pharmaceuticals (Castle Hill, NSW, Australia). All other reagents were of analytical reagent grade.

DFP and TFA were routinely prepared as isotonic solutions (approx. 140 mM) with pH adjusted to the range 7–7.5. EDTA (approx. 2 mM) was routinely added to the solutions to chelate paramagnetic ions that cause broadening of NMR spectral lines [11]. Phosphate-buffered (20 mM) isotonic sucrose (approx. 290 mM) solutions, containing DFP (20 mM), were prepared by mixing the appropriate proportion of an isotonic DFP solution to the phosphate-buffered sucrose solution. pCMBS stock solution (20 mM) was freshly prepared in phosphate-buffered (20 mM) saline (PBS), and stored in the dark at 4°C. Stock solutions of stilbene disulfonates (50 mM) were prepared in H_2O and kept in the dark to avoid photo-degradation [12]. The osmolalities of solutions were measured to be within the range 270–310 mosmol/kg, unless stated otherwise, using a Wescor vapour pressure osmometer (Model 5100C, Wescor, Logan, UT, USA).

Erythrocyte suspensions

Concentrated suspensions of human erythrocytes, in preservative solution, were supplied by the Red Cross Blood Transfusion Service, NSW, within two days of collection from healthy volunteers, or they were obtained freshly from ASLX. Fresh blood was also obtained from healthy sheep in the MacMaster Laboratory of the Commonwealth Scientific and Industrial Research Organisation, NSW; blood was collected from the common carotid artery, and it was stored at 4°C in the presence of heparin (approx. 10 U/ml). Routinely, the cells were suspended in at least three volumes of PBS and the extracellular fluid was removed by centrifugation ($3000 \times g$, 5 min, 4°C); this procedure was repeated (twice) to remove anticoagulants and plasma. The cells were then gassed with humidified CO that converts oxy- and deoxyhaemoglobin to carbonmonoxyhaemoglobin that has a stable diamagnetic nature [13]. The haematocrits (H_c) of the suspensions were determined using a haematocrit centrifuge (Clements, North Ryde, NSW, Australia); and a correction was applied to the readings to compensate for the volume of extracellular fluid trapped between the packed cells [14]. Where the cells were used to measure the equilibrium exchange-rate of DFP, they were repeatedly resuspended and sedimentated (37°C for approx. 30 min, 3-times) in a solution of phosphate-buffered (approx. 20 mM, pH approx. 7) sucrose, containing DFP (20 mM). This procedure served to fix the extracellular DFP concentration at 20 mM and it depleted Cl^- that competes with DFP for transport.

The uptake of TFA into cells was initiated by adding aliquots of an isotonic solution of TFA to a suspension to yield a final concentration of 10 mM with respect to the total volume of the sample. Inhibition of DFP transport by pCMBS and NEM was brought about by incubating (37°C) the cells that had been pre-equilibrated (37°C) in phosphate-buffered (20 mM) sucrose containing DFP (20 mM), with the inhibitors (see Results). Any non-reacted inhibitor molecules would have been removed by the repeated centrifugal washing (3-times; see above) of the treated cells in the suspending solutions (free of the inhibitors), prior to the ^{19}F-NMR studies. Similarly, the inhibition of TFA transport was achieved by incubating cells in PBS, in the presence of the inhibitors. Inhibition by the stilbene disulfonates and α-cyano-4-hydroxycinnamate was achieved by incubating cells at 37°C for approx. 10 min; for the two compounds the cells were not further washed, prior to the ^{19}F-NMR studies.

^{19}F-NMR and determination of transmembrane exchange rate-constants

^{19}F-NMR spectra were acquired on a Bruker AMX 400wb spectrometer equipped with a 5 mm ^{19}F probe tuned to 376.47 MHz (Bruker Analytische Messtechnik, Karlsruhe, Germany). A pre-acquisition delay of $\geqq 5T_1$ (longitudinal relaxation time) values allowed almost complete relaxation of magnetisation between transients. Free induction decays (FID) were digitised into 8K data points and 4 or 8 FID were summed; 2 Hz of spectral line broadening was applied by exponential multiplication of each FID, prior to Fourier transformation. The sample temperature was calibrated from the ^{1}H chemical shift difference between the OH and

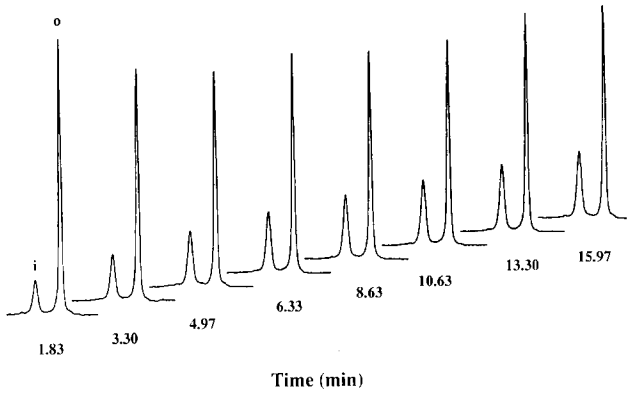

Fig. 1. ^{19}F-NMR spectra of TFA acquired in a time-course after TFA had been added to human erythrocytes suspended in PBS (20 mM (pH 7.3)) at 25°C. Four transients were acquired for each spectrum and the time indicated under each spectrum refers to the mid-point of the spectral accumulation; o and i refer to the extra- and intracellular TFA, respectively.

CH_2 resonances of ethylene glycol in a capillary inserted coaxially in the sample tube [15]. In order to minimise the rate of sedimentation of the cells in the NMR tube, the samples were not spun during spectral acquisition.

The entry of TFA into erythrocytes was monitored by sequentially acquiring a series of ^{19}F-NMR spectra (e.g., Fig. 1). A time-course typically covered the period from the initial phase of uptake until an equilibrium distribution of TFA had been established between the intra- and extracellular compartments. The well-separated ^{19}F-NMR resonances of intra- and extracellular TFA allowed its transmembrane distribution to be measured by direct spectral integration. There was no evidence of TFA taking part in metabolic reactions in the cells, thus the transmembrane exchange of the solute could be treated as an exchange between two sites. The time-dependence of the concentration of extra- and intracellular TFA can be described by:

$$[\mathrm{TFA}]_{\mathrm{extracelluar}} = [\mathrm{TFA}]_{\mathrm{total}} k_1/(k_1+k_{-1})\{1+\exp[-(k_1+k_{-1})t]\} \quad (1)$$

and

$$[\mathrm{TFA}]_{\mathrm{intracellular}} = [\mathrm{TFA}]_{\mathrm{total}} k_{-1}/(k_1+k_{-1})\{1-\exp[-(k_1+k_{-1})t]\} \quad (2)$$

where k_1 and k_{-1} are the apparent first-order rate constants for the influx and efflux, respectively. $[\mathrm{TFA}]_{\mathrm{total}}$ is the total concentration of TFA with respect to the volume of the suspensions, and $[\mathrm{TFA}]_{\mathrm{extracelluar}}$ and $[\mathrm{TFA}]_{\mathrm{intracellular}}$ denote the concentration in the extra- and intracellular compartments, respectively. Non-linear least-squares regression of Eqns. 1 and 2 onto the data of a graph of the spectral peak-areas (corrected for the H_c and volume fraction of cellular solvent water) as a function of time yielded the rate constants and their associated standard deviations (Fig. 2). The rate constants were used to obtain the membrane permeability coefficients for influx (P_1) and efflux (P_{-1}) by using the formulae:

$$P_1 = \mathrm{MCV}(1-H_c)k_1 A^{-1} H_c^{-1} \quad (3)$$

and

$$P_{-1} = \alpha \mathrm{MCV} k_{-1} A^{-1} \quad (4)$$

where α, MCV and A are the intracellular volume fraction that is freely accessible to solutes, the mean cell volume, and the mean cell-surface area (143 μm^2 for human erythrocytes [16] and approx. 61 μm^2 for sheep erythrocytes [17]). The MCV was calculated from H_c and mean cell count, obtained using a Sysmex Microcellcounter (CC 130, Toa Medical Electronics, Kobe, Japan). The relative membrane permeability is defined as the ratio of the permeability determined in the presence of transport inhibition to that obtained in the absence of inhibition.

In the absence of transport inhibition the transmembrane equilibration of DFP at 37°C is complete in a few seconds. Thus the process is too rapid to be measured by using a conventional NMR spectral time-course approach. Therefore, we used an 1D EXSY experiment [18,19] to measure the ^{19}F magnetisation exchange between the transmembrane solute populations once the equilibrium had been attained; thus we determined the rate constants of the equilibrium exchange process. In the presence of inhibitor(s) the slow membrane transport of DFP was able to be monitored using a time-course approach and its permeability was calculated using Eqns. 3 and 4.

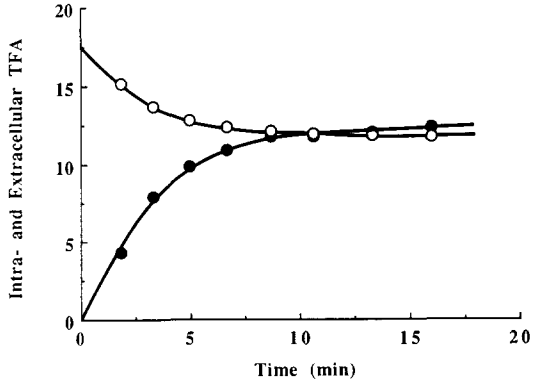

Fig. 2. Plot of relative intracellular (•) and extracellular (○) TFA concentrations (given as arbitrary unit) as a function of time of TFA transport. The arbitrary concentration values were determined from the ^{19}F-NMR spectral integrals, the mean cell volume, the haematocrit and the intracellular solvent-water volume. Non-linear least squares regression of Eqns. 1 and 2 onto the data yielded the rate constants for the equilibrium influx and efflux, respectively.

Results

Results of equilibrium exchange of TFA in human and sheep erythrocytes

The membrane permeability-coefficients of TFA (10 mM) measured in human erythrocytes were greater than those of sheep at both 21°C and 37°C (Table I). At 21°C the rates of influx and efflux in the human erythrocytes were approx. 3.6 and approx. 5.0-times greater, respectively, than those of the sheep erythrocytes; at 37°C the rates were approx. 3.0 and approx. 3.5-times greater, respectively. The above data showed that an increase in temperature from 21°C to 37°C resulted in a large increase in the permeability of TFA influx both in the human and the sheep erythrocytes.

α-Cyano-4-hydroxycinnamate inhibition of TFA and DFP transport

Pre-incubation (37°C, approx. 10 min) of erythrocytes (H_c 0.41–0.50) suspended in PBS (20 mM) with α-cyano-4-hydroxycinnamate ($\geqq 4$ mM) caused substantial inhibition of TFA transport (Fig. 3). A rapid decrease in the relative transmembrane exchange rate occurred with an increase of the inhibitor concentration. Non-linear least-squares regression of a single exponential function with an ordinate-offset onto the data, gave the residual relative membrane permeabilities of 0.04 ± 0.03 and 0.05 ± 0.02 for influx and efflux, respectively.

Incubating (37°C, approx. 10 min) erythrocytes (H_c 0.37–0.42) that had been pre-equilibrated with 20 mM DFP (in phosphate-buffered sucrose solution) with α-cyano-4-hydroxycinnamate gave rise to extensive inhibition of DFP uptake (Table II). Non-linear least squares regression of a single exponential function with an ordinate-offset onto the data, that consisted of the relative membrane permeability of efflux as a function of α-cyano-4-hydroxycinnamate concentration, gave an estimate of the residual relative membrane permeability of 0.55 ± 0.06 at maximal inhibition. The residual relative membrane permeability for the influx was determined by fitting a double exponential function with an ordinate-offset onto the graph and was 0.24 ± 0.05.

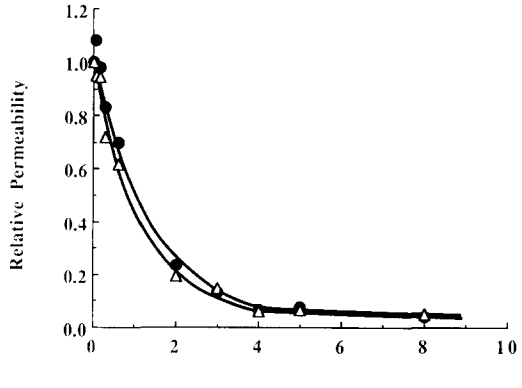

Fig. 3. α-Cyano-4-hydroxycinnamate inhibition of TFA transport at 37°C. Human erythrocytes in PBS (20 mM, pH approx. 7) were incubated (37°C, 10 min) in the presence of the inhibitor at various concentrations. Unreacted inhibitor was not removed from the cell suspensions, prior to addition of TFA. The rate constants and permeability coefficient were determined as described in Materials and Methods. Each symbol represents a single estimate of the relative membrane permeability. The curves were drawn using non-linear least squares regression of a single exponential function with an ordinate-offset: $y = (1-a)\exp(-bx) + a$ (a, $(3.7 \pm 2.6) \cdot 10^{-2}$; b, $(67 \pm 9.6) \cdot 10^{-2}$ for the influx, and a, $(5.4 \pm 2.0) \cdot 10^{-2}$; b, $(91 \pm 8.0) \cdot 10^{-2}$ for the efflux, respectively) onto the data, where y and x denote the relative membrane permeability and concentration (mM) of the inhibitor, respectively. (●) and (△) denote the relative membrane permeabilities for influx and efflux, respectively.

Silbene disulfonate inhibition of TFA and DFP transport

DNDS was seen to be a potent but incomplete inhibitor of TFA uptake. Pre-incubating (37°C, 10 min) the erythrocytes in PBS with 1 mM DNDS gave rise to a substantial inhibition of the exchange (Table III), but there was a small residual permeability (approx. 0.02) for the influx. However, the erythrocytes had shown greater relative membrane permeability (approx. 0.28 and approx. 0.20 for the influx and efflux, respectively) after incubation at the lower temperature of 21°C. In other experiments, incubating the cells with DIDS (0.5

TABLE I

Permeability coefficients of TFA in the absence of transport inhibition

Transport was initiated by adding aliquots of TFA (10 mM) to the erythrocytes suspended in PBS (20 mM). Human and sheep erythrocytes were suspended in PBS (20 mM, pH 7.4–7.6). The H_c values were within the range 0.49–0.53. TFA uptake was monitored with sequential ^{19}F-NMR spectra recorded at the indicated temperatures. The transport at each temperature was measured in cells from one human donor and one sheep. P_1 and P_{-1} denote the membrane permeability coefficients for influx and efflux, respectively, and were calculated from the rate constants determined as described in Materials and Methods. The standard deviations were calculated from those of the rate constants using a formula for propagation of errors [40], neglecting the small deviations associated with H_c and MCV measurements.

	Human erythrocytes		Sheep erythrocytes	
	P_1 (cm/s)	P_{-1} (cm/s)	P_1 (cm/s)	P_{-1} (cm/s)
37°C	$(1.5 \pm 0.1) \cdot 10^{-6}$	$(2.8 \pm 0.3) \cdot 10^{-6}$	$(5.0 \pm 0.5) \cdot 10^{-7}$	$(7.9 \pm 0.1) \cdot 10^{-7}$
21°C	$(1.2 \pm 0.2) \cdot 10^{-7}$	$(1.6 \pm 0.2) \cdot 10^{-7}$	$(3.3 \pm 0.1) \cdot 10^{-8}$	$(3.2 \pm 0.1) \cdot 10^{-8}$

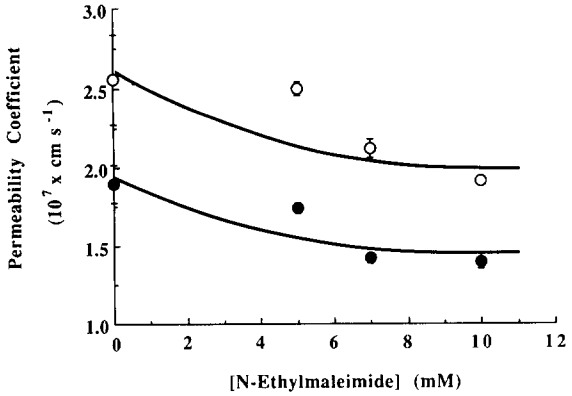

Fig. 4. Inhibition of TFA transport by NEM in human erythrocytes. The cells (H_c approx. 0.1) in PBS (20 mM (pH 7.32)) were incubated (37°C, approx. 1 h) with NEM at various concentrations. The un-reacted inhibitor was removed from the suspensions by repeated (3-times) centrifugal washing of the cells in ice-cold PBS, prior to measuring the TFA transport. (●) and (○) denote the permeability coefficients for TFA influx and efflux, respectively. The error bars were obtained from duplicate estimates. The curves were fitted empirically.

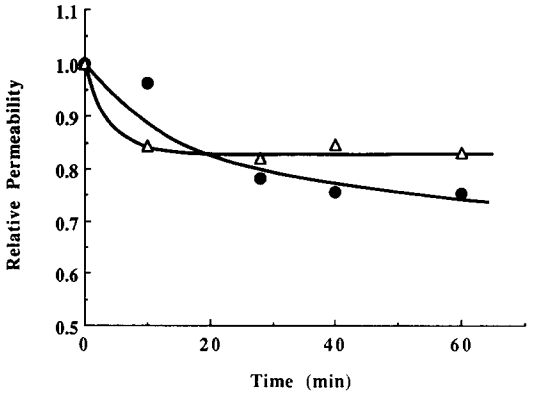

Fig. 5. Inhibition of TFA transport at 37°C in human erythroycte by pCMBS. The erythrocytes suspended (H_c approx. 0.1) in PBS (20 mM (pH 7.36)) were incubated at 37°C with pCMBS (1 mM) for various periods of time. A single exponential function with an ordinate-offset, $y = (1-a)\exp(-bx) + a$ (a, $(830 \pm 6.2)\cdot 10^{-3}$; b, $(2.9 \pm 1.2)\cdot 10^{-1}$ for efflux, and a, $(69 \pm 9.1)\cdot 10^{-2}$; b, $(3.3 \pm 2.17)\cdot 10^{-2}$ for influx, respectively) was fitted on to the data by non-linear least squares regression to obtain the relative membrane permeabilities at maximal inhibition, where y and x are the relative membrane permeability and time (min), respectively. (●) and (△) denote the relative membrane permeabilties for the equilibrium influx and efflux, respectively.

mM) at 37°C for 0.5 h resulted in complete inhibition of DFP transport. Under the same conditions of DIDS treatment, the inhibition of TFA transport was incomplete; the residual relative membrane permeabilities for influx and efflux were approx. 0.18 and approx. 0.19, respectively.

N-ethylmaleimide inhibition of TFA and DFP transport

Pre-incubating the erythrocytes (H_c approx. 0.40) in PBS (20 mM) at 37°C for approx. 1 h with NEM gave rise to a decrease in the membrane permeability for TFA (Fig. 4) that increased with an increase of the inhibitor concentration. In the presence of 7 mM NEM, the relative membrane permeabilities of TFA influx and efflux were 0.74 ± 0.05 and 0.83 ± 0.09, respectively.

NEM was a less potent inhibitor of DFP transport than α-cyano-4-hydroxycinnamate and stilbene disulfonates (Table IV). At low concentrations of NEM

TABLE II

Permeability coefficients of human erythrocytes for DFP (20 mM) at 37°C in the presence of α-cyano-4-hydroxycinnamate

Human erythrocyte suspensions (H_c 0.37–0.42) were pre-equilibrated with 20 mM DFP in phosphate-buffered (20 mM) isotonic sucrose solution before being incubated (37°C, approx. 10 min) with α-cyano-4-hydroxycinnamate of various concentrations, prior to measuring the exchange rate-constants. The rate constants for the membrane transport were determined at 37°C using ^{19}F-NMR and the modified 1D EXSY method [18,19]. P_1 and P_{-1} denote similar parameters to those of Table I. P_1/P_{1o} and P_{-1}/P_{-1o} denote the *relative* permeabilities for the influx and efflux, respectively; P_{1o} and P_{-1o} were the permeabilities obtained in the absence of transport inhibition. The results given without error estimates were from individual measurements. The standard deviations of the permeability coefficients were obtained from duplicate estimates. Those of the relative membrane permeability for influx were determined as $P_1/P_{1o}[(\sigma_{P_1}/P_1)^2 + (\sigma_{P_{1o}}/P_{1o})^2]^{1/2}$ [40], where σ_{P_1} and $\sigma_{P_{1o}}$ are the standard deviations of P_1 and P_{1o}, respectively. The values for efflux were determined similarly.

[α-Cyano-4-hydroxycinnamate] (μM)	P_1 (cm/s) ($\times 10^6$)	P_{-1} (cm/s) ($\times 10^6$)	P_1/P_{1o}	P_{-1}/P_{-1o}
0.0	12.3 ± 1.2	5.2 ± 0.9	1.0 ± 0.1	1.0 ± 0.3
5.0	6.2 ± 0.4	4.1	0.5 ± 0.1	0.80 ± 0.14
10.0	5.9 ± 0.1	4.3	0.48 ± 0.05	0.83 ± 0.15
20.0	5.6 ± 0.1	3.2	0.45 ± 0.04	0.62 ± 0.11
40.0	4.9 ± 0.2	4.1 ± 0.2	0.40 ± 0.04	0.80 ± 0.15
90.0	1.3 ± 0.3	2.5 ± 0.1	0.11 ± 0.03	0.49 ± 0.09
250	4.1 ± 0.6	3.3 ± 0.1	0.33 ± 0.06	0.65 ± 0.12
350	approx. 3.4	approx. 2.4	0.28 ± 0.03	0.47 ± 0.08
500	approx. 3.2	approx. 2.7	0.26 ± 0.02	0.53 ± 0.09

TABLE III

Permeability coefficients of TFA in the presence of DNDS

Human erythrocytes (in PBS (pH approx. 7.6)) were incubated at either 37°C or 21°C for approx. 10 min in the presence of DNDS (1 mM), prior to adding TFA (10 mM) to initiate the uptake. The human erythrocytes were from the same donors as for Table I. The transport of TFA was monitored using sequential ^{19}F-NMR spectra. The parameters P_1, P_{-1}, P_1/P_{1o} and P_{-1}/P_{-1o} are defined similarly to those of Table II. The values of P_{1o} and P_{-1o} used to calculate the relative membrane permeabilities are given in Table I. The standard deviations of the permeabilities and those of the relative membrane permeabilities were determined similarly to those of Tables I and II, respectively.

	P_1 (cm/s) ($\times 10^8$)	P_{-1} (cm/s) ($\times 10^8$)	P_1/P_{1o}	P_{-1}/P_{-1o}
37°C	3.3 ± 1.0	5.0 ± 1.1	0.02 ± 0.01	0.02 ± 0.01
21°C	3.3	approx. 3.2	approx. 0.28	approx. 0.20

(≤ 7 mM), no inhibition was evident. However, an increase of NEM concentration resulted in a significant decrease in the equilibrium-exchange rate of DFP, so that the extent of magnetisation transfer between the intra- and extracellular resonances did not allow reliable estimates of the exchange rate-constants.

pCMBS inhibition of TFA and DFP transport

The effect of pCMBS on TFA transport varied with the conditions of treatment of the cells with it. Incubating erythrocytes (37°C, approx. 40 min) at low haematocrit (approx. 0.1) with 1 mM pCMBS achieved the maximally attainable inhibition with this compound, although the inhibition was incomplete (Fig. 5). The membrane permeabilities of influx and efflux decreased to approx. 0.69 and approx. 0.83 of that determined without inhibition. An increase of haematocrit to 0.4–0.5 in the incubation led to a decrease in the extent of inhibition (data not shown).

Fig. 6A shows the steady decrease of the relative membrane permeability of DFP as a result of an increase of pCMBS concentration when the cells were treated with it at 37°C for approx. 10 min. The relative membrane permeability for influx and efflux decreased to 0.27–0.30 and 0.40–0.48, respectively, in the presence of pCMBS (≥ 5 mM), and this was the maximal inhibition achieved. Fig. 6B shows the decrease of the relative membrane permeability of TFA influx as a function of incubation time when the cells were treated with pCMBS (1 mM) at 37°C. Non-linear least squares regression of a single exponential function onto the data, with an ordinate-offset (see the legend of Fig. 6A), yielded the residual relative membrane permeability of 0.54 ± 0.12.

TFA diffusion through the lipid bilayer

Pre-treatment of the human erythrocytes with one of the inhibitors DNDS, DIDS, α-cyano-4-hydroxycinnamate, pCMBS, or NEM alone did not achieve complete inhibition of TFA transport. However, when both DNDS (1 mM) and α-cyano-4-hydroxycinnamate (4 mM) were present in the incubation medium (25°C, approx. 10 min), the uptake of TFA was negligible. In other experiments the cells (H_c approx. 0.4) in PBS were pre-incubated (37°C, 10 min) in the presence of α-cyano-4-hydroxycinnamate (≥ 4 mM), followed by further incubation with 0.5 mM DIDS (10 min); ^{19}F-NMR spectra acquired after addition of TFA to the suspensions showed no evidence of intracellular TFA, thus showing a complete inhibition of the transport pathways. Addition of butanol, up to 50 mM to cells (H_c 0.36–0.42) that had been pre-treated with both inhibitors, did not lead to detectable TFA uptake. However, a significant increase in the uptake was found

TABLE IV

Permeability coefficient of DFP at 37°C in the presence of NEM

Human erythrocytes were suspended in PBS (20 mM (pH 7.3), H_c 0.34–0.41) followed by incubation at 37°C (approx. 60 min) with various concentrations of NEM. The cells were then washed in about 3 volumes of ice-cold PBS to remove NEM that had not reacted with the cells. The cells were further incubated in sucrose solution (pH 7.3) containing DFP (20 mM), prior to measuring the exchange rate-constants using ^{19}F-NMR and a modified 1D EXSY method [18,19]. The parameters P_1, P_{-1}, P_1/P_{1o} and P_{-1}/P_{-1o} have the same meanings as those in Table II and were calculated as described in Materials and Methods. The standard deviations of the permeability coefficients were obtained from four estimates. Those of the relative membrane permeabilities were determined similarly to those of Table II.

[NEM] (mM)	P_1 (cm/s) ($\times 10^6$)	P_{-1} (cm/s) ($\times 10^6$)	P_1/P_{1o}	P_{-1}/P_{-1o}
0.0	5.9 ± 0.8	4.3 ± 0.2	1.0 ± 0.2	1.0 ± 0.1
5.0	7.5 ± 0.8	5.8 ± 1.1	1.3 ± 0.3	1.3 ± 0.2
7.0	6.2 ± 0.3	4.5 ± 0.4	1.1 ± 0.2	1.0 ± 0.1

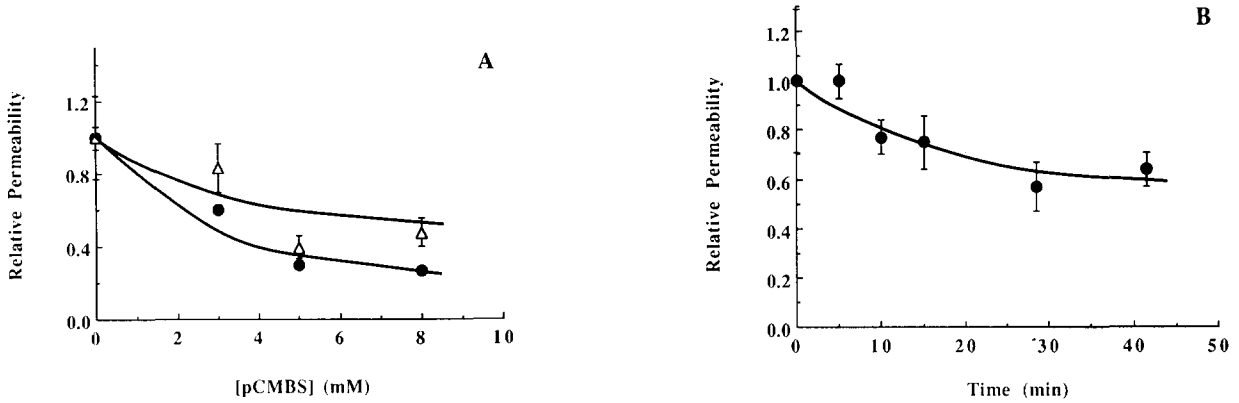

Fig. 6. (A) The pCMBS-concentration-dependent inhibition of DFP transport in human erythrocyte at 37°C. The cells pre-equilibrated in phosphate-buffered (20 mM (pH 7.51)) sucrose solution containing DFP (20 mM) were further incubated (37°C, 10 min) with pCMBS of various concentrations. The unreacted pCMBS was not removed from the suspensions (H_c 0.38–0.46), prior to ^{19}F-NMR measurement DFP transport. (●) and (△) denote the relative membrane permeability for influx and efflux, respectively. The curves were fitted empirically. The error bars denote standard deviations calculated using the method given in Table II. (B) relative membrane permeability of DFP influx (37°C) measured in human erythrocytes pre-incubated (37°C) with pCMBS (1 mM) for various periods of time. The unreacted inhibitor was removed by repeated (3-times) centrifugal washing of the cells in ice-cold inhibitor-free medium, prior to the ^{19}F-NMR experiments. The rate constants of DFP transport were determined using the modified 1D EXSY method. Non-linear least squares regression of a single exponential function with an ordinate-offset, $y = (1 - a)\exp(-bx) + a$ (a, $(5.4 \pm 1.2) \cdot 10^{-1}$; b, $(5.3 \pm 3.5) \cdot 10^{-2}$) onto the data yielded the relative membrane permeability at maximal inhibition, where y and x denote the relative membrane permeability and time (min) of incubation, respectively.

to be linearly correlated with further increases of butanol concentration ($\geqq$ 140 mM). This was accompanied by haemolysis, indicating that the membranes had become leaky.

Discussion

London and Gabel [10] first reported a substantial, but incomplete, inhibition of TFA uptake when human erythrocytes were pre-treated with the anion-transport inhibitor SITS. It was therefore suggested that TFA entered the cells via band-3 and simple diffusion via the lipid bilayer. This suggestion was based on the implicit assumption that stilbene disulfonates do not inhibit other possible protein-mediated pathways. Studies by Deuticke et al. [20], Jennings and Adams-Lackey [21] and more recently by Poole and Halestrap [6] have all shown that stilbene disulfonates bind to membrane proteins that are responsible for the transport of lactate and pyruvate in human, rabbit and rat erythrocytes. These findings raised questions on the specificity of the inhibitors, which for a long time have been considered to be specific to inhibition of band-3 [22,23]. In the present work, we differentiated between the effects on the rate of transport of TFA by the inhibitors, α-cyano-4-hydroxycinnamate, NEM and stilbene disulfonates to identify the membrane pathways of TFA transport.

Inhibition by α-cyano-4-hydroxycinnamate

α-cyano-4-hydroxycinnamate competes with lactate and pyruvate in binding to the monocarboxylate transporter: in human erythrocytes the K_m for lactate influx is 9.14 ± 0.53 mM and the K_i for the cinnamate is 0.064 ± 0.006 mM [24]. Small relative membrane permeabilities of 0.04 ± 0.03 and 0.05 ± 0.02 for TFA influx and efflux were obtained in the presence of saturating amounts of the inhibitor ($\geqq$ 4 mM). The value of the relative membrane permeability for influx was the same order of magnitude as that obtained in the presence of 1 mM DNDS (0.01) that inhibited DFP exchange completely.

α-cyano-4-hydroxycinnamate is claimed to be a specific inhibitor of lactate and pyruvate transport in mitochondria [1]. However, it also inhibits anion transport mediated by band-3 of human, rat and rabbit erythrocytes [4], but little is known about the exact mechanism of its association with band-3.

In the present work an effect of α-cyano-4-hydroxycinnamate on band-3 was demonstrated by the partial inhibition of the rapid equilibrium exchange of DFP which is known to exchange via band-3 [19]. The maximal extent of inhibition left the relative membrane permeabilities for DFP influx and efflux of 0.23 ± 0.05 and 0.55 ± 0.06, respectively (Table II). The inhibition with α-cyano-4-hydroxycinnamate concentration $\geqq$ 500 μM resulted in the decrease of ^{19}F-NMR magnetisation transfer to an amount which became too small to allow reliable estimates of the values of the exchange rate-constants for DFP. In view of the simultaneous inhibition by α-cyano-4-hydroxycinnamate of band-3, the much greater decrease of the relative membrane permeability for TFA pointed to the involvement of an additional transporter, namely the monocarboxylate transporter, in mediating the transmembrane exchange of TFA.

Inhibition by stilbene disulfonates

In agreement with the findings of Deuticke et al. [20], on the inhibition of lactate and pyruvate transport in human erythrocyte by H_2DIDS, Jennings and Adams-Lackey [21] reported the concentration-dependent inhibition of the monocarboxylate transporter at higher inhibitor concentrations than those which were required for band-3 inhibition of rabbit erythrocytes. Polyacrylamide gel electrophoresis showed a protein band of approx. 43 kDa as the major H_2DIDS-labelled protein; it was therefore identified as the putative monocarboxylate transporter. The gel-band of approx. 43 kDa from human erythrocytes was only weakly labelled by H_2DIDS, but this still suggested that the monocarboxylate transporter in these cells might be inhibited by it. More recently, Poole and Halestrap [8] presented evidence of inhibition of the monocarboxylate transporters of rabbit, rat and guinea-pig erythrocytes by DIDS by measuring the binding of anti-DIDS antibody to the transporter; their results led to the identification of the transport protein in these cells.

Consistent with the previous study, in the present work DNDS was found to be a potent but incomplete inhibitor of TFA uptake. Pre-incubation of the cells with 1 mM DNDS at 37°C led to significantly smaller residual relative membrane permeabilities than that of cells treated at 21°C (Table III). Complete inhibition of band-3 can be achieved by treating erythrocytes with even lower concentrations of stilbene disulfonates [25]; Poole and Halestrap [8] reported complete inhibition of band-3 using only 10 μM DIDS at 10°C. Our conditions of DNDS treatment (1 mM at 21°C or 37°C) were thus expected to inhibit band-3 completely. At 7°C the apparent K_i of DNDS inhibition of lactate transport, which is predominantly mediated by the monocarboxylate transporter, is approx. 1.12 mM [5,20]. This reveals a much more potent inhibition of band-3 than of the monocarboxylate transporter by the reagent. Thus a higher residual TFA transport, when the cells were treated with DNDS at lower temperature (21°C) is able to be attributed to the lesser extent of inhibition of the monocarboxylate transporter. This is also supported by the observation of the incomplete inhibition of TFA transport (relative membrane permeabilities approx. 0.18 and approx. 0.19 of equilibrium influx and efflux, respectively) when the cells were treated with 0.5 mM DIDS (37°C, 0.5 h) alone, and the complete 'blockage' when the erythrocytes were first treated with α-cyano-4-hydroxycinnamate ($\geq$ 4 mM, 37°C, approx. 10 min) and then incubated with DIDS (0.5 mM, 37°C, approx. 10 min).

Diffusion of TFA via the lipid bilayer

Pre-treatment of the human erythrocytes with $\geq$ 4 mM α-cyano-4-hydroxycinnamate and stilbene disulfonates achieved complete inhibition of TFA uptake. In view of the incomplete inhibition of monocarboxylate transporter by stilbene disulfonates alone, and that of band-3 by α-cyano-4-hydroxycinnamate alone, the residual uptake of TFA reported by London and Gabel [10] may be attributed to the incomplete inhibition of the monocarboxylate transporter by SITS alone. Butanol is known to increase membrane fluidity and, thus, increase simple diffusion via the lipid bilayer [26]. The lack of change of the membrane permeability to TFA when the cells were pre-treated with up to 140 mM butanol indicates that under our conditions negligible amounts of TFA enter via the lipid bilayer. With higher concentrations of butanol ($\geq$ 140 mM), the membranes became permeable to TFA, but the cells haemolysed, thus indicating a change of the membrane structure. Therefore, if it is accepted that no simple lipid-mediated diffusion occurs with TFA, then the residual TFA transport (approx. 0.18 and approx. 0.19 for influx and efflux, respectively) observed after complete inhibition of band-3 (as assessed by its effect on DFP) is attributed to the monocarboxylate transporter.

Inhibition by sulfhydryl reagents

Covalent association of NEM with sulfhydryl groups in the membrane leads to marked inhibition of pyruvate transport [24]. NEM has also been shown to bind to the sulfhydryl groups on band-3 of human erythrocytes, 'overlapping' with the binding site(s) of DIDS [27,28]. The association with band-3 is accompanied by the inhibition of transmembrane exchange of Cl^-. Also, the inhibition of water transport in erythrocytes by NEM has led to the suggestion that band-3 is an important route for water diffusion [29–31]. We observed marked inhibition of TFA transport while there was no inhibition of DFP transport. Thus, the residual relative membrane permeability (0.74 ± 0.05 and 0.83 ± 0.09 for TFA influx and efflux, respectively) represents the contribution from band-3 to the overall transport of TFA. This leaves a relatively small fraction (0.25 ± 0.02 and 0.17 ± 0.02 for influx and efflux, respectively) as being via the monocarboxylate transporter. The TFA transport via the monocarboxylate transporter might be partially responsible for the apparent difference of membrane permeabilities of human and sheep erythrocytes observed without transport inhibition (Table I).

Deuticke [4] has reviewed the data on monocarboxylate transporter-mediated transport of a series of acetate derivatives, and concludes that mono- and di-substitution of α-H by halogen atoms enhances the transport, whereas tri-substitution (e.g., TFA) gives rise to a reduced rate of transport. In view of the smaller contribution of the monocarboxylate transporter to the overall flux of TFA (than to that of lactate and pyruvate [20]), our results are consistent with the structural requirements of the substrates noted by Deuticke [4].

We observed significant changes of MCV of the cells treated with the pCMBS; the volumes of the cells used in TFA transport measurements (Fig. 5) were 0.15–0.30-times greater than the normal value (approx. $8.4 \cdot 10^{-18}$ m^3) [32], whereas those of the cells used in DFP transport were 0.15–0.27-times smaller than the normal value. The volumes of the cells used as control also differed from normal values; they were approx. 0.18-times greater in the TFA transport measurement, and 0.1–0.2-times smaller in the DFP transport measurement. The cells used in TFA transport measurements were suspended in slightly hypotonic medium (osmolality approx. 280 mosmol/kg), whereas those used to measure DFP exchange were in the hypertonic sucrose (osmolalities ranged from 310 to 380 mosmol/kg). The change of the mean cell volumes may thus be attributed to variation of the osmolality of the medium. However, the possible ion and metabolite leakage caused by pCMBS treatment can no be excluded.

The interpretation of the pCMBS inhibition of TFA transport is complicated largely by the 'broad spectrum' of inhibition of a number of transport pathways by this reagent. In this study, pCMBS inhibited DFP transport via band-3; pCMBS reacts with the six sulfhydryl groups of band-3 of erythrocyte ghosts [33–35], whereas NEM only reacts with five of the six groups and does not react with the one that is located on the 17-kDa fragment [27,34]. This band-3 fragment links the membrane domain to the cytoplasmic domain, and is known to be involved in the translocation of anions [23,36,37]. Binding of pCMBS to this suflhydryl group is suggested to cause a conformational change of band-3, and thus is partly responsible for the inhibition of anion transport.

In conclusion, by differentiating the inhibition of band-3 from that of the monocarboxylate transporter, it is concluded that the transmembrane exchange of TFA is mediated primarily by band-3, but a significant fraction of the exchange was via the monocarboxylate transporter. Under the conditions used, transport by simple diffusion via the lipid phase of the bilayer is negligible. A further general conclusion is that ^{19}F-NMR provides a valuable method for the facile measurement of transport in erythrocytes of solutes that show the 'split peak' phenomenon, such as occurs with a range of fluorinated compounds [9,38,39].

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