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Fluoride transmembrane exchange in human erythrocytes measured with ^{19}F NMR magnetization transfer

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Abstract. ^{19}F NMR spectra of sodium fluoride in suspensions of human erythrocytes were seen to yield separate resonances for the F^- populations inside and outside the cells. Selective saturation of the magnetization of the intracellular population gave rise to transfer of that saturation to the extracellular population. The extent of magnetization transfer was high and it was blocked by the capnophorin (band 3) anion exchange inhibitor 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS). A series of magnetization-inversion transfer experiments was carried out for the range of intracellular fluoride concentrations of 11 m*M* to 136 m*M* and analysed using one-dimensional 'overdetermined' exchange analysis. This yielded an estimate of the equilibrium exchange Michaelis constant and maximal velocity of 27 ± 3 m*M* and $180 \pm 5 \times 10^{-16}$ mol cell^{-1} s^{-1}, respectively. There was no alteration of exchange flux of fluoride at an intracellular concentration of 49 m*M* in the presence of 50 m*M* glucose; thus suggesting no interaction between glucose and anions in capnophorin-mediated exchange of solutes.

Key words: Erythrocyte – F^- exchange – ^{19}F NMR

Introduction

The exchange of chloride and bicarbonate ions across the erythrocyte membrane is of extreme importance in respiration. The rates of exchange are high with intra-erythrocyte residence life times of the ions being much less than 1 s. The exchange occurs via the membrane protein capnophorin. The kinetics of the exchange of chloride have been relatively well characterised (e.g. Dalmark 1975; Brahm 1977); but those of the chemically related fluoride ion have not been extensively studied. And yet, the ^{19}F nucleus is the second most NMR-receptive stable nucleus, so we set out to assess if $^{19}F^-$ would be a useful probe of capnophorin function.

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Previous investigators may have been deterred from using F^- as a probe of Cl^- exchange because of its known inhibition of glycolysis, principally via inhibition of enolase. Nevertheles, we demonstrated during the present work that, even in relatively high concentrations (~ 150 m*M*) of NaF, human erythrocytes maintain their integrity for many hours. The ^{19}F NMR spectrum of F^- in a suspension of human erythrocytes contains two resonances, one each from the intra- and extra-cellular populations of the ion. This observation enabled the application of NMR magnetization transfer techniques to measure the rates of transmembrane exchange that occur under conditions of thermodynamic equilibrium (e.g., Kuchel 1990).

The above mentioned NMR 'split-peak' phenomenon has been observed for a series of phosphoryl compounds (e.g., Kirk and Kuchel 1988) and more recently some fluorinated compounds (London and Gabel 1989; Potts et al. 1990; Xu et al. 1990). There do not appear to be any reports of the observation of resonances from intracellular $^{19}F^-$ in any cell type (Selinsky et al. 1988). We surmise that their observation in the present work is a result of our use of much higher concentrations of the ion than may have been used in previous studies. It is also evident that the intracellular resonance is very broad, but in spite of some experimental drawbacks which this causes it was possible to characterise the Michaelis-Menten parameters of fluoride efflux. In addition, since the exchange was inhibited by a stilbene reagent we deduced that it occurs via capnophorin.

Experimental

4,4′-Dinitrostilbene-2,2′-disulfonic acid (DNDS) was obtained from Pfaltz and Bauer, Inc. (Waterbury, CT, 06708, USA). All other compounds were of Analytical Reagent grade. Freshly drawn venous blood, from the one donor (BEC), was washed twice in ice-cold isotonic saline. For the final series of experiments, fluoride-containing solutions contained 10 m*M* phosphate, 1 m*M* ethylenediamine tetra-acetic acid, sodium fluoride 10–230 m*M* and

various amounts of sucrose to adjust the osmolality of the solutions to 401 ± 5 mOsm/kg. The solutions were adjusted to pH 7.2. One ml of erythrocytes was incubated with 12 ml of washing solution at 37 °C for 30 min before centrifugation. The procedure was repeated three times to allow for equilibration of fluoride and efflux of chloride (Jennings 1982). Half an ml of cell suspension, haematocrit ~0.8, was pipetted into 5 mm o.d. NMR tubes. For the initial saturation transfer experiments (see text) the above procedure was followed for a washing solution containing 40 m*M* sodium fluoride, except that the osmolality was adjusted with sucrose to 300 mOsm/kg and the haematocrit of the cell suspension was 0.94. Capnophorin was inhibited by addition of 0.5 mg of DNDS to the cell suspension in the NMR tube. Osmolalities were measured using a Wescor vapour pressure osmometer (Wescor Inc. Logan, UT 84321 USA). Cell counts were obtained on a Sysmex Microcellcounter CC-130 (Toa Medical Electronics Co., Kobe, Japan) and haematocrits were measured using a Clements microhaematocrit centrifuge (H. J. Clements Pty. Ltd. North Ryde, NSW, Australia).

^{19}F NMR spectra were obtained at 37 °C on a Varian XL/VXR-400 NMR spectrometer operating at 376.47 MHz (9.4 tesla) in the Fourier mode. Initial saturation transfer measurements were carried out using the DANTE pulse sequence (Morris and Freeman 1978) on one sample with a spectral width of 3000 Hz and averaging 8 transients: an interpulse delay of 333 μs was used. The procedure for obtaining data for the 'overdetermined' one-dimensional exchange analysis was based on the two-dimensional EXSY pulse sequence (Engler et al. 1988) with non-selective $\frac{\pi}{2}$ pulses and the appropriate phase cycling (with phases denoted by the subscripts): $\frac{\pi}{2_x} - t_1 - \frac{\pi}{2_x} - t_m - \frac{\pi}{2_{x,y,-x,-y}}$ – acquire (t_2), where t_1 is the evolution time, t_m is the mixing time, and t_2 the acquisition time. A series of spectra was acquired with different evolution times varying from 0 to $1/2\Delta\nu$: the increments were $1/8\Delta\nu$, where $\Delta\nu$ is the frequency difference in Hz between the peaks of the exchanging species. Also, the mixing times between the second and third pulse were either 0.0 and 0.06 s. Typically 64–128 transients were averaged into 16 K data points with a spectral width of 5000 Hz. Because the 'dead-time' of the receiver, which is the time between the last $\frac{\pi}{2}$ pulse and the start of data acquisition, had a value of 179 μs, there was a significant loss of signal intensity for broad peaks. Therefore, peak integrals were corrected by using the relationships $T_2^* = 1/\pi\nu_{1/2}$, where T_2^* is the apparent transverse relaxation time, $\nu_{1/2}$ is the peak width in Hz at half-height, and $I_t = I_0 \exp(-t/T_2^*)$; where I_t is the measured integral, I_0 the corrected integral and t the receiver dead-time. The corrected intensities of the spectral peaks, together with those from a "fully relaxed" spectrum, were then used to form the so called exchange matrix, which was analysed by the 'overdetermined' method of Bulliman et al. (1989); this method has the virtue of yielding estimates of the standard deviations of the computed exchange rate-constants.

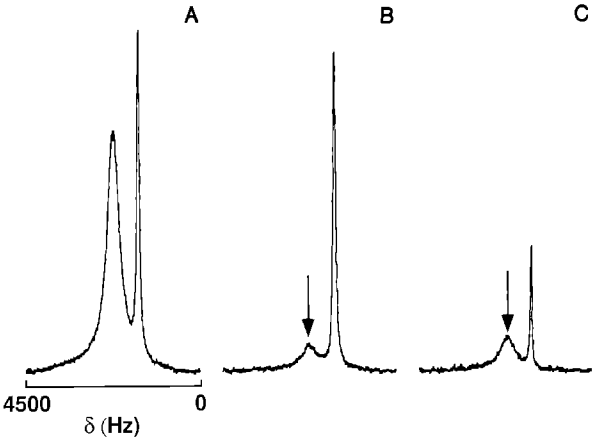

Fig. 1 A–C. ^{19}F NMR spectra obtained from human erythrocytes in 40 m*M* NaF at 37 °C. The sharp low frequency resonance and broad high frequency resonance are from the extra- and intra-cellular fluoride populations, respectively. **A** "Fully relaxed" control spectrum with no selective irradiation. **B** Spectrum obtained with selective irradiation applied at the frequency indicated by the arrow, namely that of the intracellular fluoride nuclear population, in the presence of the capnophorin inhibitor DNDS. **C** Selective irradiation at the frequency indicated by the arrow, of the intracellular fluoride nuclear population in the absence of inhibition of capnophorin

Non-linear regression analysis, such as that for fitting the function-line to the data in Fig. 4, was carried out using a previously published procedure (e.g., Potts et al. 1990).

Results and discussion

Erythrocytes washed in fluoride-containing medium gave rise to ^{19}F NMR spectra consisting of two peaks with maximum ordinates separated by ~650 Hz; there was a broad intra-cellular peak and a sharp extra-cellular peak at lower frequency (Fig. 1 A). The ionic species giving rise to these peaks are in slow exchange across the erythrocyte membrane. This may be assessed qualitatively from a saturation transfer experiment. Saturation of the magnetization of the broad intracellular peak, by using a DANTE pulse sequence, gave rise to decreased intensity of the narrow peak (Fig. 1 C) compared with the control experiment (Fig. 1 A). Inhibition of capnophorin with DNDS prevented this saturation transfer as shown in Fig. 1 B. The greater extent of saturation of the intracellular peak, compared with that in Fig. 1 C, is presumably due to removal of the exchange contribution to the relaxation from the extracellular peak.

Because of the short longitudinal relaxation time of the magnetization of the intracellular nuclei (e.g., 0.036 s at a 42 m*M* intracelluar fluoride concentration) it was *impossible* to saturate totally the intracellular magnetization without significant off-resonance saturation of the extracellular magnetization. Thus quantitative measurement of the equilibrium exchange rate constants could not be determined by using this technique.

The broadness of the intracellular peak is attributed to the fluoride ions being in rapid exchange, on the *NMR*

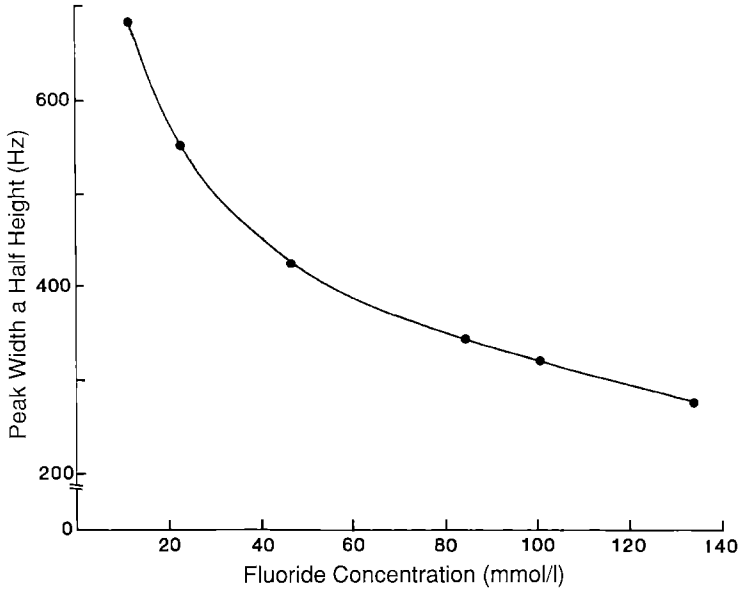

Fig. 2. The dependence of the line width at half height from the intracellular fluoride nuclei, as a function of concentration, in ^{19}F NMR spectra of suspensions of human erythrocytes. Other details are given in the Experimental section

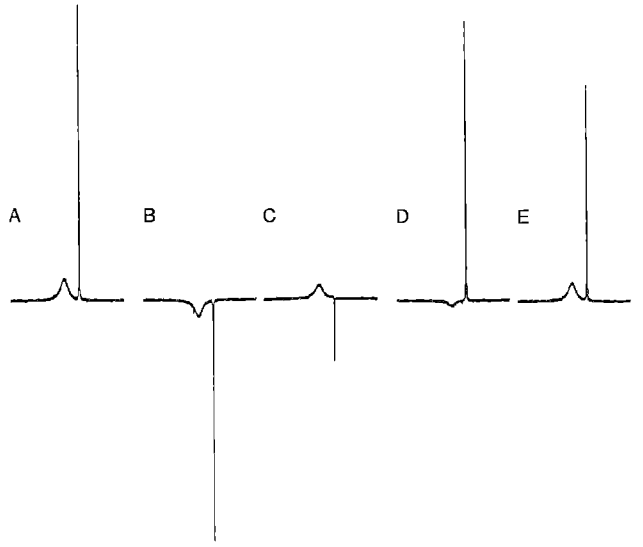

Fig. 3A–E. Five of the 11 spectra obtained for the one-dimensional 'overdetermined' exchange analysis of human erythrocytes containing 46 m*M* intracellular fluoride. **A** The 'fully relaxed' equilibrium-magnetization spectrum; **B** The spectrum obtained with the inversion-transfer pulse sequence using an evolution time of 0.0 s and a mixing time of 0.0 s. **C** Same as **B** except with a mixing time of 0.06 s. **D** The evolution time was $1/2\Delta\nu$ (769 μs) with a mixing time of 0.0 s. **E** The evolution time was the same as for the previous spectrum but the mixing time was 0.06 s

time-scale, amongst unbound fluoride and several intracellular binding sites in which the ions have different rotational correlation times. Evidence for this may be obtained from Fig. 2, which is a graph of peak-width at half-height versus intracellular fluoride concentration. The interpretation is that, as the fluoride concentration increases the proportion of unbound intracellular fluoride also increases as the result of saturation of the available binding sites. The peak-width of unbound intracellular fluoride should be comparable to the extracellular fluoride peak-width because the erythrocyte micro-viscosity is only approximately twice that of the extracellular medium (Endre et al. 1983). Rapid exchange of the intracellular species between different binding sites may be inferred from the inability to "hole-burn" the intra-cellular signal, using the DANTE pulse sequence (Morris and Freeman 1978); this shows that the intracellular signal behaves as a single 'complex' resonance rather than the superposition of several 'simple' resonances.

Quantitative measurements of the fluoride equilibrium efflux rate constants were obtained using the 'overdetermined' (Bulliman et al. 1989) one-dimensional NMR spin-exchange experiment (Engler et al. 1988). An example of the spectra obtained with an intracellular fluoride concentration of 46 m*M* is given in Fig. 3. The equilibrium ("fully relaxed") spectrum is shown in Fig. 3A. Figure 3D, E shows spectra obtained for a delay, between the first and second pulse, of $1/2\Delta\nu$ and mixing times of 0.0 and 0.06 s, respectively. Evidence of inversion transfer from the intracellular to the extracellular peak may be seen from the relative diminution of the extracellular peak in Fig. 3E compared with Fig. 3D.

In normal human erythrocytes the intracellular chloride concentration is ~70 m*M* (Henry et al. 1974). Limited washing of erythrocytes in a medium with a fluoride concentration that is lower than this does not result in total substitution of fluoride for chloride. However, incubation of erythrocytes at 37 °C in a hypertonic medium results in uni-directional efflux of chloride as potassium chloride. The efflux is associated with a decrease in cell volume that is a consequence of the transmembrane osmotic equilibrium. The cell volume, determined from the cell count and haematocrit, varied monotonically from 72–80 fl for fluoride concentrations varying from 10–230 m*M* in the washing medium. The volume of these cells in isotonic medium was 85 fl. Using the factor 0.717 (Raftos et al. 1990) as the volume-fraction of water in human erythrocytes in isotonic medium, a value of 24 fl was obtained for the volume of cell 'solids'; this value is assumed, to a first approximation, to remain constant

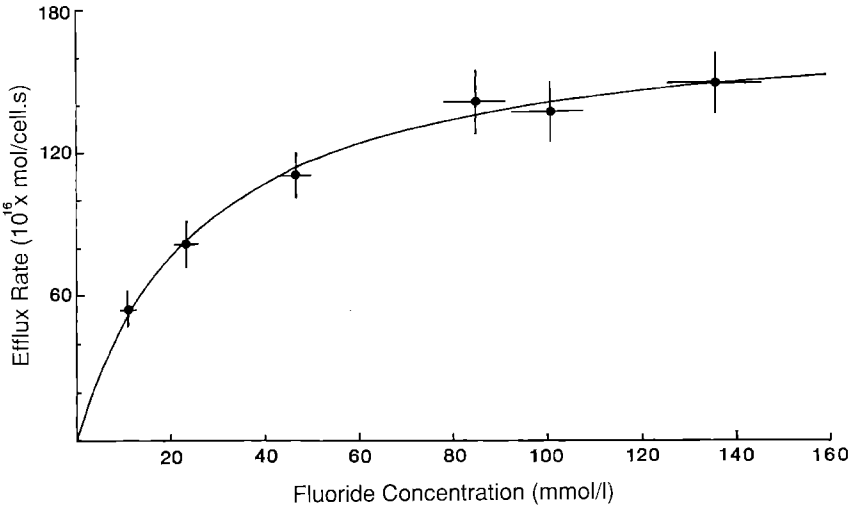

Fig. 4. Michaelis-Menten-type plot of rate of fluoride efflux under equilibrium exchange conditions in human erythrocytes as a function of the intracellular fluoride concentration. The rates of efflux were obtained from 'overdetermined' equilibrium exchange analysis as illustrated in Fig. 3 with 6 different intra-cellular fluoride concentrations. The values for the error bars were computed as described in the Results and discussion

with changes in total cell volume, thus enabling the water volume of the cells to be calculated. As the haematocrit of the cell suspension is known, the relative volume of the intra- and extra-cellular compartments can be determined. Thus, combining the ratio of the two compartment volumes with the integral ratio of an equilibrium spectrum, and the known extracellular fluoride concentration, allowed an estimate of the free intra-cellular fluoride concentration. This is known to be less than the total intracellular fluoride concentration as some fluoride binds irreversibly to intracellular components, such as calcium, and does not take part in exchange (Tosteson 1959). The intra- to extra-celluar fluoride concentration ratio was 0.99 ± 0.2.

It has been shown that the self-exchange flux of ions is independent of cell volume in erythrocytes (Dalmark 1975) and erythrocyte ghosts (Funder and Weith 1976), and the flux depends only on the concentration of the ions and the number of transporters in each cell. Flux parameters are thus given in terms of the units 'per cell' or 'per kg of cell solids', 1 kg being equivalent to 3.1×10^{13} cells (Brahm 1977). The rate of efflux of fluoride that occurred as a function of intra-cellular fluoride concentration is shown in Fig. 4. The exchange kinetics can be described by the Michaelis-Menten equation with a K_m of 27 ± 3 m*M* and a V_{max} of $180 \pm 5 \times 10^{-16}$ mol cell^{-1} s^{-1}.

An example of the error analysis used for Fig. 4 is given below for the sample with 46.3 m*M* intracellular fluoride: The cell volume was determined to be 74.9 ± 0.3 fl (average of 10 measurements); the water volume was thus 50.9 ± 0.3 fl. The coefficient of variation of the measured NMR peak integral was assumed to be the same as the fractional root mean square error of a peak height, obtained from the measurement of the signal-to-noise ratio (Kuchel et al. 1988). Combining the volume and integral errors gave an estimate of intracellular fluoride as 46.3 ± 3.5 m*M*, which for this point is represented by the horizontal error bar in Fig. 4. The 1-D spin exchange experiment yields the first-order rate constants for fluoride self-exchange. Theoretically these may be determined from any combination of two of the five pairs of spectra with different delays between the first and second $\frac{\pi}{2}$ pulses. There are 10 different possible combinations. The 'overdetermined' method of analysis may be regarded as giving a mean value, obtained from 10 measurements, of these first-order rate constants and thus a standard deviation is also obtained. For the present sample the mean value was determined to be 4.68 ± 0.02 s^{-1}. Combining this with the errors in cell volume and concentration gave an efflux rate of $101.3 \pm 9.5 \times 10^{-16}$ mol cell^{-1} s^{-1}. The error in this estimate is represented by the vertical bar in Fig. 4.

The maximal velocity of chloride self-exchange, determined using radioactive isotope exchange, at 38 °C is 670×10^{-16} mol cell^{-1} s^{-1} (1.25×10^2 mol kg^{-1} min^{-1}; Brahm 1977). Thus the fluoride transport rate, determined here, is 0.26 times the rate of chloride at approximately the same temperature. Tosteson (1959), from a single experiment using a radioactive tracer method with a medium containing 127 m*M* fluoride, obtained an efflux rate of 32.9×10^{-16} mol cell^{-1} s^{-1} (2300×10^{-12} mol cm^{-2} s^{-1}; area of erythrocyte 1.43×10^{-6} cm^2) at 23 °C. For chloride efflux a value of 131×10^{-10} mol cm^{-2} s^{-1} was obtained at the same temperature thus giving a fluoride/chloride efflux-rate ratio of 0.18, a result which is comparable to that obtained at 37 °C in the present work.

It has been suggested that capnophorin is also the major glucose transporter in the human red cell and that the glucose transport activity of band 4.5 is attributable to a capnophorin component (Langdon and Holman 1988; May 1987). To test if glucose and anion transport occur via the same site, the fluoride efflux rate was measured in the presence of 50 m*M* glucose: a value of 108×10^{-16} mol cell^{-1} s^{-1} was obtained with 49 m*M* intracellular fluoride. From Fig. 4, a value of $116 \pm 15 \times 10^{-16}$ mol cell^{-1} s^{-1} would be expected if no competition existed between the two solutes. The result suggests that if capnophorin is the transporter for anions and glucose the transport channels are separate.

In conclusion, fluoride exchange across the human erythrocyte membrane is rapid, but it can be measured

directly by using magnetization transfer methods, and these procedures may prove useful in probing the molecular mechanism of capnophorin.

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