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SHORT COMMUNICATION

HAGFISH (*EPTATRETUS STOUTI*) ERYTHROCYTES SHOW MINIMAL CHLORIDE TRANSPORT ACTIVITY

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Capnophorin (Band 3) is the major red cell transport protein, present in human erythrocyte membranes at 1×10^6 copies per cell. Under physiological conditions, the transporter is capable of moving $50\,mol\,(Cl^-/HCO_3^-)\,l\,cells^{-1}\,min^{-1}$ (Knauf, 1979), this high rate being necessary for carriage of CO_2 in normal respiration (Wieth *et al.* 1982). In the present paper we demonstrate that, in contrast to the situation in all other vertebrate species studied except the lamprey (Ohnishi & Asai, 1985), capnophorin activity in hagfish red cells is very limited, amounting to only $40\,\mu mol\,Cl^-$ transported $l\,cells^{-1}\,min^{-1}$ at 11 °C, the environmental temperature of this species. Five experimental approaches were used to characterize this transporter in hagfish red cells: pH regulation in lightly buffered medium; $^{36}Cl^-$ uptake; $^{36}Cl^-$ efflux; effects of H_2DIDS (dihydro-4,4-diisothiocyanostilbene 2,2-disulphonic acid), a specific capnophorin inhibitor (Knauf, 1979); and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE).

Hagfish (*Eptatretus stouti* Lockington) were trapped at 90–110 fathoms (=165–201 m) in Trevor Channel, Bamfield, British Columbia, and maintained unfed in running sea water until bled from the subcutaneous sinus into heparin. Flounders (*Parophrys vetulus* Girard) were caught by trawling in Trevor Channel at 70 fathoms (=128 m). Hagfish red cells were washed three times in a medium containing (in $mmol\,l^{-1}$) NaCl, 500; KCl, 10; glucose, 5·5; Mops, 10 (pH 7·5 at 11 °C). The buffy coat was discarded. The medium for flounder erythrocytes was similar, but contained $150\,mmol\,l^{-1}$ NaCl. Washed cells were stored overnight at 1 °C and used within 48 h of collection. pH experiments were performed at 1 °C in lightly buffered

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(1·5 *vs* 10 mmol l^{-1} MOPS (3-[*N*-morpholino]propane sulphonic acid)) modified medium. Cells at a haematocrit of 40 % were equilibrated at pH 7·3 and KOH (0·4 μequiv ml^{-1}) was added externally with rapid mixing. Extracellular pH was monitored *via* a Radiometer pH electrode system M64/EK 2401C. Influxes of $^{36}Cl^-$ (1 μCi ml^{-1}) at 1 °C and 11 °C were measured in triplicate initially by a phthalate separation system and subsequently by washing cell pellets four times in ice-cold medium (Young & Ellory, 1982). Efflux was measured by loading the cells for 24 h at 11 °C with $^{36}Cl^-$ (2 μCi ml^{-1}) followed by washing on ice and incubation at 11 °C, four duplicate samples being taken at 1-h intervals. The rate constant for $^{36}Cl^-$ efflux was calculated by non-linear curve fitting of counts appearing in the supernatant *vs* time. H_2DIDS was purchased from Molecular Probes Inc., Junction City, Oregon, USA, and was dissolved (20 mmol l^{-1}) in dimethyl sulphoxide (DMSO) and subsequently diluted 1000-fold in medium for use at a final concentration of 20 μmol l^{-1}. $^{36}Cl^-$ was obtained from Amersham International, Oakville, Ontario, Canada.

Fig. 1 compares the effects of an extracellular alkaline challenge in hagfish and flounder red cells, monitoring external pH over 10 min. It is clear that the flounder red cells showed the expected buffering of added alkali *via* Cl^-/OH^- exchange (ΔpH is only 0·08), a response which was substantially inhibited by H_2DIDS (maximum $\Delta pH = 0{\cdot}42$). In contrast, hagfish red cells showed a very limited

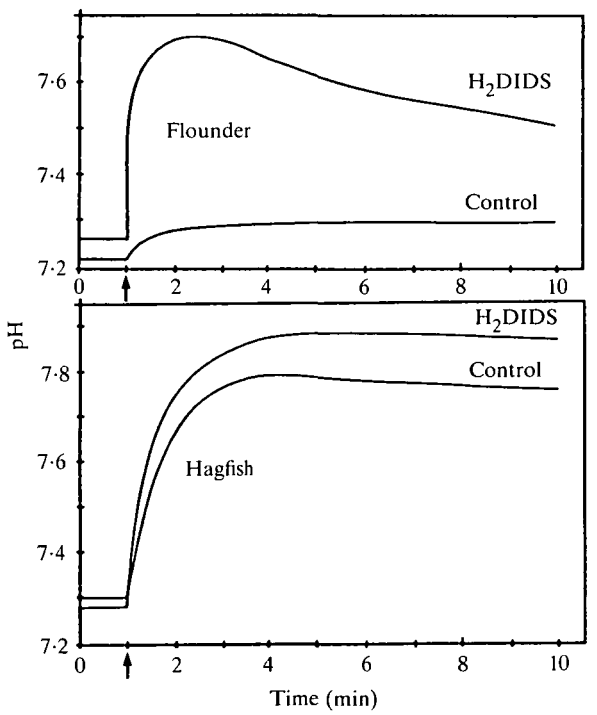

Fig. 1. Responses of hagfish and flounder red cells to an alkaline challenge. Arrows indicate the addition of KOH (0·4 μequiv ml^{-1}). The temperature was 1 °C. Other experimental details are given in the text.

buffering capacity and a modest H_2DIDS effect. Thus, in control hagfish red cells the maximum ΔpH was 0·48, increasing to a ΔpH of 0·59 for cells treated with H_2DIDS. Addition of alkali in the absence of cells produced a ΔpH of 0·60. These experiments were repeated a number of times with similar results, i.e. that the effect of H_2DIDS is significant, although small, the mean ΔpH in four experiments being $0·083 \pm 0·009$. Hagfish red cells, therefore, show a very limited ability to buffer an extracellular OH^- challenge, which would be consistent with a low activity of the Band 3 anion-exchange transporter. The low buffering capacity of hagfish blood has also been observed by Wells *et al.* (1986). This was interpreted to be a consequence of a low haemoglobin content rather than a lack of Band 3 activity. Our comparisons between flounder and hagfish red cells were carried out using suspensions of comparable haematocrit.

To determine whether Band 3 activity is indeed low in these red cells, $^{36}Cl^-$ uptake at $[Cl^-] = 510\,mmol\,l^{-1}$ was measured at 1° and 11 °C. Initial experiments, using oil-separation, revealed that fluxes were slow, and subsequently the washing methodology was used to remove extracellular radioactivity (Young & Ellory, 1982) (Fig. 2). Uptake of $^{36}Cl^-$ was linear with time and amounted to $5·8\,mmol\,l\,cells^{-1}\,h^{-1}$ at 11 °C, the environmental temperature of the fish. At 1 °C, the corresponding rate was $2·2\,mmol\,l\,cells^{-1}\,h^{-1}$, giving a Q_{10} of 2·6. In a survey of 18 fish, the mean rate of $^{36}Cl^-$ transport (±S.E.M.) was $8·3 \pm 0·9\,mmol\,l\,cells^{-1}\,h^{-1}$ (range 3·0–18·6 mmol l $cells^{-1}\,h^{-1}$). All subsequent influxes were performed over 1 h at 11 °C. Addition of H_2DIDS resulted in a substantial inhibition of uptake. In five separate experiments, the mean flux was $5·46 \pm 1·23\,mmol\,l\,cells^{-1}\,h^{-1}$; $+H_2DIDS$, $3·19 \pm 0·78\,mmol\,l$ $cells^{-1}\,h^{-1}$; Δ, $2·27 \pm 0·55\,mmol\,l\,cells^{-1}\,h^{-1}$; percentage of activity remaining was $41·6 \pm 4·0$. Efflux experiments over 6 h revealed a single exponential yielding a rate constant of $0·078\,h^{-1}$ decreasing to $0·030\,h^{-1}$ in the presence of H_2DIDS. Altering cell volume by swelling [addition of water, 25 % (v/v)] or shrinking [addition of 25 % (v/v) $1\,mol\,l^{-1}$ sodium methylsulphate] did not alter the rate of $^{36}Cl^-$ influx.

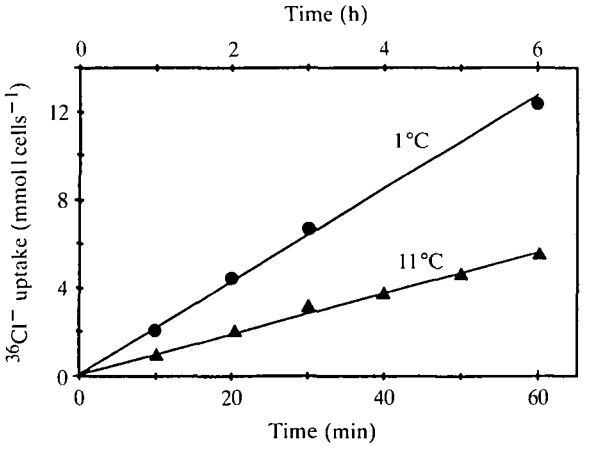

Fig. 2. Time course of chloride uptake by hagfish red cells. Uptake of $^{36}Cl^-$ at 1 °C (●, 1–6 h) and 11 °C (▲, 10–60 min) was measured as described in the text.

Finally, hagfish red cell ghost membranes were prepared by hypotonic lysis and centrifugation, solubilized in SDS and run by conventional PAGE electrophoresis (Thompson & Maddy, 1982). In marked contrast to human membrane preparations, those from hagfish were deficient in capnophorin. The small amount of hagfish protein in this region of the gel ran as a sharp band which comigrated with the leading edge of human Band 3 (Fig. 3). In some other separations, this hagfish polypeptide ran significantly ahead of human Band 3. Interestingly, hagfish red cell membranes were also deficient with respect to spectrin (Bands 1 and 2), some other cytoskeleton proteins (Bands 2.1, 4.1, 4.2) and glyceraldehyde-3-phosphate dehydrogenase (Band 6) (nomenclature of Steck, 1974). This may be related to the fact that Band 3 is the normal membrane anchor for these extrinsic membrane proteins (see e.g. Gratzer, 1981).

We conclude that hagfish red cells express very limited Cl^- transport activity, in marked contrast to red cells from other vertebrate species. The fact that H_2DIDS has a significant inhibitory effect on both influx and efflux of Cl^- makes it likely that capnophorin is present in membranes from these cells, but in relatively small amounts compared with human red cells. Thus at 10°C human red cells exhibit a capnophorin transport activity of $1{\cdot}5\,mol\,l\,cells^{-1}\,min^{-1}$ (Brahm, 1977) compared with the present H_2DIDS-sensitive Cl^- influx rate of approx. $2\,mmol\,l\,cells^{-1}\,h^{-1}$. If the turnover rates of the transporter in hagfish and human red cells at 11°C are comparable, the observed H_2DIDS-sensitive transport activity would be accounted for by about 20 copies $cell^{-1}$ in contrast to the human site density of $1{\cdot}2\times10^6$. Other transport activities in the hagfish red cell include significant Na^+ pump activity (approx. $0{\cdot}5\,mmol\,K^+\,l\,cells^{-1}\,h^{-1}$ at $10\,mmol\,l^{-1}\,K^+$ and 11°C compared with $1{\cdot}5\,mmol\,l\,cells^{-1}\,h^{-1}$ at $5\,mmol\,l^{-1}\,K^+$ and 37°C in human red cells), but rapid glucose [approx. $15\,mmol\,l\,cells^{-1}\,h^{-1}$ at $0{\cdot}1\,mmol\,l^{-1}$ 3-*O*-methyl-D-glucose and 10°C (Ingermann, Hall, Bissonnette & Terwilliger, 1984)] and amino acid transport [$160\,mmol\,l\,cells^{-1}\,h^{-1}$ at $0{\cdot}2\,mmol\,l^{-1}$ L-alanine and 11°C (D. A. Fincham, M. W. Wolowyk & J. D. Young, in preparation)]. We considered the unlikely possibility that some Cl^- enters the hagfish red cells as an 'accidental' substrate of an amino acid transporter, as in the complementary transport of glycine as an accidental substrate of Band 3 (Young, Jones & Ellory, 1981). However, addition of $10\,mmol\,l^{-1}$ glycine, L-alanine or L-lysine did not inhibit Cl^- uptake (data not shown). As indicated above, only half the measured Cl^- uptake was *via* a DIDS-sensitive route. Obvious candidates for the residual flux include KCl and/or NaKCl cotransport, and amino-acid-linked fluxes. At present, therefore, we are unable to identify the true basal permeability to Cl^- in this cell.

Myxodemes are the most primitive vertebrates and represent the only fish to have evolved entirely in sea water. It is intriguing to speculate whether the low anion permeability of hagfish red cells is truly primitive, or represents a secondary loss of Band 3 transport activity. Carlsson, Kjellstrom & Antonsson (1980) have shown the occurrence of carbonic anhydrase in hagfish red cells, indicating a present (or previous) role for the Jacobs–Stewart cycle in this species, which would favour a secondary loss of activity. In view of the habitat and behaviour of this species, which

lives buried in silt for most of its life, it seems possible that rapid changes in blood pH may not occur and the system may have been reduced to a vestigial role. Our results seem superficially in conflict with those of Ohnishi & Asai (1985) who reported the absence of capnophorin from lamprey red cells. However, their methodology (i.e. without flux measurements) would not be sensitive enough to identify low transport

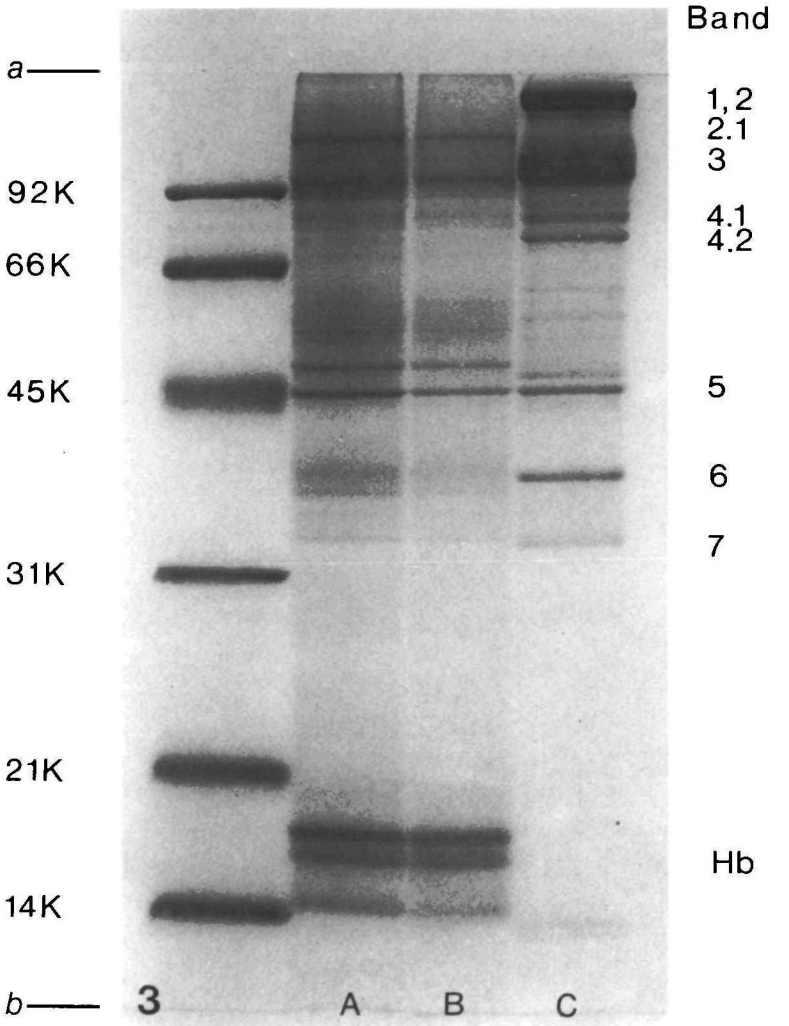

Fig. 3. Membrane protein composition of hagfish and human red cells. Nucleus-free plasma membranes from hagfish red cells were prepared by hypotonic lysis in 3 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$ and 5 mmol l^{-1} Mops (pH 7·0). Absence of nuclei (removed in a low-speed spin) was confirmed by phase-contrast light microscopy. Human erythrocyte ghosts were prepared by the method of Dodge, Mitchell & Hanahan (1963). Electrophoresis samples (A and B, hagfish membranes; C, human membranes) (30 μg of protein) were run on a 12% polyacrylamide slab gel and stained with Coomassie blue. The positions of the stacking gel–running gel interface and tracking dye are indicated by *a* and *b*, respectively. 92 K, for example, 92 000 M_r standard; Hb, haemoglobin.

activity and it is likely that lamprey red cells may also express limited Band 3 transport activity (see also Nikinmaa, Kunnamo-Ojala & Railo, 1986). We note that Ohnishi & Asai (1985) also failed to detect capnophorin activity in flounder and carp red cells. In contrast, our experiments with flounder red cells (Fig. 1) clearly demonstrate the presence of rapid H_2DIDS-sensitive Cl^-/OH^- exchange.

One obvious attraction of the hagfish red cell lies in the study of Cl^--related secondary active transport systems. At present it is not known whether these cells express the Cl^--dependent β and *gly* systems for amino acid transport; however, they do show bumetanide-sensitive K^+ transport (0·7–1·4 mmol l cells^{-1} h^{-1} at 10 mmol l^{-1} K^+ and 11 °C, unpublished result), suggesting that these cells may be an ideal system for studying the stoichiometry and ionic interactions of secondary active transport.

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