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Comparison of murine band 3 protein-mediated Cl^- transport as measured in mouse red blood cells and in oocytes of *Xenopus laevis*

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Murine band 3 protein was expressed in oocytes of *Xenopus laevis* after microinjection of the mRNA from the spleens of anemic mice. The $^{36}Cl^-$ efflux from the oocytes was compared with the chloride fluxes measured in murine red cells. In both oocytes and red cells, the band 3-mediated chloride transport showed the following features: the selective inhibitor of band 3-mediated anion transport, 4,4′-dinitrostilbene-2,2′-disulfonate exerts its effects only when applied to the outside and not when applied to the inside of the membrane. The $K_{1/2}$ for inhibition by external 4,4′-dinitrostilbene-2,2′-disulfonate was of the order of 1.5 to 2.0 μmol/l. Flufenamate and persantine also produce similar inhibitory effects. Decreasing the pH from 7.4 to 6.0 leads to some inhibition. It is concluded that essential features of the mode of action of murine erythroid band 3 protein in the plasma membrane of the oocyte are similar to the mode of action in the bilayer of the red blood cell of the mouse.

Introduction

In previous work from this laboratory, two essentially different methods have been employed for the incorporation of the anion transport protein of the red blood cell into foreign lipid bilayers; (i) Reconstitution of the isolated protein into artificial bilayers [1] and (ii) biosynthetical insertion of the protein into the lipid bilayer of the oocytes of *Xenopus laevis* after translation of microinjected mRNA encoding the genetic information for the synthesis of erythroid band 3 protein [2].

The present paper is concerned with a further appraisal of the latter technique. In our previous studies, we used mRNA prepared from the spleens of anemic mice. We demonstrated the successful biosynthesis of the band 3 protein by immunoprecipitation with antibodies against murine erythroid band 3 and showed its functionality by measurements of $^{36}Cl^-$ flux and its inhibition by certain stilbene disulfonates which are known to be specific inhibitors of band 3 protein-mediated anion transport [2].

Using the murine band 3-dotted oocytes, properties of the band 3 protein could be studied which can not be investigated easily in red cells. Thus the potential dependence of the band 3-mediated Cl^- equilibrium exchange was determined by measuring $^{36}Cl^-$ fluxes under the voltage clamp in single oocytes [3]. Even though the electrical potential does not act as a driving force for this 'electrically silent' process (for review, see Ref. 4), a dependence of the rate of Cl^-/Cl^- exchange on potential was observed. This was interpreted in terms of ping-pong kinetics assuming potential-dependent variations of the

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ratio between inward-oriented and outward-oriented Cl^- loaded transporters. According to this interpretation, differences of the electrical momentum of these two conformeric states of the transporter are responsible for the potential dependence of the exchange rate [3].

The application of these results and other findings on band 3-mediated transport studied in oocytes to the behavior of the band 3 molecule in the lipid bilayer of the red cell membrane of the mouse requires the demonstration of similarities of the behaviour of the band 3 protein in the plasma membrane of *Xenopus* oocytes and mouse red cells.

In the present article we compare observations on some characteristic features of mouse band 3-mediated anion exchange in the oocytes of *Xenopus* with the anion exchange in the red cells of the mouse.

Materials and Methods

mRNA expression in Xenopus oocytes

mRNA was prepared and purified from the spleens of anemic mice according to Ref. 2. Instead of the laborious RNA-extraction procedure used in that reference the RNA was isolated by the caesium chloride method of Glisin et al. [5]. Removal of the oocytes from females of *Xenopus laevis,* microinjection into stage VI oocytes and translation was performed as described in Refs. 2 and 6.

Measurement of anion flux in oocytes

After microinjection of mRNA and incubation in Barth's medium (Barth's medium: in mmol/l: 88 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 5.0 Hepes, (pH 7.6)) for 16–40 hours, the oocytes are ready for measurements of anion efflux. A single oocyte is microinjected with $^{36}Cl^-$ (about 5 nCi in about 50 nl of 200 mmol/l NaCl) and placed on the mica-window of a Geiger-Müller tube which forms the bottom of a perfusion chamber. The chamber is perfused with radioactivity-free Barth's medium to remove continuously the $^{36}Cl^-$ that is released by the oocyte. The radioactivity in the oocytes is recorded as a function of time [3,6]. The absence of leaks in the oocyte is checked by occasional perfusion periods with Barth's medium containing 500 μM/l DNDS (4,4′-dinitrostilbene-2,2′-disulfonate). The composition of the perfusion medium (i.e. inhibitor concentration, pH) is changed according to the respective aims of the various experiments. Details are presented in the course of the description of the individual experiments in Results below. Fluxes were calculated from the time-course of release of $^{36}Cl^-$ from the oocytes as described in Refs. 3 and 6.

Measurement of anion flux in red cells

Red cell ghosts from murine erythrocytes were prepared as described for human red cell ghosts [7] by hemolysis at 0 °C in a solution containing 4 mM $MgSO_4$, 0.8 mM CH_3COOH (pH 6.0). For resealing the medium was made 130 mM NaCl, 20 mM EDTA, 1 mM Na_2SO_4 (pH 7.4) and the suspension was incubated for 45 min at 37 °C. SO_4^{2-} efflux was measured as described in Ref. 8. Cl^- exchange was determined at 0 °C using the inhibitor stop technique of Ku et al. [9] with 80 μM H_2DIDS (dihydrodiisothiocyanatostilbenedisulfonic acid) and 300 μM flufenamate as transport inhibitors in the stop medium. Calculation of exchange rates was performed with a non-linear least-squares fit routine. The limited time resolution of the inhibitor stop technique does not permit measurements of chloride transport at 20 °C while the survival of the oocytes requires that measurement of chloride flux is performed at 20 °C. NMR studies with human red cells have demonstrated that Cl^- binding to the transfer site of band 3 is virtually independnet of temperature (Glibowicka, Schuster, Aranibar, Rüterjans and Passow, unpublished data).

Results

After the expression of the murine band 3 protein in the oocyte's plasma membrane, $^{36}Cl^-$ was microinjected and the efflux of the radioactivity was studied. Fig. 1 shows that in oocytes that had not been injected with the specific mRNA, there is no observable $^{36}Cl^-$ efflux. If, however, the oocyte had received mouse spleen mRNA 2–3 days prior to the flux measurements, a rapid $^{36}Cl^-$ efflux was observed (Fig. 1). This efflux can be inhibited by DNDS (500 μmol/l) indicating that

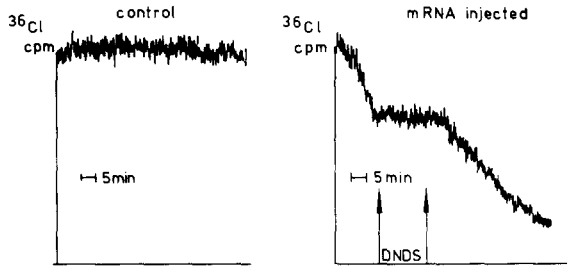

Fig. 1. Time-course of $^{36}Cl^-$ release from *Xenopus* oocytes. (Left) Control oocyte (no mRNA injected). (Right) Oocyte after microinjection of mouse spleen mRNA two days before the efflux experiment. Efflux can be reversibly inhibited by DNDS (500 μM/l). The arrows indicate the beginning and the end of the perfusion period with DNDS.

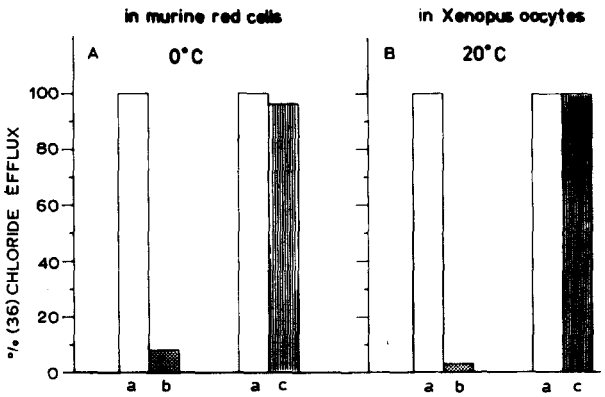

Fig. 2. Sidedness of action of DNDS on band 3-mediated Cl^- transport in murine red cells and *Xenopus* oocytes. Cl^- efflux in oocytes was measured as depicted in Fig. 1 in Barth's medium (pH 7.6). Cl^- efflux in red cells was determined in Cl^- medium (130 mM NaCl, 20 mM EDTA (pH 7.4)), as described in Ref. 6. a, control; b, 100 μmol/l DNDS outside; c, 100 μmol/l DNDS inside.

the induced increase of $^{36}Cl^-$ efflux is mediated by the band 3 protein.

In the human red cell, stilbene disulfonate derivatives are known to inhibit anion exchange only if they are applied to the external medium [10]. Fig. 2 demonstrates that DNDS has no effect on band 3-mediated anion exchange if it is enclosed in red cell ghosts or microinjected into *Xenopus* oocytes containing mouse band 3.

External DNDS is a competitive inhibitor of chloride exchange [11]. At 130 mmol/l NaCl in the medium, the $K_{1/2}$ value for DNDS inhibition is about 4 μmol/l in human red cells [12]. In murine red cells, the inhibition of anion equilibrium exchange by DNDS is a biphasic process. About 75% of the inhibition takes place with the $K_{1/2}$ value indicated in Fig. 3 (2 μmol/l), the remainder with a $K_{1/2}$ value which is much larger. Similar observations are made with the oocytes, although in these cells the more sensitive portion of the curve comprises 80–90% of the total flux. The $K_{1/2}$ value for the more sensitive portion is about 1.5 μmol/l and hence rather similar to the corresponding $K_{1/2}$ value in the red cells. Fig. 4 exemplifies the experimental procedure used for the determination of the concentration-efficiency curve shown in Fig. 3. The inhibitor-free perfusion medium is changed after 10–20 min to a medium containing the inhibitor at the desired concentration and the flux is recorded for another 10–20 min. After several perfusion periods at a range of DNDS concentrations, the medium is changed to Barth's medium containing DNDS at the maximally inhibitory concentration of 500 μmol/l. The rate of efflux at maximum inhibition serves as a measure of the $^{36}Cl^-$ efflux via transport systems other than band 3 or via leaks. This residual anion

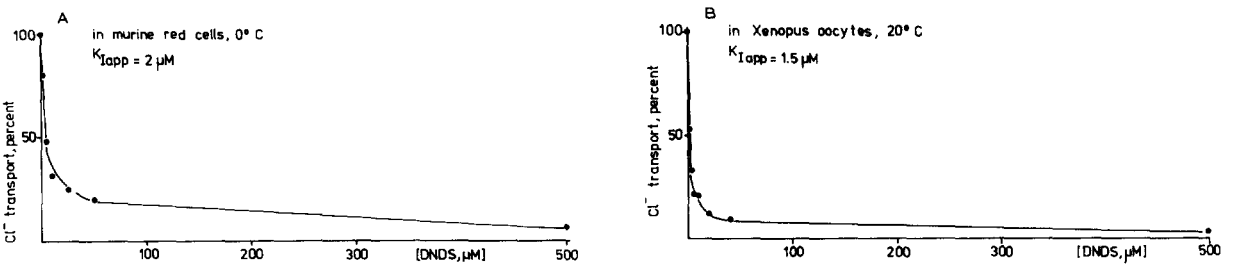

Fig. 3. Effect of external DNDS on band 3-mediated Cl^- equilibrium exchange in murine red cells and *Xenopus* oocytes. (Oocytes in Barth's medium, pH 7.6; red cells in 130 mM NaCl, 20 mM EDTA (pH 7.4)).

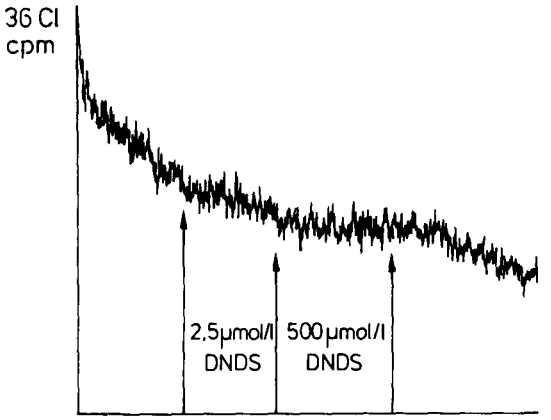

Fig. 4. Time-course of Band 3-mediated Cl^- release from mRNA-injected *Xenopus* oocytes. Perfusion of the $^{36}Cl^-$-releasing oocyte with Barth's medium containing DNDS at 0, 2.5, 500 and 0 µmol/l. Respective rate constants (in 10^{-2} min^{-1}) are: 0 µM DNDS: $k = 1.60$; 2.5 µM DNDS: $k = 0.63$; 500 µM DNDS: $k = 0.02$. Rate constants were calculated by $k = \ln(y_2/y_1)\cdot(t_2 - t_1)^{-1}$, where y_1 and y_2 represent radioactivity in the oocyte at times t_1 and t_2, respectively. Curves of this type were made to establish the flux-concentration relationship for DNDS inhibition of Cl^- transport as shown in Fig. 3. The arrows indicate the beginning and the end of the perfusion period with the two DNDS-containing solutions.

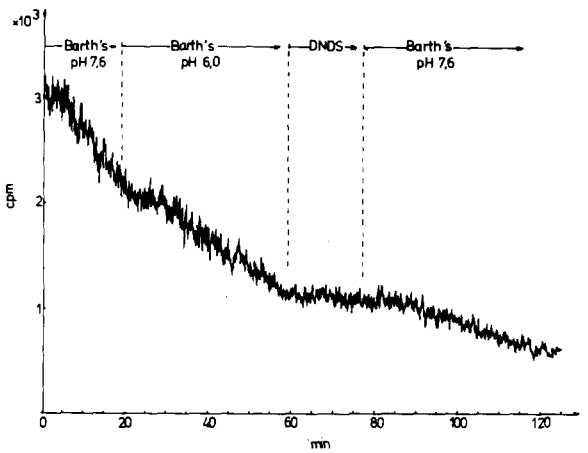

Fig. 5. pH-dependence of Band 3-mediated $^{36}Cl^-$ efflux from mRNA-injected *Xenopus* oocytes. pH of the Barth's perfusion medium was changed from pH 7.6 to pH 6.0 and after inhibition with 500 µM DNDS back to pH 7.6 (20 °C).

efflux rate is subtracted from the rate of efflux observed at the intermediate concentrations of the inhibitor.

Persantine and flufenamate are inhibitors of chloride transport in human red cells [13,14]. As shown in Table I these agents also produce inhibition of mouse band 3-mediated Cl^- transport in both mouse red cells and *Xenopus* oocytes. The inhibition in the oocytes is similar to the inhibition in the red cells.

In mouse red cells, chloride transport decreases when the pH is reduced from 7.6 to pH 6.0. In the oocyte, the band 3-mediated Cl^- transport also decreases with decreasing pH (Fig. 5, Table II). Comparing the flux measurements in oocytes and red cells, one should notice that in the oocytes, the

TABLE I

EFFECT OF PERSANTINE AND FLUFENAMATE ON BAND 3-MEDIATED Cl^- EFFLUX IN MURINE RED CELLS AND *XENOPUS* OOCYTES

Oocytes in Barth's medium, pH 7.6, 20 °C; red cells in 130 mM NaCl, 20 mM EDTA (pH 7.4), 0 °C. The figures in brackets indicate the number of experiments.

Xenopus oocytes	Percent transport
Control	100 ±5.2 (4)
100 µM flufenamate	5.48±1.5 (3)
50 µM persantine	15.4 ±5.5 (3)
Red cells	**Percent transport**
Control	100
100 µM flufenamate	4.35
50 µM persantine	19.29

TABLE II

pH-DEPENDENCE OF BAND 3-MEDIATED Cl^- EFFLUX IN MICROINJECTED *XENOPUS* OOCYTES AND IN MURINE RED CELL GHOSTS

Oocytes in Barth's medium at 20 °C, red cell ghosts in 130 mM NaCl, 20 mM EDTA at 0 °C. The figures in brackets indicate the number of experiments.

Xenopus oocytes	Percent transport
pH 6.0	54.1 ±14.3 (10)
pH 6.8	60.5 ±11.4 (8)
pH 7.2	66.5 ±20.7 (10)
pH 7.6	100 ±21.4 (20)
Red cells	**Percent transport**
pH 6.0	52.14±18.57 (4)
pH 6.5	92.14±17.14 (4)
pH 7.0	98.57±17.01 (4)
pH 7.5	100 ±15.71 (6)
pH 8.0	104.29±16.07 (4)

intracellular pH can be assumed to remain constant during changes of external pH, while in red cells, intracellular pH changes more or less parallel to extracellular pH.

Conclusions

Although the comparison of the erythroid band 3-mediated chloride transport in *Xenopus* oocytes and murine red cells is far from exhaustive, it is clear that a number of typical features can be observed in both systems. It seems justified, therefore, to postulate that the mode of operation of the transport system is essentially similar in the lipid bilayers of the two types of cells.

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