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Potential Dependence of the "Electrically Silent" Anion Exchange across the Plasma Membrane of *Xenopus* Oocytes Mediated by the Band-3 Protein of Mouse Red Blood Cells

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Summary. Mouse erythroid band-3 protein was incorporated into the plasma membrane of *Xenopus* oocytes by microinjection of poly(A)$^+$-mRNA from spleens of anemic mice. Subsequently, the efflux of microinjected ^{36}Cl was continuously followed in single oocytes in a perfusion chamber the bottom of which was formed by the window of a Geiger-Müller tube. During the flux measurements, the membrane potential was clamped to different holding potentials. The efflux increased over the voltage range of -10 to -100 mV by a factor of about 1.5. Since the membrane potential cannot act as a driving force of anion exchange, it is suggested that the observed slight potential dependence is related to a recruitment of the anion-loaded transport protein by the electrical field, thereby changing the steady-state distribution between inwardly and outwardly facing anion binding sites of the transport molecules.

The experimental data are discussed in terms of ping-pong kinetics, assuming that the potential dependence is primarily due to an effect of the electrical field in the membrane on the rate-limiting interconversion of inwardly and outwardly oriented anion binding sites. The results are compatible with the assumption that in the oocyte membrane the substrate-loaded band-3 molecules are preferentially inwardly oriented, and that the transition from the inwardly to the outwardly oriented conformation is associated with a reorientation of an effective charge of 0.1 elementary charge.

During progesterone-induced maturation of the oocytes, several endogenous transport systems change their activity drastically. The mouse band-3 protein in the oocyte membrane also undergoes activity changes; however, these changes do not seem to involve direct regulation by specific metabolic processes. They can be explained as a consequence of the depolarization of the membrane potential associated with the maturation process.

Key Words anion exchange · expression · erythrocyte · *Xenopus* oocyte · voltage clamp · chloride flux · maturation

Introduction

The band-3-mediated anion transport in red blood cells consists essentially of an anion exchange which does not contribute to the electrical conductance of the membrane; it is "electrically silent" (for recent reviews *see* Jennings, 1985; Knauf, 1986; Passow, 1986). For this reason, electrical potential differences across the erythrocyte membrane cannot act directly as a driving force for the band-3-mediated anion exchange. Nevertheless, the rate of the electrically silent anion exchange could conceivably depend on membrane potential. The potential could affect anion binding and/or subsequent conformational changes of the transport protein that lead to the anion transfer across the membrane. In previous work from several laboratories, attempts have been made to study potential dependence of anion exchange using valinomycin-induced K^+ permeability as the source for the generation of a potential gradient and different K^+ concentrations in large volumes of external medium to clamp the membrane potential at various levels. The results obtained suggest that there exists little if any potential dependence (Gunn & Frölich, 1979; Frölich, Leibson & Gunn, 1983; Knauf et al., 1984). However, by its very nature, this technique is not very accurate, and the results may be affected by the presence of the ionophore in the membrane. The present paper serves to elucidate whether or not band-3-mediated, electrically silent anion exchange varies with membrane potential using a more direct approach which avoids the use of ionophores.

Changes of membrane potential can most directly be produced by means of voltage clamp using intracellular microelectrodes. The red blood cells are, however, too small for impalement with microelectrodes. In addition, even if voltage clamp were achieved by whole-cell patch-clamp techniques, it would be impossible to measure the electrically silent anion exchange in a single red cell. Attempts to use planar lipid bilayers with incorporated band-3 protein were unsuccessful (M. Tosteson and H. Passow, *unpublished*). We decided, therefore, to perform our studies with band-3 protein which had been inserted biosynthetically into the plasma membrane of oocytes of *Xenopus laevis*.

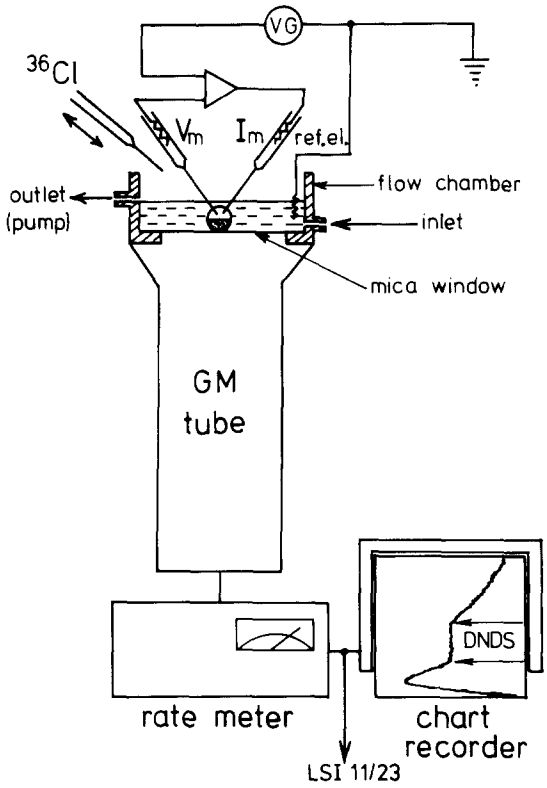

Fig. 1. Experimental setup for measuring ^{36}Cl efflux mediated by the band-3 protein in *Xenopus* oocytes under voltage clamp. After microinjection with mRNA and incubation, an oocyte is placed in a perfusion chamber mounted on a GM tube and injected with ^{36}Cl. During perfusion with ^{36}Cl-free solution the ^{36}Cl (in cpm) in the oocyte is measured under voltage clamp by a rate meter. The counting rate is recorded by a chart recorder, or digitized by a computer and stored on magnetic disk. VG: voltage generator; V_m: voltage-measuring electrode; *ref.el.*: reference electrode; I_m: current-delivering electrode; ^{36}*Cl*: micropipette for injection of ^{36}Cl; *LSI 11/23*: computer for sampling and evaluating the data

In previous work from this laboratory it was shown that messenger RNA from the spleens of anemic mice is translated by the oocytes into functional band-3 protein (Morgan et al., 1985). The oocytes acquire a component of anion flux across the plasma membrane which they do not normally possess. This component can be inhibited by stilbene disulfonates, like the band-3-mediated anion transport in the red blood cells of the mouse (M. Raida and H. Passow, *unpublished*). The large size of the oocytes allows flux measurements on single cells that can simultaneously be subjected to conventional voltage clamp. In the present paper we shall demonstrate that the chloride fluxes measured under voltage clamp slightly decrease over the voltage range from −100 to −10 mV. The theoretical interpretation of the data suggests that during the rate-limiting step of the interconversion of inwardly and outwardly facing binding sites of the complex between the monovalent anion and the transport protein an effective charge of 0.1 of one elementary charge is reoriented within the membrane. This leads to a slight but significant voltage dependence of the distribution between inwardly and outwardly facing binding sites of the transport proteins.

Studies of the voltage dependence of the band-3-mediated anion exchange are not only necessary for the characterization of the molecular mechanism of the anion transport process. They may also help to understand the previously observed physiological changes of transport activity during oocyte maturation (Richter, Jung & Passow, 1984). During meiotic maturation the activity of the inserted band-3 protein from the mouse red cells decreases by about 40%. This decrease is primarily a consequence of the depolarization that is associated with the maturation process (Belle, Ozon & Stinnakre, 1977; Wallace & Steinhardt, 1977). A similar regulatory mechanism has been demonstrated for the endogenous Na-alanine cotransport in the oocytes (Jung, Lafaire & Schwarz, 1984). In contrast to these two transport systems, several other endogenous transport proteins are most likely regulated by biochemical modification. This supports the view (Richter et al., 1984) that no common mechanism is involved in the regulation of endogenous transport systems, but that each system is regulated individually.

Short reports on part of this work have been published (Grygorczyk, Schwarz & Passow, 1987; Schwarz et al., 1987).

Materials and Methods

Females of the clawed toad *Xenopus laevis* were anesthetized on ice and parts of the ovary were removed. Full-grown oocytes arrested in the prophase of the first meiotic division (type V or VI after Dumont (1972), diameter 1.2–1.4 mm) were selected after removal of the enveloping tissue by treatment of the ovarial pieces with collagenase (1.5 U/ml Barth's solution (*see below*)) and subsequent washing with calcium-free Barth's solution. Meiotic maturation of the selected oocytes was induced by treatment with 5 μM progesterone for 5 to 10 hr. The maturation process leads to conversion of the oocytes arrested in the prophase of the first meiotic division to oocytes arrested in the metaphase of the second meiotic division.

Poly(A)$^+$-mRNA was prepared essentially as described by Braell and Lodish (1982) from the spleens of mice that had been made anemic by repeated injections with phenylhydrazine (for details *see* Morgan et al., 1985). Each batch of mRNA was tested prior to use in cell-free translation systems using reticulocyte lysates. For most injections density-gradient-fractionated mRNA was used which yielded translation products of the size of the band-3 protein. The mRNA was filtered before microinjection by centrifugation through a polycarbonate filter (pore size 0.2 μm) in an Eppendorf centrifuge for 5 sec. The mRNA was injected using a Bachofer microinjection pump (for details *see*

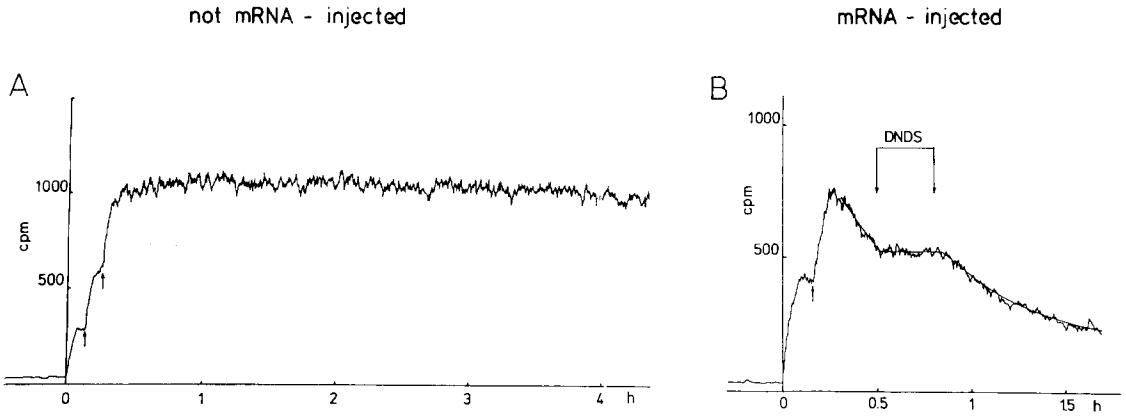

Fig. 2. (*A*) ^{36}Cl efflux from a control oocyte which had not been injected with mRNA. Three portions, each of about 25 nl of ^{36}Cl, were consecutively injected; the first injection was made at zero time, the other two at times indicated by the arrows. The figure represents ^{36}Cl content of the oocyte as a function of time. The ^{36}Cl released from the oocyte into the ^{36}Cl-free perfusate amounted to about 2.5% per hour. (*B*) ^{36}Cl efflux from a mRNA-injected oocyte which had been incubated for 2 days before the flux measurement. Two portions of 50 nl of ^{36}Cl were injected, the first at zero time and the second at the time indicated by the arrow. The rapid efflux is nearly completely inhibited by 0.4 mM DNDS and returns to the original value when perfusion with DNDS-free solution is resumed. The figure indicates ^{36}Cl content of the oocyte as a function of time. The smooth line was drawn by hand. In both experiments *A* and *B* the time constant of the rate meter was set to 50 sec

Grygorczyk et al., 1987). The microinjected oocytes were incubated for at least 16 hr and up to several days. The temperature was 18 to 20°C during the first 16 hr and 10 to 12°C thereafter.

For influx measurements oocytes were placed into Barth's solution which contained radioactively labled chloride (^{36}Cl). After incubation for 3 to 4 hr, the oocytes were washed and placed individually into vials for liquid scintillation counting. For efflux measurements under voltage-clamp conditions (*see* Fig. 1), an oocyte was placed into a perfusion chamber which was directly mounted on the window of a GM (Geiger-Müller) tube, and the cell was microinjected with 50 to 75 nl of ^{36}Cl (about 5 nCi in 200 mM NaCl, the cell volume is about 1 μl). The radioactivity remaining in the oocyte was measured by means of a rate meter. The data were continuously recorded on a paper recorder and stored in digitized form on magnetic disk. For studying the voltage dependence of the ^{36}Cl-efflux, on-line experiments were performed by means of PDP 11/23 computer (Hof, 1986); the membrane potential was clamped during the experiment at a desired level by conventional two-microelectrode techniques. Every 30 sec the counting rate was sampled for half a second and an average value was stored on magnetic disk.

The composition of the Barth's solution was (in mM): 90 NaCl, 1 KCl, 0.8 $MgSO_4$, 0.3 $Ca(NO_3)_2$, 0.4 $CaCl_2$, 5 N-2-hydroxylethylpiperazine-N'-ethansulphonic acid (HEPES, pH 7.4), 0.08 penicillin, and 0.03 streptomycin. To inhibit the band-3-mediated anion transport 0.4 mM 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) was used.

Results

Kinetics of the Band-3-Mediated Anion Exchange in the Oocytes

After expression of microinjected mRNA during the incubation period and after microinjection of ^{36}Cl, chloride efflux from the oocytes is measured in the flow chamber. In contrast to control oocytes which had not been injected with mRNA (Fig. 2*A*), a rapid ^{36}Cl-efflux is observed (Fig. 2*B*). When the Barth's medium in the perfusion chamber is replaced by Barth's solution containing 0.4 mM DNDS, the efflux from the microinjected oocytes is nearly completely inhibited. The efflux is resumed when the DNDS-containing medium is again replaced by Barth's solution without DNDS. Similar observations were made with other typical inhibitors of band-3-mediated anion transport such as persantine (20 μM) and flufenamate (100 μM) (*unpublished data*).

The efflux of ^{36}Cl from mRNA-injected oocytes slows down with time. The time course of release of ^{36}Cl does not follow a single exponential (Fig. 3, curve *a*), but comes closer to a single exponential when ^{36}Cl is microinjected together with DNDS (Fig. 3, curve *b*). Apparently the deviations from a single exponential are related to an intracellular redistribution of the ^{36}Cl which is prevented by the injection of DNDS. Unequal distribution of the injected ^{36}Cl is also supported by the observation that the counting rate depends on the orientation of the oocytes with respect to the window of the GM tube; higher activity of ^{36}Cl is found in the animal hemisphere compared to the vegetal hemisphere. Regardless of the interpretation of the effect of injected DNS, it is obvious that intracellular DNDS produces no inhibition of ^{36}Cl-efflux; this contrasts with the prompt inhibition by extracellular DNDS and corresponds to previous observations on the

sidedness of the action of stilbene disulfonates in human (Kaplan et al., 1976) and murine erythrocyte ghosts (M. Raida and H. Passow *(unpublished)*). All experiments described below were done after simultaneous injection of ^{36}Cl and the DNDS.

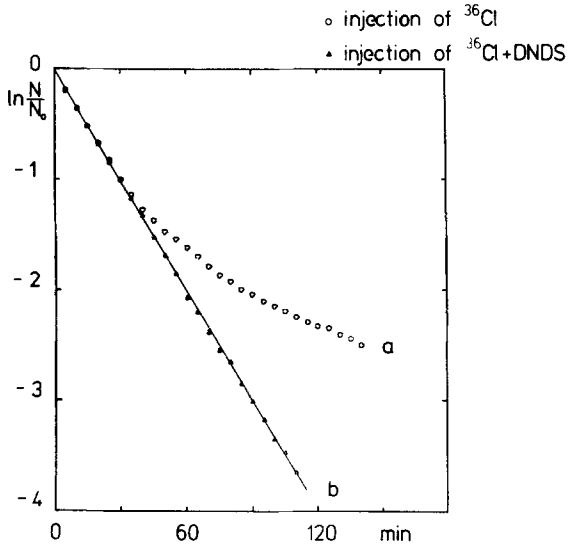

Fig. 3. ^{36}Cl efflux from mRNA-injected oocytes. The figure indicates ^{36}Cl content as a function of time in a semilogarithmic plot. After mRNA injection and incubation, the oocytes were injected with 50 nl of ^{36}Cl (curve *a*) or with 50 nl of (^{36}Cl + DNDS) (curve *b*). The amount of injected DNDS corresponds to a final concentration in the cytoplasm of the oocyte of about 0.4 mM. For better illustration, the curves were normalized to the same initial slope represented by the solid line. In both oocytes the membrane potential during the flux measurements was clamped to −90 mV

Potential Dependence of the Band-3-Mediated Anion Exchange

The voltage dependence of ^{36}Cl-efflux was studied according to the protocol depicted in Fig. 4. A first determination of efflux was made after setting the membrane potential to a reference level of −70 mV. The membrane potential was then changed to some other value, in the example depicted in Fig. 4 to −20 mV; the measurements of efflux were continued, first in the absence and then in the presence of DNDS in the perfusate. The presence of the inhibitor served to establish the influence of leaks and other physiological flux components which would permit ^{36}Cl-efflux without participation of band-3 protein. Still maintaining the membrane potential at the selected level of −20 mV, the measurements were continued again in the absence of DNDS to demonstrate the reversibility of the inhibition. A new dose of ^{36}Cl was now injected into the same oocyte. The efflux measurements were continued to make sure that this additional microinjection did not change the rate of efflux compared to the previously determined value. Thereafter the whole sequence of measurements could be repeated at some other potential than −20 mV. In this manner, one is able to determine the rate constants of ^{36}Cl-efflux at a number of different potential values in the same oocyte (Table).

Rate constants determined in the same oocyte are directly comparable. However, this is not necessarily the case with rate constants determined in different oocytes since the number of band-3 molecules successfully inserted into the plasma membrane of these oocytes may vary. To eliminate this

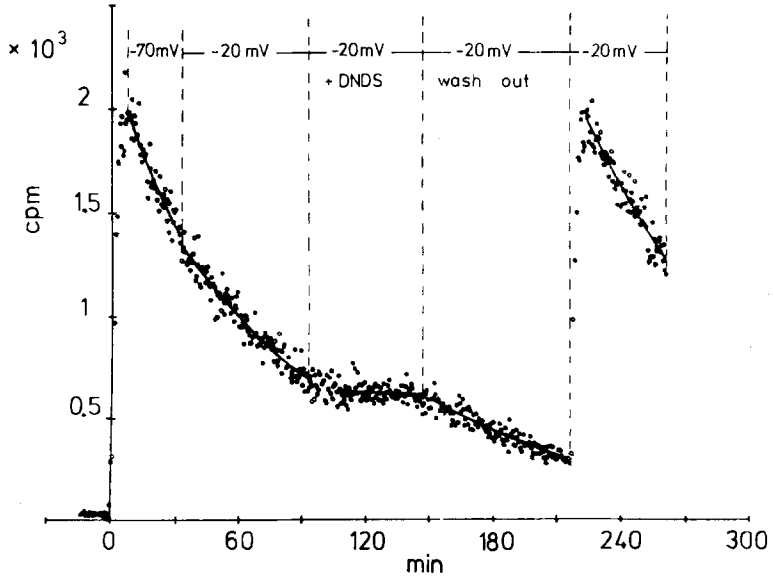

Fig. 4. Time course of a typical, computer-controlled flux experiment in a single, mRNA-injected oocyte under voltage clamp. The data points represent the counting rates of the GM tube sampled every 30 sec (*see* Materials and Methods). At time zero, 50 nl of (^{36}Cl + DNDS) were injected into the oocyte. After an equilibration period of about 7 min, the membrane potential was clamped to −70 mV, and the flux experiment was started. Membrane potential and perfusion medium were changed as indicated in the figure (*see* text). The time constant of the ratemeter was set to 10 sec. The solid lines represent least-squares fits of the exponential $N = No \exp(-kt)$ to the data points of each sampling period. The calculated values of k and No (±SEM) are listed in the Table

and other possible sources of variability amongst different oocytes, the rate constants were determined at the selected membrane potentials and expressed as a fraction of the rate constant determined at the reference potential of −70 mV in the same oocyte. A linear plot of the normalized rate constants of the ^{36}Cl efflux versus voltage can be approximated a straight line with a slope of about -3.3×10^{-3} mV^{-1} (Fig. 5*a*). This slope is significantly different from zero ($P > 0.999$, in Student's *t*-test) and indicates that decreasing the potential by −100 mV leads to an increase of anion efflux by about 1.5 times. Thus, the electrically silent anion efflux characterized by its sensitivity to DNDS varies slightly with membrane potential.

Changes of the Band-3-Mediate Anion Exchange during Oocyte Maturation

During meiotic maturation of the oocytes the activity of endogenous transport systems is downregulated (*see* Richter et al., 1984). The effect of maturation on the band-3-mediated anion exchange was determined by measuring ^{36}Cl influx in oocytes before and after progesterone-induced meiotic maturation. Control oocytes not injected with the mRNA also show some ^{36}Cl uptake, indicating an endogenous capacity for chloride transport already described by Richter et al. (1984); but this component is only slightly sensitive to DNDS (*see* Morgan et al., 1985), indicating anion transport by a mechanism that does not involve band 3. In the specific experiment represented in Fig. 6, the rate of ^{36}Cl uptake in the mRNA-injected oocytes is about twice the control value. The mRNA-induced extra uptake of ^{36}Cl is completely abolished by DNDS. After progesterone-induced maturation, the endogenous, DNDS-insensitive ^{36}Cl uptake is inhibited by about 85%. The large mRNA-induced component is also inhibited, but inhibition amounts to no more than about 40% (*see* Fig. 6*A*). During oocyte maturation, the membrane potential depolarizes from about −70 mV to nearly zero mV (Fig. 6*B*, *see also* Belle et al., 1977; Wallace & Steinhardt 1977). According to Fig. 5 this potential change could account for the major part of decrease of the anion transport mediated by the band-3 protein.

Table. Potential dependence of the fitted parameters of $N = No \cdot \exp(-kt)$ for the data presented in Fig. 4

mV	−70	−20	−20 +DNDS	−20 washout	−20 after Cl reinjection
k (sec^{-1})	(2.39 ± 0.15) 10^{-4}	(1.87 ± 0.05) 10^{-4}	(9.77 ± 1.18) 10^{-6}	(1.81 ± 0.07) 10^{-4}	(1.84 ± 0.08) 10^{-4}
No (cpm)	2029 ± 24	1352 ± 12	630 ± 14	610 ± 8	2090 ± 23

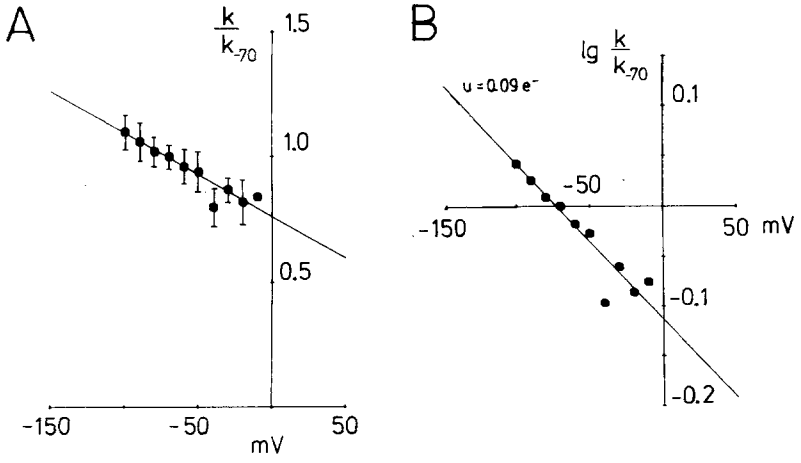

Fig. 5. (*A*) Normalized rate constants (k) for ^{36}Cl efflux from mRNA-injected oocytes versus membrane potential. Each data point represents a k value measured at a selected membrane potential divided by the rate constant k_{-70} obtained in the same oocyte at the reference potential of −70 mV. The bars represent the maximal error of such ratios when k and k_{-70} are estimated by the fit procedure with accuracies of ±SD (*see* e.g. the Table). The fitted straight line has a slope of $(-3.33 \pm 0.29) \times 10^{-3}$. (*B*) The same data as in *A* but in a semilogarithmic plot. The line represents a fit of $\ln(k/k_{-70}) = uy + \ln(k^o/k_{-70})$ with $u = -0.089 \pm 0.008$

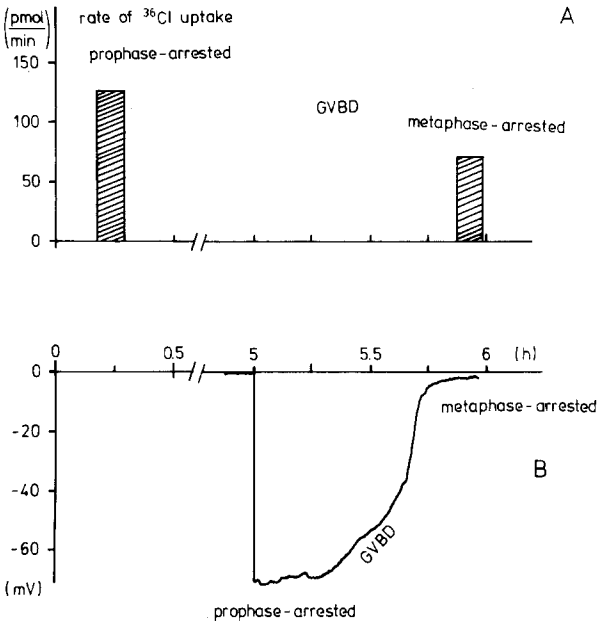

Fig. 6. Changes of band-3-mediated Cl transport and membrane potential during maturation of *Xenopus* oocytes. The progesterone-induced transition from prophase arrested oocytes to metaphase arrested was verified by observation of the germinal vesicle breakdown (*GVBD*). Maturation was initiated by addition of 5 μM progesterone to the bath solution at zero time. (*A*) mRNA-induced extra uptake of Cl. (*B*) Membrane potential. The registration of membrane potential was started 5 hr after application of the progesterone. The time scales of *A* and *B* are identical

Discussion

The present experiments confirm the previous observation (Morgan et al., 1985) that mRNA of erythroid tissue from anemic mice is successfully translated in the oocytes of *Xenopus laevis* and that the synthesized band-3 protein is expressed in a functional state. It further shows that band-3-mediated chloride fluxes can be measured in single oocytes under voltage clamp, and that the electrically silent anion exchange exhibits a slight but significant potential dependence. It finally demonstrates that the rate of the anion exchange mediated by the erythroid band-3 protein of mouse decreases during the progesterone-induced meiotic maturation of the *Xenopus* oocyte; this decrease can be explained as a consequence of the change of membrane potential during the maturation process.

Recruitment of Band-3 Conformers by the Electrical Field

Effects of Membrane Potential on Ping-Pong Kinetics

Anion transport across the membrane of human red cells obeys ping-pong kinetics (Fig. 7; for reviews *see* Knauf, 1986; Passow, 1986). The anion (at concentration a') is bound at the internal membrane surface to the unloaded inwardly oriented protein r. The resulting complex of anion and protein ar undergoes a conformational change to as. This is associated with a reorientation of the anion binding site which enables the bound anion to dissociate at the external membrane surface. The unloaded outwardly oriented transport protein s cannot revert to the conformation r; there is no "slippage." For a return journey, binding of an external anion (at concentration a'') is required. Thus, the protein mediates anion exchange but no anion net flow. Nevertheless, such electrically silent anion exchange mechanism could vary with potential if either the anion binding to r or s, or the conformational changes between ar and as are both potential sensitive. In the following, we shall interpret our results on the mouse band-3 transport in the *Xenopus* oocytes in terms of such a ping-pong kinetics.

Potential Dependence of j_{max} and $K_{1/2}$ in Terms of Ping-Pong Kinetics without Slippage

When measured at equal concentrations of the same anion species at both membrane surfaces (i.e., $a' = a'' = a$), the ping-pong mechanism gives rise to simple saturation kinetics of the exchange flux j. This flux can be described by two parameters, j_{max} and $K_{1/2}$. They depend in turn on the kinetic parameters which define the ping-pong kinetics as represented in Fig. 7 (*see* Passow, 1986):

$$j = j_{max} \frac{a}{K_{1/2} + a} \tag{1}$$

where

$$j_{max} = \frac{k_{12}\,\overline{RS}}{1+q},\quad K_{1/2} = \frac{K_{101} + q\,K_{11}}{1+q},\quad q = \frac{k_{12}}{k_{21}}.$$

q represents the distribution ratio between ar and as, $K_{1/2}$ is the apparent affinity constant, and $\overline{RS}$ is the sum of all forms of the transport protein.

In these equations, the constants k_{12}, k_{21}, K_{11}, K_{101} could vary in response to potential-dependent conformational changes of the transport protein. For the sake of simplicity, we assume that the mass law constants K_{11} and K_{101} are potential independent but that the rate-limiting conformational changes $ar \rightleftharpoons as$ depend on membrane potential:

$$k_{12} = k_{12}^{o} \cdot \exp(uy)$$

and

$$k_{21} = k_{21}^{o} \cdot \exp(-vy). \tag{2}$$

Here k^o_{12} and k^o_{21} designate potential-independent rate constants, $y = EF/RT$, and u and v represent "effective" charges of the protein in the conformations *ar* and *as*, respectively. The term "effective" charge represents the actual electrostatic charge that can be reoriented by the membrane potential.

Inserting Eq. (2) into Eq. (1) yields:

$$j_{max} = j^o_{max} \frac{1 + q^o}{\exp(-vy) + q^o \exp(uy)} \tag{3}$$

with

$$j^o_{max} = \frac{k^o_{12} k^o_{21}}{k^o_{12} + k^o_{21}} \overline{RS} = \frac{k^o_{12}\, \overline{RS}}{1 + q^o}$$

and

$$K_{1/2} = \frac{K_{101} + q^o\, K_{11} \exp((u + v)y)}{1 + q^o \exp((u + v)y)}. \tag{4}$$

In these equations q^o represents the distribution ratio between anion-loaded conformers of the transport protein with inwardly and outwardly oriented anion binding sites at zero membrane potential:

$$q^o = \frac{k^o_{12}}{k^o_{21}}. \tag{4a}$$

Equations (3), (4) and (4*a*) yield the following limiting cases:

$$\begin{array}{llll} y \to +\infty & j_{max} \to 0 & q \to 0 & K_{1/2} \to K_{101} \\ y = 0 & j_{max} = \dfrac{k^o_{12}}{1 + q^o} RS & q = q^o = \dfrac{k^o_{12}}{k^o_{21}} & K_{1/2} = \dfrac{K_{101} + q^o K_{11}}{1 + q^o} \\ y \to -\infty & j_{max} \to 0 & q \to \infty & K_{1/2} \to K_{11}. \end{array} \tag{5}$$

When the distribution equilibrium between inwardly and outwardly directed anion-loaded transporters at $y = 0$ favors one of the two conformations, one obtains two further limiting cases:

(i) $q^o \ll 1$

(ii) $q^o \gg 1$.

Correspondingly, equations for the anion flux read:

$$\text{(i)}\ j_{max} = j^o_{max} \exp(uy),\ j^o_{max} = k^o_{12}\, \overline{RS} \tag{6a}$$

$$\text{(ii)}\ j_{max} = j^o_{max} \exp(-vy),\ j^o_{max} = k^o_{21}\, \overline{RS}. \tag{6b}$$

In the human red cell at the membrane potential close to zero mV, the anion binding site of the anion-loaded transport protein is primarily orientated to the cytoplasm, i.e., $k_{12} \ll k_{21}$ (for review *see* Passow, 1986). If one assumes a similar situation in the mouse red cell, Eq. (6a) would apply.

For the evaluation of our data we have assumed

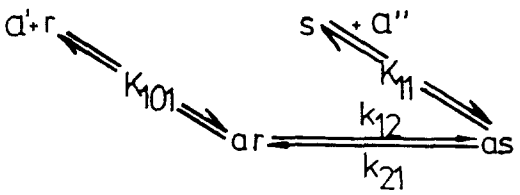

Fig. 7. Ping-pong kinetics of anion exchange (without slippage). K_{11} and K_{101} represent dissociation constants for the reactions $as \rightleftharpoons a + s$ and $ar \rightleftharpoons a + r$, respectively. k_{12} and k_{21} are the potential-dependent rate constants for the transitions indicated in the figure. a' and a'' indicate internal and external chloride concentrations, respectively. For further details, *see* text

$a' = a'' = a \gg K_{1/2}$. These assumptions are conjectural since $K_{1/2}$ in mouse red cells is unknown. The data for human red cells, which could perhaps be used as an approximation, are in the range of 20 to 65 mM, depending on the laboratory where the measurements have been made (reviewed by Passow, 1986). They pertain to 0°C while the measurements in the oocytes have been made at 20°C where the $K_{1/2}$ value may be higher (Knauf & Brahm, 1986). in Barth's solution a is about 90 mM, and in the cytosol of *Xenopus* oocytes a amounts to 60 mM or more (Kusano, Miledi & Stinnakre, 1982). Although neither the condition $a' = a''$ nor $a \gg K_{1/2}$ is strictly fullfilled, it seems instructive to evaluate the data in terms of the theoretical predictions for j_{max}. Below we shall first discuss the potential dependence of j_{max} and evaluate our data in terms of Eq. (3). We will then add a few comments on the theoretical consequences of Eq. (4) about the potential dependence of $K_{1/2}$.

Potential Dependence of j_{max} and Evaluation of the Data. As shown above [*see* Eq. (5)] the flux j_{max} tends toward zero for $y \to \pm\infty$, and passes through a maximum somewhere between these extremes. This effect of potential is the result of changing the equilibrium position between *ar* and *as*, the anion-loaded band-3 molecules with inwardly and outwardly oriented binding sites, respectively. This is analogous to the "recruitment" previously described to occur under the influence of suitable ion gradients across the red cell membrane (for reviews *see* Knauf, 1986; Passow, 1986). Evidently, the occurrence of a maximum is due to recruitment by the membrane potential of all band-3 molecules to one ($k_{12} \ll k_{21}$, $q \to 0$) or the other ($k_{12} \gg k_{21}$, $q \to \infty$) membrane surface which would lead to a complete inhibition of transport. Over the potential range studied in our experiments we observe only an increase of the rate of transport with decreasing membrane potential. Apparently, the potential range that could be covered did not suffice to reach the expected maximum.

The measurements of the potential dependence of j_{max} do not allow one to determine the preferential

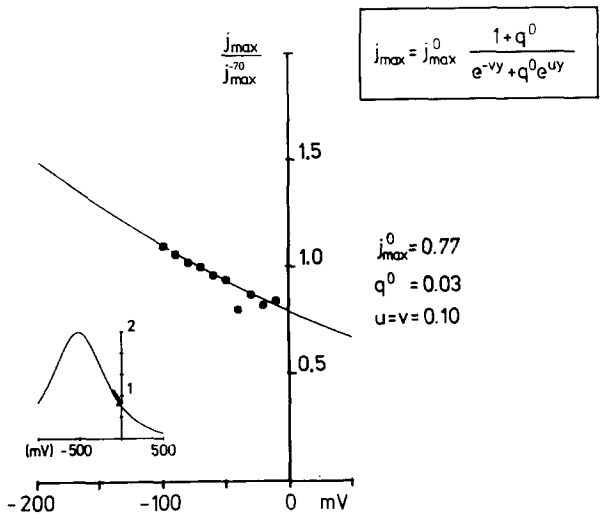

Fig. 8. Same data as in Fig. 5. The solid line represents a fit of Eq. (3) to the data points based on the assumption $u = v$; the fitted parameters are (±SEM): $j_{max} = 0.77 \pm 0.01$, $q^o = 0.031 \pm 0.024$, $u = v = -0.099 \pm 0.006$. The inset represents the same data but shows the fitted curve also at potentials outside the experimentally covered range

orientation of the band-3 protein since the same relationship between j_{max} and membrane potential is obtained if q^o is replaced by $1/q^o$ and the signs of the effective charges u and v are inverted [*see* Eq. (3)]. For an unequivocal evaluation of the data one needs, therefore, to know whether q^o is larger or smaller than 1.0. This requires additional and independent information, which is not supplied by the experiments described in this paper. From flux measurements on human red cells it is known that at equal anion concentrations inside and outside the red cell the anion-loaded transport protein is preferentially inwardly oriented, i.e., $q < 1$ (reviewed by Knauf, 1986; Passow, 1986). It is unknown whether this is also true for the mouse band-3 protein in the oocyte membrane. However, the concentrations at which the competitive inhibitor DNDS give 50% inhibition of chloride exchange are about the same in mouse red cells and in the oocytes and amount to 1.5 and 2 μM, respectively (M. Raida and H. Passow, *unpublished*); these values are close to the 4 μM reported for human red cells. These similarities suggest that in the oocytes, as in red cells, the number of anion-loaded band-3 molecules that are inwardly directed exceeds the number of the outwardly directed molecules. The discussion that follows below is based on this assumption.

If $q^o \ll 1$, then the potential dependence of the exchange flux may be described by Eq. (6a):

$$j = j^o_{max} \exp(uy).$$

Thus, in a semilogarithmic plot of j_{max} versus y one would expect to obtain a straight line with a slope that depends on the effective charge of the loaded transport protein. The experimental data in fact distribute around a straight line in this presentation (Fig. 5*B*). This supports the view that the anion-loaded transport molecules show a preferential distribution under the experimental conditions. From the slope we can estimate that the electrical field in the membrane acts on an effective charge of the transport molecule of about −0.09 unit charge. Of course, the data do not allow one to distinguish whether such negative charge is moved during the conformational transition $ar \rightarrow as$ from inside to outside or whether an equally sized positive charge is moved in the opposite direction.

The simplifications described do not permit an estimate of the distribution ratio q^o of anion-loaded outwardly and inwardly oriented conformers of the band-3 protein at zero-mV potential. Such an estimate can, however, be obtained by fitting Eq. (3) to the data. If one assumes $u = v$ one obtains $q^o = 0.03$ (*see* Fig. 8). This value agrees with the assumption that the majority of the binding sites of the loaded transport molecules are oriented to one of the two membrane surfaces.[1] Using Eq. (3) it is also possible to calculate the effective charge that is redistributed in the electrical field; the result $u = v = -0.10$ (*see* legend to Fig. 8) agrees with the value derived from the semilogarithmic plot of the data (Fig. 5*B*). It should be emphasized that identical fits are obtained for $q^o = 1/0.03 \approx 32$ and $u = v = +0.11$. However, if one agrees that the preferential orientation is inward as stipulated above, then one would infer $q^o = 0.03$ and $u = v = -0.10$ to be correct. This means that during the conformational change of the anion-loaded transport protein from the inwardly to outwardly oriented conformer, 0.1 of a negative unit charge moves outward or 0.1 of a positive charge moves inward.

One should notice that the term "effective charge" does not simply indicate the net charge of the transport protein at the substrate binding site. Instead it refers to the total spatial charge configuration of the loaded transport protein that is subjected to changes of the electrical field in the membrane. The contribution of the bound anion to the electrical momentum depends on its location relative to other

[1] The inset in Fig. 8 also indicates the potential dependence expected at voltages outside the range that could be covered experimentally. Its shows the predicted maximum and suggests that it is located at about −430 mV, a value not accessible to voltage clamp. The assumption $u = v$ used in the calculations is purely conjectural and has neither an empirical nor a theoretical basis.

charged groups in the transport protein. Hence the low numerical value of the effective charge calculated from the experimental data does not necessarily imply a nearly complete local balance of positive and negative charges at the substrate binding site as one would expect, for example, after chloride binding to the positive charge of an arginine residue. Thus the effective charge tells something about the charge distribution within the transport molecule as a whole and refers to the conformational change that it rate-limiting under the given set of experimental conditions.

Potential Dependence of $K_{1/2}$. In conclusion of this discussion, it should be pointed out that not only j_{max} but also $K_{1/2}$, the half-saturation concentration, varies with membrane potential [*see* Eq. (4)]. This is also true if the dissociation constants of chloride binding to the inward- and outward-facing conformers of the transport protein, K_{11} and K_{101}, respectively, are potential independent [*see* Eqs. (1) and (4), Fig. 9]. When the electrical field recruits all transport molecules to the inner surface, $K_{1/2}$ yields the limiting value K_{101}; when it recruits all transport molecules to the opposite surface, $K_{1/2}$ approaches the limiting value K_{11}. With the knowledge of numerical values for the parameters u, v and q^o, it is possible to describe explicitly the potential dependence of $K_{1/2}$, provided numerical values for K_{11} and K_{101} are known from independent measurements. $K_{1/2}$ is only independent of potential if $K_{11} = K_{101}$ [*see* Eq. (4)]. In our voltage-clamp experiments we could cover the potential range of -100 to -10 mV; this would not be sufficient to detect a possible potential dependence of $K_{1/2}$.

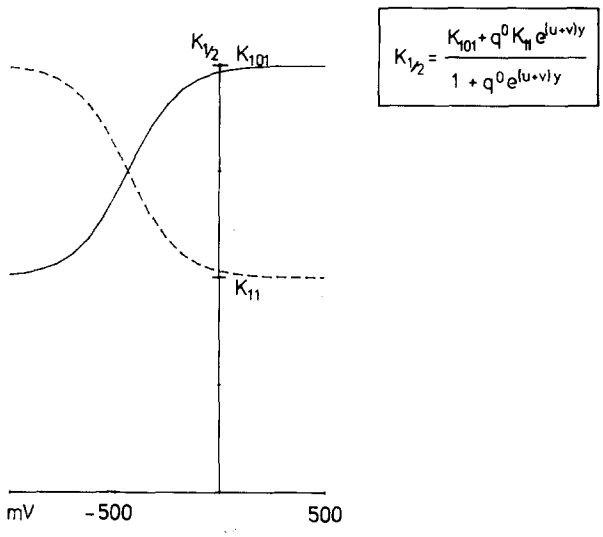

Fig. 9. The solid line represents the potential dependence of $K_{1/2}$ predicted by Eq. (4) with the parameters given in Fig. 8 and assuming $K_{101} = 2\ K_{11}$. The same extended range is shown as in the inset of Fig. 8. The numerical values for K_{11} and K_{101} were arbitrarily chosen to provide an example for the predictions made by Eq. (4). The values of K_{11} and K_{101} used for the calculations are indicated on the ordinate. For $K_{11} = K_{101}$ there is no potential dependence of $K_{1/2}$. The broken line is obtained if K_{101} is replaced by K_{11}, and K_{11} by K_{101}

The Membrane Potential Modulates the Activity of Band-3-Mediated Transport during Meiotic Maturation

The study of the potential dependence is not only a necessary requisite for the characterization of the molecular mechanism of the band-3-mediated anion transport. It is also of interest for the understanding of the physiological changes of transport processes which take place during oocyte maturation. The development of the full-grown oocytes is arrested in the prophase of the first meiotic division. The oocytes may remain in this resting state in the ovarial follicles up to several months. Upon exposure to progesterone the further maturation of the oocytes can be induced; this leads to the completion of the first meiotic division and the eventual shedding of the oocytes. During this maturation process, the activities of many endogenous transport systems of the oocyte plasma membrane are drastically reduced (Richter et al., 1984).

The decrease of the band-3-mediated chloride influx by about 40% during progesterone-induced maturation is similar to the changes expected to occur in response to the observed depolarization of the membrane potential from about -70 mV before maturation to nearly zero mV after maturation (*compare* Figs. 5 and 6). Thus the activity change can be understood as a consequence of the change of membrane potential which accompanies the maturation process. This suggests that the intrinsic properties of the band-3 protein remain unaltered and that this foreign transport protein is not subjected to regulation by biochemical modification of its activity. It resembles in this respect the behavior of the endogenous Na-alanine cotransport system (Jung et al., 1984); the activity of this transport system also depends on membrane potential, and its decrease of activity during oocyte maturation can also be accounted for as a result of the depolarization. This behavior differs from that of the sodium-potassium pump. In the oocytes, the potential dependence of the pump activity (Lafaire & Schwarz, 1986) would require an increase of the pump activity during the maturation-induced depolarization. In contrast, the pump becomes completely inactivated and completely loses the capacity for ouabain binding (Richter et al., 1984). Thus the downregulation of the pump requires the participation of a specific biochemical mechanism. Although this has not yet

been studied in sufficient detail, one may suspect that this is also true for the inhibition of nucleoside transport, which also accompanies the maturation process (Richter et al., 1984). Thus the decrease of the different transport activities during oocyte maturation cannot be explained as the result of one single global effect but represents the operation of separate mechanisms on the individual transport systems.

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