

Effects of Membrane Potential on Electrically Silent Transport

Potential-independent Translocation and Asymmetric Potential-dependent Substrate Binding to the Red Blood Cell Anion Exchange Protein

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ABSTRACT Tracer anion exchange flux measurements have been carried out in human red blood cells with the membrane potential clamped at various values with gramicidin. The goal of the study was to determine the effect of membrane potential on the anion translocation and binding events in the catalytic cycle for exchange. The conditions were arranged such that most of the transporters were recruited into the same configuration (inward-facing or outward-facing, depending on the direction of the Cl^- gradient). We found that the membrane potential has no detectable effect on the anion translocation event, measured as $^{36}\text{Cl}^-$ - Cl^- or $^{36}\text{Cl}^-$ - HCO_3^- exchange. The lack of effect of potential is in agreement with previous studies on red cells and is different from the behavior of the mouse erythroid band 3 gene expressed in frog oocytes (Grygorczyk, R., W. Schwarz, and H. Passow. 1987. *J. Membr. Biol.* 99:127–136). A negative potential decreases the potency of extracellular SO_4^{2-} as an inhibitor of either Cl^- or HCO_3^- influx. Because of the potential-dependent inhibition by SO_4^{2-} , conditions could be found in which a negative intracellular potential actually accelerates $^{36}\text{Cl}^-$ influx. This effect is observed only in media containing multivalent anions. The simplest interpretation of the effect is that the negative potential lowers the inhibitory potency of the multivalent anion by lowering its local concentration near the transport site. The magnitude of the effect is consistent with the idea that the anions move through 10–15% of the transmembrane potential between the extracellular medium and the outward-facing transport site. In contrast to its effect on extracellular substrate binding, there is no detectable effect of membrane potential on the competition between intracellular Cl^- and SO_4^{2-} for transport sites. The lack of effect of potential on intracellular substrate binding suggests that the access pathway leading to the inward-facing transport site is of lower electrical resistance than that leading to the extracellular substrate site.

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INTRODUCTION

The transport of HCO_3^- , Cl^- , and other inorganic anions across the red blood cell membrane is catalyzed by the major transmembrane protein known as band 3 or capnophorin (for comprehensive review, see Passow, 1986). Early work using cation ionophores demonstrated that most of the tracer Cl^- flux catalyzed by band 3 does not contribute the membrane conductance (Hunter, 1971; Knauf et al., 1977). Band 3 does mediate a small conductive Cl^- flux, but in the presence of 100–150 mM Cl^- on both sides of the membrane, <0.1% of the exchange flux carries net current (Knauf et al., 1977; Fröhlich et al., 1983; Knauf et al., 1983; Fröhlich, 1984).

The anion exchange flux through band 3 is believed to take place by way of a ping-pong mechanism, in which the exchange partners take turns crossing the membrane. At least at low temperatures (but possibly not at higher temperatures), substrate anion binding is much more rapid than translocation (Falke et al., 1985). The influx half of the catalytic cycle consists of binding of an extracellular anion to an outward-facing site, inward translocation by way of a conformational change, and release into the intracellular medium. Before any other anion can be transported inward, the cycle must be completed by the binding and outward translocation of an intracellular anion (see Knauf, 1979, 1989; Jennings, 1982; Fröhlich and Gunn, 1986; Passow, 1986). In the absence of detailed structural information, the substrate binding sites have kinetic definitions: the outward-facing binding site is defined as the location of the substrate anion immediately before the rate-limiting inward translocation event.

This article concerns the effects of membrane potential on the elementary events in the catalytic cycle for anion exchange. The work was stimulated by the finding of Grygorczyk et al. (1987) that the mouse band 3 gene product, when expressed in *Xenopus* oocytes, catalyzes a $^{36}\text{Cl}^-$ - Cl^- exchange flux that is affected significantly by membrane potential. The magnitude of the observed effect of potential is consistent with the idea that the anion translocation event involves the movement of 0.2 charges through the transmembrane potential drop,¹ or a full charge through 20% of the potential drop, or any combination of charge and fractional potential drop, which, when multiplied together, equals 0.2.

The effects of potential observed by Grygorczyk et al. (1987) are quite different from the results of similar experiments in intact red cells and resealed ghosts. Gunn and Fröhlich (1979) and Fröhlich et al. (1983) found no significant effect of membrane potential on the maximum Cl^- - Cl^- exchange flux. Milanick and Gunn (1984) measured the effect of membrane potential on the net influx of SO_4^{2-} into Cl^- -loaded cells. They found that at acid pH, where the entire SO_4^{2-} influx consists of

¹ The text of the Grygorczyk et al. (1987) paper states that the effects of potential are consistent with the movement of 0.1 equivalent charges through the transmembrane potential drop. However, the derivation used by Grygorczyk et al. did not consider that the potential should affect the translocation rate only during the uphill phase of the reaction in each direction. If the barrier is symmetric, there should be a factor of 0.5 in the exponential terms for the effect of potential on the inward and outward translocation rates (see Läger, 1987). Therefore, the data of Grygorczyk et al. are consistent with the equivalent of 0.2, not 0.1, charges moving through the transmembrane field.

$\text{H}^+\text{-SO}_4^-$ cotransport (Jennings, 1976), there is no detectable effect of potential on the SO_4^- influx. In agreement with the data of Milanick and Gunn (1984), we have recently reported (Jennings, 1989*a, b*) that valinomycin-mediated changes in membrane potential do not affect the maximum SO_4^- influx at acid extracellular pH. These findings are consistent with the idea that, when band 3 is operating in the exchange mode, the anion translocation event involves essentially no net charge (<0.1 elementary charges) moving through the membrane potential. Similar conclusions were reached by Wieth et al. (1980), who showed that valinomycin does not affect the tracer influx of Cl^- from a low Cl^- medium into high Cl^- ghosts. In summary, there are several indications that membrane potential has essentially no effect on the anion translocation event in the native red cell membrane, but no systematic study of this question has been published. Moreover, as pointed out clearly by Grygorczyk et al. (1987), the lack of effect of potential on coupled exchange can be misleading if the experiments cover only conditions in which the inward-facing and outward-facing states are symmetrically distributed.

A separate but related issue is the effect of potential on the apparent affinity of band 3 for substrate anions and anionic inhibitors. We recently reported (Jennings, 1989*a, b*) that, although membrane potential does not affect SO_4^- translocation, it does have a detectable effect on the apparent affinity of band 3 for SO_4^- binding to the outward-facing transport site. The SO_4^- influx data are consistent with the idea that there is an access channel leading from the bulk extracellular medium to the outward-facing substrate site. (Again, the "substrate binding site" is by definition the location of the transported anion immediately before the rate-limiting inward translocation event.)

Our finding of an effect of potential on the apparent binding affinity for extracellular SO_4^- differs from results obtained with monovalent anions. Fröhlich et al. (1983) found no effect of potential on the $K_{1/2}$ extracellular Cl^- at fixed intracellular Cl^- . Although the $K_{1/2}$ reflects not only the actual binding affinity but also the asymmetry in the translocation rates (Fröhlich and Gunn, 1986; Passow, 1986; Knauf, 1989), the lack of effect of potential on the $K_{1/2}$ for Cl^- suggests that the extracellular substrate site does not lie within the transmembrane field. In addition, Knauf (1989) cites unpublished data in which variations of the membrane potential have no effect on the apparent binding affinity of iodide for the external transport site.

The goal of this work was to examine, in intact red cells, the effects of membrane potential on monovalent anion exchange, measured under conditions designed to optimize the chances of detecting any effects that may be present. We find that the only measurable effect of potential is on the inhibitory potency of multivalent extracellular anions (e.g., SO_4^-); the results suggest that SO_4^- experiences $\sim 10\%$ of the transmembrane potential in moving from the extracellular bulk medium to the outward-facing transport site. In contrast, the inhibition by SO_4^- of the intracellular Cl^- binding is not detectably voltage dependent. There appears, then, to be a larger potential drop leading to the outward-facing transport site than to the inward-facing site. There is no detectable potential dependence of the translocation event for either Cl^- , HCO_3^- , or H^+/SO_4^- .

MATERIALS AND METHODS

Materials

Human blood was drawn into EDTA and generally used after no more than 4 d of storage at 4°C as whole blood; there was no detectable effect of storage on the anion flux. Gramicidin D was obtained from Calbiochem-Behring Corp., La Jolla, CA (lot 500113) and was used as a 10 µg/ml ethanolic stock. ⁸⁶RbCl was purchased from DuPont/New England Nuclear (Boston, MA). H³⁶Cl was purchased from ICN Biomedicals Inc. (Irvine, CA) and neutralized with NaOH. Bovine serum albumin (fraction V) and phloretin were from Sigma Chemical Co. (St. Louis, MO). Media containing *N*-methyl-D-glucamine (NMG) as the only cation were prepared from the appropriate acids (H₂SO₄, HCl, MOPS acid), titrated to pH 7.0 (room temperature) with NMG base, which was purchased from Sigma Chemical Co. Other salts and buffers were purchased from either Sigma Chemical Co. or Fisher Scientific Co. (Pittsburgh, PA).

Preparation of Cells

For many of the experiments, most of the intracellular Cl⁻ was replaced with SO₄⁻. The reason for loading cells mainly with SO₄⁻ is that the total anion conductance is lower in SO₄⁻-loaded cells than in all-Cl⁻ cells (Knauf et al., 1977), and gramicidin therefore has a larger effect on membrane potential. To remove most of the Cl⁻, cells were suspended in 20 vol of 110 mM K₂SO₄ and incubated 10 min at 37°C. The half-time for Cl⁻ efflux into a Cl⁻-free SO₄⁻ medium is <1 min at this temperature (Parpart and Hoffman, 1954). The suspension was then washed twice in 20 vol of 80 mM K₂SO₄, 30 mM KCl, 10 mM MOPS, pH 7.0. (The pH was measured at room temperature; the pH of the solution at 0°C was ~7.2; at 37°C the pH was ~6.9.) Before each spin the suspension was incubated 10 min at 37°C to allow equilibration of the Cl⁻ and SO₄⁻. In several of the preparations the intracellular Cl⁻ concentration was estimated with ³⁶Cl⁻ and found to be between 25 and 30 mM, as expected from the fact that the Donnan ratio is near unity at this pH (e.g., Gunn et al., 1973). For loading cells with different Cl⁻ concentrations (e.g., 5 mM), the same procedure was used, except that the final two washes were in 5 mM KCl, 97 mM K₂SO₄, 10 mM MOPS, pH 7.0.

For the experiments in Fig. 2 in which the intracellular Cl⁻ concentration was lowered by raising intracellular pH, whole blood was diluted with 20 vol of 165 mM KCl, and K₂CO₃ was added to raise extracellular pH to ~8.5 (room temperature). In the presence of 2–3 mM HCO₃⁻, the intracellular pH rises rapidly in response to the extracellular alkalization. After 5 min at room temperature cells were centrifuged and washed three times in unbuffered 165 mM KCl and finally suspended in 165 mM KCl containing 0.2% serum albumin and chilled to 10°C for gramicidin treatment (see below).

Gramicidin Treatment and Estimation of Membrane Potential from Rb Flux

To verify that gramicidin treatments had the expected effect on membrane potential, fluxes of ⁸⁶Rb⁺ were measured under the conditions of the anion exchange experiments. For ⁸⁶Rb⁺ loading, cells were incubated 2 h at 37°C in HEPES-buffered physiological saline (150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM Na-phosphate, 1 mM CaCl₂, 10 mM HEPES, pH 7.5 at 22°C, 10 mM glucose) plus ⁸⁶RbCl (30 µCi/ml cells). Cells were then washed in K₂SO₄ and/or KCl for anion loading as described above. There is only slight ⁸⁶Rb⁺ loss during the 30 min of incubations for SO₄⁻ loading (which is, of course, before exposure to gramicidin).

For all experiments (either with ⁸⁶Rb⁺ or ³⁶Cl⁻) using gramicidin, cells were pretreated with gramicidin (1 µg/ml cells; 3% hematocrit) for 10 min at 10°C immediately before the flux experiment. The pretreatment media contained 2 mg/ml serum albumin; this amount of albumin lowers the gramicidin-induced conductance somewhat, but albumin helps minimize

mechanical lysis during rapid resuspension of cold, packed cells. The pretreatment was always in media containing the Cl^- and SO_4^- concentrations and pH with which the cells had been equilibrated. For the cells that had been preloaded with $^{86}\text{Rb}^+$, the gramicidin pretreatment was in a medium containing NMG as the only cation. (In a K or Na medium all the tracer would exit the cells during the pretreatment.) For the $^{36}\text{Cl}^-$ flux experiments the pretreatment was in a K^+ medium. All flux experiments were initiated by adding 0.12 ml cells to 5 ml flux medium; the medium contained 0.2% ethanol and 0.1 μg gramicidin.

Cl Efflux

After the gramicidin pretreatment the cells were centrifuged and the supernate was removed to leave a pellet of ~80% hematocrit. To each milliliter of pellet, 15 μl of 100 mM Na^{36}Cl (~1.5 μCi) was added, and the pellet was mixed by gentle swirling in the bottom of a 40-ml tube. The pellet was then chilled on ice and the efflux of $^{36}\text{Cl}^-$ was initiated by adding 0.12 ml of the 80% suspension to 5 ml of flux medium. As in the $^{86}\text{Rb}^+$ efflux experiments, the flux medium contained 0.2% ethanol and 0.1 μg gramicidin.

For preliminary experiments, the method of Ku et al. (1979) was used to measure ^{36}Cl efflux. In this method samples of a stirred suspension are taken with a repeating syringe and plunged into a DIDS-containing stop solution, then centrifuged, and a known amount of supernate is counted. For most of the experiments, however, a different stop method was used in which the pellets rather than the supernates were counted. The rationale for this somewhat more laborious method is that, for rapid fluxes, most of the samples are taken after the transport is 75% complete, thus requiring the determination of small differences between large numbers. Counting pellets is more accurate because the final (equilibrium) pellet radioactivity is much smaller than that of the time points, and difference formation is not such a problem. For the pellet-counting method, 0.8-ml samples of suspension were plunged into 9 ml of ice-cold 100 mM K_2SO_4 , 20 mM citrate, pH 5.5, containing 100 μM phloretin. Phloretin stops the exchange immediately (Wieth et al., 1973) and is less expensive than DIDS. The tubes were centrifuged 2 min at 4000 rpm, the supernates were discarded, and the radioactivity in the pellets was determined after lysis and precipitation of protein with trichloroacetic acid (Jennings, 1980).

Cl Influx

The above method for counting pellets was also used to determine $^{36}\text{Cl}^-$ influx. Cells were prepared and pretreated with gramicidin exactly as for the efflux measurements. The influx of $^{36}\text{Cl}^-$ was initiated by adding 0.12 ml of 80% suspension to 5 ml of flux medium. The medium consisted of combinations of K^+ and NMG salts of 100 mM SO_4^- or 150 mM CH_3SO_3^- , buffered with 10 mM MOPS. The medium contained no HCO_3^- (20 min N_2 bubbling) and no added carbonic anhydrase, and contained 100 μM Cl^- with a trace (0.03 μCi) of $^{36}\text{Cl}^-$. At the beginning of the experiment, then, there was a 300-fold outward gradient of Cl^- for the cells loaded with 30 mM Cl^- .

RESULTS

Clamping the Potential with Gramicidin

Fröhlich et al. (1983) showed that gramicidin is a more appropriate ionophore than valinomycin for the purpose of altering the membrane potential of red cells at low temperatures. To ensure that gramicidin does in fact induce large potential changes under the conditions of our $^{36}\text{Cl}^-$ transport experiments, we used $^{86}\text{Rb}^+$ as a test

ion. Fig. 1 A shows the time course of efflux of $^{86}\text{Rb}^+$ from cells pretreated with $1\ \mu\text{g}$ gramicidin/ml cells for 10 min at 10°C and then centrifuged and resuspended in a medium containing $150\ \text{mM}\ \text{Cl}^-$, $10\ \text{mM}$ MOPS, and either NMG^+ as the only cation, or $135\ \text{mM}\ \text{NMG}^+$ and $15\ \text{mM}\ \text{K}^+$. The time course of $^{86}\text{Rb}^+$ efflux is clearly much faster into the medium containing $15\ \text{mM}\ \text{K}^+$. Assuming that the efflux of $^{86}\text{Rb}^+$ follows the constant field equation, we estimate that the membrane potential in the K^+ -free medium is about $-130\ \text{mV}$. Thus, as expected, gramicidin induces a cation conductance that is much higher than the Cl^- conductance.

Many of our $^{36}\text{Cl}^-$ flux experiments were performed in the presence of an outward Cl^- gradient. Under these conditions the Cl^- conductance is high when the intracellular Cl^- concentration is $100\ \text{mM}$ (Fröhlich et al., 1983); gramicidin-mediated changes in membrane potential therefore tend to be smaller. To minimize the total anion conductance in the presence of an outward Cl^- gradient, most of the

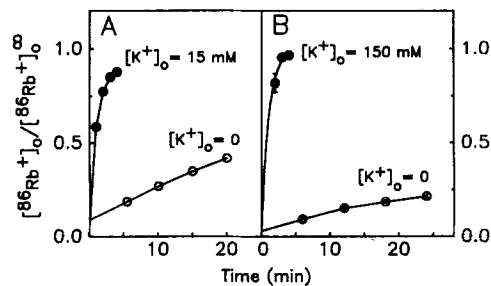


FIGURE 1. Membrane potential changes induced by gramicidin and K^+ gradient. Cells were loaded with $^{86}\text{Rb}^+$ and pretreated with gramicidin (see Methods) before being resuspended in media containing varying concentrations of K^+ . The medium also contained gramicidin ($0.02\ \mu\text{g}/\text{ml}$). A, Cells containing $150\ \text{mM}\ \text{Cl}^-$ suspended in a $150\ \text{mM}\ \text{Cl}^-$ medium

buffered with $10\ \text{mM}$ MOPS, $\text{pH}\ 7.2$ (0°C). *Open symbols*, K^+ -free NMG^+ medium; *solid symbols*, $15\ \text{mM}\ \text{K}^+$ substituted for equivalent amount of NMG^+ . B, Cells containing $30\ \text{mM}\ \text{Cl}^-$, $80\ \text{mM}\ \text{SO}_4^-$ were suspended in media containing $30\ \text{mM}\ \text{Cl}^-$, $120\ \text{mM}\ \text{CH}_3\text{SO}_3^-$, and either $150\ \text{mM}\ \text{K}^+$ or zero K^+ (NMG^+) as indicated. The data obtained in K^+ -free media include two effluxes each in media containing $30\ \text{mM}\ \text{Cl}^-$ and no Cl^- (CH_3SO_3^- replacement). The presence of an outward Cl^- gradient has no detectable effect on the initial efflux of $^{86}\text{Rb}^+$, indicating that the membrane potential is still strongly negative in the presence of an outward Cl^- gradient. All data are represented as the extracellular $^{86}\text{Rb}^+$ relative to that at infinite time, determined by lysing the cells in trichloroacetic acid.

intracellular Cl^- was replaced by SO_4^- . Under these conditions ($30\ \text{mM}\ \text{Cl}^-$, $80\ \text{mM}\ \text{SO}_4^-$), in either the presence or absence of an outward Cl^- gradient, gramicidin induced major changes in potential (Fig. 1 B). The magnitudes of the $^{86}\text{Rb}^+$ fluxes indicate that the membrane potential is approximated by the K^+ Nernst potential for all values of extracellular K^+ above $\sim 1\ \text{mM}$ (see Appendix).

Lack of Effect of Potential on Anion Exchange under Symmetric Conditions

In light of the potential-dependent exchange fluxes observed by Grygorczyk et al. (1987), we decided to reexamine the effect of potential on $^{36}\text{Cl}^-$ exchange under symmetric conditions (0°C , $\text{pH}\ 7.2$, $150\ \text{mM}\ \text{Cl}^-$ on both sides of the membrane). The data in Fig. 2 show that, as found earlier by Gunn and Fröhlich (1979) under somewhat different conditions, there is no detectable effect of potential on Cl^- self-exchange. Three other experiments were performed with the same extracellular

medium (pH 7.2) but with cells equilibrated at an intracellular pH of ~ 8.2 . The reason for loading cells at higher pH is to lower the intracellular Cl^- concentration to a value closer to that of the oocytes used by Grygorczyk et al. (1987). At the higher intracellular pH, the rate constant for tracer efflux is higher because of the smaller internal Cl^- compartment (Gunn et al., 1973). In contrast to the behavior of mouse band 3 expressed in oocytes, there is no effect of membrane potential on the exchange flux, even with the inward Cl^- gradient.

As described in detail by Grygorczyk et al. (1987), experiments such as those in Fig. 2 can be interpreted only if there is independent information on the distribution of inward-facing and outward-facing transport systems (see also Lauser, 1987). Even if there is net charge movement or dipole reorientation in the translocation event, there still exists a membrane potential at which the exchange flux is maximal. Departure of the potential from this value, in either direction, inhibits the exchange flux, but the effects of potential near the optimum are small. Human red cell band 3 is functionally asymmetric; in the presence of symmetric Cl^- the majority of the

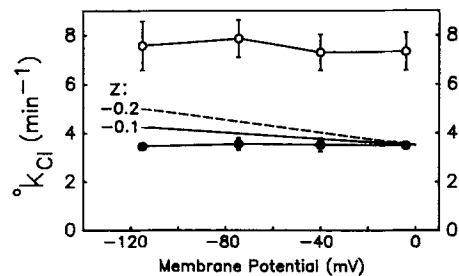


FIGURE 2. Lack of effect of membrane potential on ^{36}Cl -Cl exchange in a medium containing 150 mM Cl^- . The rate constant for $^{36}\text{Cl}^-$ efflux was measured in cells containing 150 mM Cl^- , intracellular pH 7.0-7.2 (solid symbols), or ~ 75 mM Cl^- , intracellular pH 8.0-8.2 (open symbols). Cells were pretreated with gramicidin (See Methods), and gramicidin was

present in the flux medium, which contained 150 mM Cl^- buffered with MOPS at pH 7.2 (0°C). The membrane potential was varied by substituting NMG^+ for K^+ . The potentials were estimated from the Nernst potentials for K^+ . Data represent the mean (\pm SD) of six fluxes on three preparations of cells (solid symbols) or four to five fluxes on three preparations of cells (open symbols).

transporters are inward-facing (Frohlich and Gunn, 1986; Passow, 1986; Knauf, 1989). Fig. 2 includes the calculated potential dependence of the exchange flux if either 0.2 or 0.1 equivalent charges move through the transmembrane potential; the calculation is similar to that by Grygorczyk et al. (1987) except that we assumed that 10% rather than 3% of the transporters face inward (the exact fraction of inward-facing states makes little difference over this range of potentials). Our experiments thus provide no indication that there is net charge movement in the translocation event; the data are consistent with a completely electroneutral translocation event.

Lack of Effect of Potential in the Presence of a Large Inward Cl Gradient

To examine the effect of potential under conditions of a more extreme inward Cl^- gradient, cells were loaded at pH 7.2 with 97 mM SO_4^{2-} , 5 mM Cl^- , and the efflux of $^{36}\text{Cl}^-$ was measured in the same 150 mM Cl^- medium as was used in the experiments described above. The rate constant for the efflux is quite large under these conditions, but it is possible to obtain reasonably accurate data by counting pellets

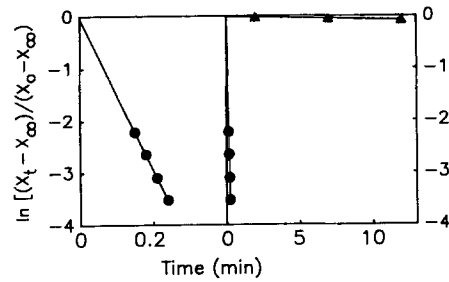


FIGURE 3. Time course of $^{36}\text{Cl}^-$ efflux in the presence of a large inward Cl^- gradient. Cells were loaded with 97 mM SO_4^- , 5 mM Cl^- , pH 7.2, and the efflux of $^{36}\text{Cl}^-$ was measured in 150 mM KCl, 10 mM MOPS, pH 7.2 (0°C). The phloretin stop method was used (see Methods) with a repeating syringe. The time course of a typical efflux is shown on the left, and the

same data are replotted on a slower time scale on the right, along with the time course (*triangles*) of $^{36}\text{Cl}^-$ loss from cells loaded identically but resuspended directly in stop solution that had been supplemented with the appropriate amount of flux solution.

rather than supernates (Fig. 3). In three experiments, we found essentially no effect of membrane potential on $^{36}\text{Cl}^-$ efflux in the presence of a large inward Cl^- gradient (Fig. 4). As in the other experiments, the cells were pretreated with gramicidin in the anion loading medium. Therefore, even though each flux measurement lasted only a few seconds, the pretreatment allowed gramicidin to have its full effect on the membrane potential. It is noteworthy that the $^{36}\text{Cl}^-$ efflux is potential independent even in the presence of a large excess of divalent intracellular competitor, indicating that potential does not affect the local concentration of anions near the inward-facing transport site (see below).

Anion Exchange in the Presence of a Large Outward Cl Gradient

The remainder of the experiments in this paper were performed under conditions of a large outward Cl^- gradient ($[\text{Cl}^-]_i/[\text{Cl}^-]_o > 100$). The main advantage of these conditions is that the algebraic expression for the anion exchange flux is relatively simple. In addition, we wished to examine the possibility that the outward-facing substrate site lies sufficiently deep in the membrane that the local anion concentration near the site is affected by the transmembrane potential. The goal in the design of the experiments, then, is to arrange conditions such that an anion exchange flux can be measured in the presence of a large outward Cl^- gradient, and the membrane potential can still be varied over a wide range with gramicidin.

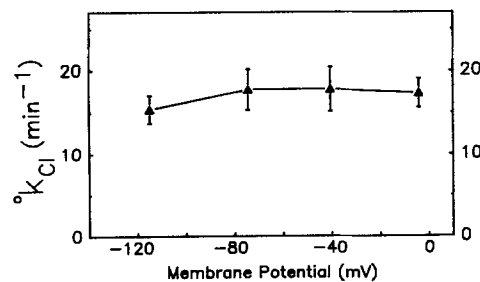


FIGURE 4. Lack of effect of potential on $^{36}\text{Cl}^-$ efflux into from low Cl^- cells into 150 mM Cl^- medium. Cells were loaded with 5 mM Cl^- , 97 mM SO_4^- , pH 7.2, and resuspended in media containing 150 mM Cl^- , buffered with 10 mM MOPS, pH 7.2 (0°C). The cells were pretreated with gramicidin, and gramicidin was present in the flux media. The mem-

brane potential was varied by substituting K^+ for NMG^+ . Data shown are the means (\pm SD) of six to eight fluxes from four preparations of cells.

Consider a situation in which the extracellular medium contains a very low concentration of a rapidly transported anion X^- , with no competing extracellular anions. As long as nearly all the transporters are facing outward, the algebraic expression for the unidirectional influx of X^- is quite simple:

$$J_x = Tk_x[X^-]_o / ([X^-]_o + K_x). \quad (1)$$

In Eq. 1 T is the total number of transporters per milliliter of cells, k_x is the unimolecular rate constant for inward translocation of X^- , and K_x is the concentration of X^- in the bulk solution at which half the outward-facing sites would be occupied with X^- in the absence of competing anions. The modifier (self-inhibitory) site (Dalmark, 1976; Knauf, 1979; Knauf and Mann, 1986) is ignored here.

According to Eq. 1, the anion exchange flux in the presence of very low extracellular substrate concentration is dependent on both the affinity and the inward translocation rate constant. Therefore, any observed effect of potential could be caused by an alteration in either parameter. The situation is improved by the presence of a high concentration of nonpenetrating (or slowly penetrating) extracellular competitor Y^- . Under these conditions, the transporters are still recruited into the outward-facing configuration, and the only difference is that now the expression for the influx of x^- includes the effects of the competitor Y^- :

$$J_x = \frac{Tk_x[X^-]_o/K_x}{(1 + [X^-]_o/K_x + [Y^-]_o/K_y)}. \quad (2)$$

At sufficiently high concentrations of Y^- , Eq. 2 can be approximated by:

$$J_x = Tk[X^-]_oK_y/[Y^-]_oK_x. \quad (3)$$

Eq. 3 contains as unknown parameters the inward translocation rate constant for X^- and the dissociation constants for X^- and Y^- to the outward-facing transport site. A priori, all these parameters could depend on potential. However, if the effect of membrane potential on substrate anion binding is simply a consequence of geometry (e.g., distance through an access channel), then the effect should be the same for all monovalent anions. Since the apparent dissociation constant for Y^- is in the numerator and that for X^- is in the denominator, any effect of membrane potential on the local concentration of X^- should be cancelled by the presence of the competing monovalent anion Y^- . That is, with full outward recruitment and a high concentration of monovalent competitor, it should be possible to study the effect of potential on the inward translocation rate constant.

The conditions chosen for the transport experiments are as follows. Cells were equilibrated with a medium containing 80 mM SO_4^{2-} , 30 mM Cl^- . The reason for SO_4^{2-} loading is that the SO_4^{2-} conductance of the membrane is known to be lower than the Cl^- conductance (Knauf et al., 1977). In a predominantly SO_4^{2-} medium, the effects of cationophores on the membrane potential are larger. The choice of 30 mM intracellular Cl^- is somewhat arbitrary. One of us showed some years ago that suspending cells loaded with 30 mM Cl^- and 80 mM SO_4^{2-} into a Cl^- -free SO_4^{2-} medium causes recruitment of >95% of the transporters into the outward-facing state, as judged by tracer SO_4^{2-} efflux (Jennings, 1980). Thus, the 30 mM intracellu-

lar Cl^- is a compromise between the goal of full outward recruitment of transport systems and that of a minimum anion conductance. (Fig. 1 *B* shows that the membrane potential can be clamped by gramicidin even in the presence of an outward Cl^- gradient. Fig. 4 shows that intracellular SO_4^- itself has no voltage-dependent effects on Cl^- transport.)

Outward Recruitment

In our previous studies on the outward recruitment of transporters by the Cl^- gradient, the major extracellular anion was SO_4^- (Jennings, 1980, 1982). For the present purposes it was desirable to use a monovalent anion; CH_3SO_3^- was chosen because it is essentially impermeant at 0°C , as indicated by the very slow efflux of $^{36}\text{Cl}^-$ into a CH_3SO_3^- medium (Fig. 5). According to the ping-pong model, the reason that the efflux is slow in the absence of extracellular Cl^- is that nearly all the transporters have been recruited into the outward-facing state and hence are unavailable for efflux.

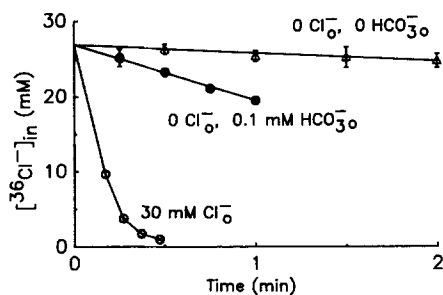


FIGURE 5. Time course of $^{36}\text{Cl}^-$ efflux into CH_3SO_3^- media, demonstrating the slow permeation rate of CH_3SO_3^- relative to Cl^- or HCO_3^- . Cells containing 30 mM Cl^- , 80 mM SO_4^- were loaded with $^{36}\text{Cl}^-$, and the efflux was measured at 0°C , pH 7.2, in media containing 120 mM KCH_3SO_3 , 30 mM KCl (open circles); 150 mM KCH_3SO_3 , 0.1 mM HCO_3^- , and 20 $\mu\text{g}/\text{ml}$ carbonic anhydrase

(solid circles); or 150 mM KCH_3SO_3 bubbled with N_2 for 30 min to remove atmospheric $\text{CO}_2/\text{HCO}_3^-$ (open triangles). Data represent mean and range of two determinations at each time point.

Cl-HCO₃ Exchange

The point of the foregoing is to establish conditions in which essentially all the transporters are in the outward-facing configuration and in which the membrane potential can also be varied over a wide range with gramicidin. The next step is to measure a band 3-mediated monovalent anion exchange flux in the presence of the outward Cl^- gradient. One way to do this is to include a small (but reproducible) amount of HCO_3^- in the extracellular medium along with carbonic anhydrase to accelerate the hydration-dehydration reactions such that transport is rate limiting. Intracellular $^{36}\text{Cl}^-$ leaves the cell in exchange for HCO_3^- , and CO_2 is recycled by the mechanism described years ago by Jacobs and Stewart (1942). Fig. 5 shows that the inclusion of HCO_3^- and carbonic anhydrase accelerates $^{36}\text{Cl}^-$ efflux into the CH_3SO_3^- medium, but the efflux is still slow compared with the efflux into a 30 mM Cl^- medium. Thus, most of the transporters are still facing outward, and anion exchange can be measured as Cl^- - HCO_3^- exchange.

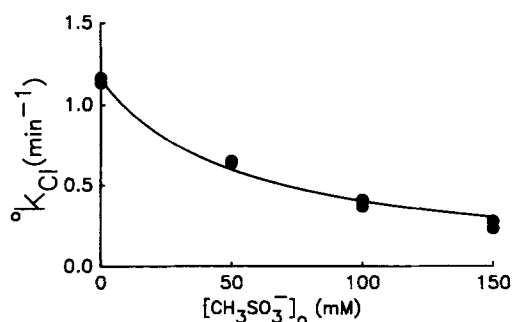


FIGURE 6. Inhibition by CH_3SO_3^- of $^{36}\text{Cl}^-$ efflux into medium containing 0.1 mM HCO_3^- and 20 $\mu\text{g}/\text{ml}$ carbonic anhydrase. Cells were loaded as in Fig. 5, and the efflux of $^{36}\text{Cl}^-$ was measured in media containing 0–150 mM KCH_3SO_3 , substituted for K-glutamate, in all cases buffered with 10 mM MOPS, pH 7.2 (0°C). Single experiment with two fluxes at each concentration.

Inhibition of Cl Exchange by Extracellular Methanesulfonate

Although CH_3SO_3^- itself is transported much more slowly than is Cl^- or HCO_3^- , it is a reasonably potent inhibitor of anion influx. Fig. 6 shows the effect of extracellular CH_3SO_3^- (substituted for glutamate) on the efflux of Cl^- into a medium containing carbonic anhydrase and 0.1 mM HCO_3^- . Glutamate was chosen as a substitute anion because we had found previously (Jennings, 1989a) that it appears to inhibit anion influx less than does gluconate, which is a commonly used replacement but is known to inhibit anion exchange with an IC_{50} of ~ 50 mM (Knauf and Mann, 1986). It is not possible to infer a precise K_1 for CH_3SO_3^- without knowledge of the affinity of the outward binding site for glutamate, but the data are consistent with a CH_3SO_3^- dissociation constant of ~ 20 mM. Accordingly, most of the outward-facing sites will be occupied with CH_3SO_3^- at an extracellular concentration of 150 mM, and the approximation represented by Eq. 3 is applicable to within ~ 10 –20%.

No Effect of Potential in Methanesulfonate Medium

Fig. 7 shows the time course of $^{36}\text{Cl}^-$ efflux from cells loaded as described above and suspended in a 150 mM methanesulfonate medium containing 100 μM HCO_3^- . The

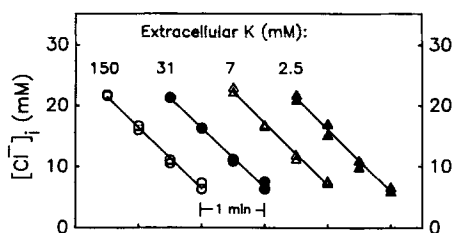


FIGURE 7. Lack of effect of membrane potential on Cl^- - HCO_3^- exchange in the presence of an outward Cl^- gradient. Cells were loaded with 30 mM Cl^- , 80 mM SO_4^- as in Figs. 5 and 6, and the efflux was measured in medium containing 150 mM CH_3SO_3^- , 0.1 mM HCO_3^- , and 20

$\mu\text{g}/\text{ml}$ carbonic anhydrase, buffered with 10 mM MOPS, pH 7.2 (0°C). Cells were pretreated with gramicidin, and gramicidin was present in the medium as in Fig. 1. The K^+ concentration was varied as indicated. Data shown are from a single experiment representative of three others. Note that the time course of the efflux is not exponential.²

² As shown previously (Jennings, 1980, 1982), the time course of anion heteroexchange is not in general an exponential under conditions of full outward recruitment of transporters. The net exchange flux is constant for over half the time course because the influx half-cycle is rate limiting, and extracellular substrate concentration does not change significantly.

extracellular K^+ concentration was varied by NMG^+ concentration was varied by NMG^+ replacement. As previously, the cells were pretreated with gramicidin at 10°C for 10 min, and gramicidin was also included in the flux medium. Extracellular K^+ in the presence of gramicidin has no detectable effect on the $^{36}\text{Cl}^-$ - HCO_3^- exchange flux in a CH_3SO_3^- medium. Thus, as is true for inward recruitment (Fig. 2), membrane potential has no detectable effect on the anion translocation event under conditions of outward recruitment.

Membrane Potential Does Have Effects in the Presence of Sulfate

The conditions of the experiment in Fig. 7 were chosen to minimize any possible effect of potential on the binding of anions to the outward-facing substrate site: if a negative potential inhibits HCO_3^- binding, it should also inhibit CH_3SO_3^- binding, and the effects should cancel. To examine the effect of potential on anion binding to the substrate site, the same experiment was performed in a SO_4^- medium (Fig. 8). As in Fig. 7, the medium contained $100\ \mu\text{M}\ \text{HCO}_3^-$ and $20\ \mu\text{g}$ carbonic anhydrase/ml.

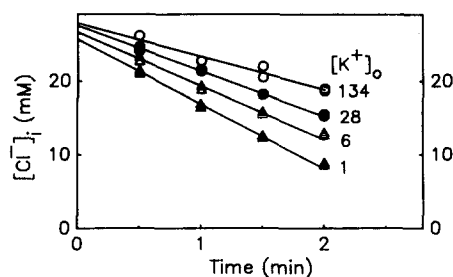


FIGURE 8. Effect of membrane potential on Cl^- - HCO_3^- exchange in the presence of an outward Cl^- gradient in a $100\ \text{mM}\ \text{SO}_4^-$ medium. Cells were loaded with $30\ \text{mM}\ \text{Cl}^-$, $80\ \text{mM}\ \text{SO}_4^-$ as in Figs. 5–7, and the efflux was measured in medium containing $100\ \text{mM}\ \text{SO}_4^-$, $0.1\ \text{mM}\ \text{HCO}_3^-$, and $20\ \mu\text{g}/\text{ml}$ carbonic anhydrase, buffered with $10\ \text{mM}\ \text{MOPS}$, pH 7.2

(0°C). Cells were pretreated with gramicidin, and gramicidin was present in the medium as in Fig. 2. The K^+ concentration was varied as indicated. Data shown are from a single experiment representative of five others.

The $^{36}\text{Cl}^-$ efflux at zero membrane potential is slightly slower in the SO_4^- medium than in the CH_3SO_3^- medium, presumably because SO_4^- is a slightly better competitor for extracellular HCO_3^- . In contrast to the results in Fig. 7, a negative membrane potential accelerates the $^{36}\text{Cl}^-$ efflux when the major extracellular anion is SO_4^- . Hyperpolarization of 80 – $100\ \text{mV}$ accelerates the initial $^{36}\text{Cl}^-$ efflux by a factor of 1.5 – 2.3 (range, six experiments). The accelerated efflux is not a conductive flux. If Cl^- conductance were this large, the initial $^{86}\text{Rb}^+$ efflux (Fig. 1 B) would be much larger than that observed. Specifically, the net Cl^- efflux into medium containing $1\ \text{mM}\ K^+$ is $\sim 10\ \text{mmol}/\text{liter}$ cell water per min (Fig. 8, *solid triangles*). Under similar conditions, the net conductive K^+ ($^{86}\text{Rb}^+$) efflux is only $\sim 1\ \text{mmol}/\text{liter}$ cell water per min (data not shown; one experiment, identical to that in Fig. 1 B, except with extracellular SO_4^- rather than CH_3SO_3^-). The acceleration must therefore represent an effect of potential on an electrically silent exchange process.

Potential Effects in the Presence of Extracellular Citrate

Citrate is often used as a substitute anion in both transport and binding studies on band 3 (Gunn and Fröhlich, 1979; Verkman et al., 1983; Schnell and Besl, 1984; Falke and Chan, 1986). Although 25 mM citrate has no effect of Cl^- self-exchange at pH 7.8 (Gunn and Fröhlich, 1979), the divalent form of citrate does appear to inhibit anion binding (Kaufmann et al., 1986). We find that, under our standard conditions of Cl^- - HCO_3^- exchange, 100 mM citrate has a major effect on the exchange rate: the ^{36}Cl efflux from the same cells is about four times as rapid into 250 mM sucrose as into 100 mM K_3 citrate, with the same 0.1 mM HCO_3^- and 20 μg carbonic anhydrase/ml. Although part of the effect of citrate could be related to the major change in ionic strength, the substantial inhibition indicates that extracellular citrate may be able to bind with low affinity to the substrate site. These results are in agreement with those of Kaufmann et al. (1986). Fig. 9 shows that, as is true in a SO_4^- medium, but not in CH_3SO_3^- , the ^{36}Cl - HCO_3^- exchange rate is accelerated by a

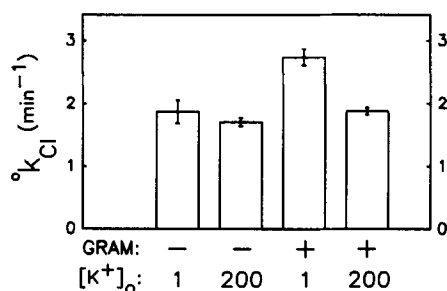


FIGURE 9. Effect of membrane potential on Cl^- - HCO_3^- exchange in the presence of an outward Cl^- gradient in a 100 mM citrate medium. Cells were loaded with 30 mM Cl^- , 80 mM SO_4^- as in Figs. 5-8, and the efflux was measured in medium containing 100 mM citrate, 0.1 mM HCO_3^- , and 20 $\mu\text{g}/\text{ml}$ carbonic anhydrase, buffered with 10 mM MOPS, pH 7.2

(0°C). Gramicidin treatment was as previously described. The K^+ concentration was varied as indicated. Data are the mean ($\pm\text{SD}$) of six fluxes on three preparations of cells. The Cl^- efflux is considerably faster and is more nearly exponential in the citrate medium than in SO_4^- or CH_3SO_3^- , because citrate is a weaker inhibitor of HCO_3^- influx. The rate constants for efflux were derived from the slopes of semi-log plots of the efflux time course.

negative membrane potential in a citrate medium. Therefore, the potential effect is not specific to SO_4^- but applies to at least one other multivalent anion.

Tracer Chloride Influx

As an independent confirmation that a negative potential accelerates monovalent anion exchange in a medium containing 100 mM SO_4^- and 0.1 mM rapidly permeant substrate anion, we measured $^{36}\text{Cl}^-$ influx from a low Cl^- medium into cells initially containing 30 mM Cl^- and 80 mM SO_4^- , i.e., loaded exactly as for the efflux experiment but without the intracellular tracer. The net efflux of Cl^- is slow compared with the tracer influx, and it is relatively easy to measure influx, because the intracellular compartment is large compared with the extracellular compartment. This kind of experiment was first done by Wieth and co-workers (1980).

A negative membrane potential in a SO_4^- medium accelerates $^{36}\text{Cl}^-$ influx (Fig. 10). Cells were prepared exactly as in the previous figures, and the influx of $^{36}\text{Cl}^-$ was measured from a medium containing 0.1 mM total Cl^- and no HCO_3^- or

carbonic anhydrase. The intracellular radioactivity increases with time over the first minute as the $^{36}\text{Cl}^-$ equilibrates between the intracellular and extracellular compartments. At much later times the activity declines slowly as the net efflux of Cl^- proceeds. The data show clearly that a negative membrane potential accelerates $^{36}\text{Cl}^-$ influx from the SO_4^- medium. A hyperpolarization of 80–100 mV accelerates the influx by a factor of 1.5–2.1 (range, eight preparations of cells; 16 pairs of fluxes; three to four time points each). In contrast, no acceleration (slight inhibition) was observed when the same experiment was performed in a 150 mM CH_3SO_3^- medium (four experiments, data not shown). The effect of potential in the SO_4^- medium is novel in that a negative intracellular potential accelerates the influx of a negative ion. This effect is observed only in the presence of a high concentration of extracellular divalent anion and a low concentration of extracellular monovalent substrate anion.

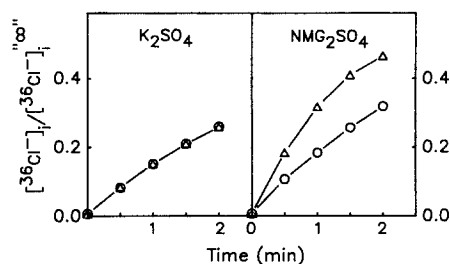


FIGURE 10. Acceleration of $^{36}\text{Cl}^-$ influx by a negative intracellular potential. Cells were loaded with 30 mM Cl^- , 80 mM SO_4^- as in Figs. 5–9, suspended in media containing 0.1 mM $\text{Cl}^-/^{36}\text{Cl}^-$ and 100 mM K_2SO_4 or NMG_2SO_4 as indicated, and buffered with 10 mM MOPS, pH 7.2 (0°C). Circles, No gramicidin present and no

pretreatment; triangles, gramicidin treatment as in the previous figures. Plotted is the intracellular radioactivity relative to that expected if the initial intracellular pool of Cl^- equilibrated with the extracellular isotope. In reality there is a slow net efflux of Cl^- , but the amount of net loss over the first 2 min is only ~10%.

DISCUSSION

The main findings in this paper are that:

1. Membrane potential has no detectable effect on the anion (Cl^- or HCO_3^-) translocation event intact human red blood cells.
2. The inhibition by extracellular SO_4^- of either Cl^- or HCO_3^- influx is voltage dependent; the results are consistent with our earlier report that SO_4^- experiences ~10% of the transmembrane potential drop in moving from the extracellular medium to the outward-facing substrate binding site.
3. The inhibition by intracellular SO_4^- of Cl^- efflux is not detectably dependent on voltage.

These three findings are discussed separately.

Electrically Silent Translocation Event

The finding that the anion translocation event is not detectably dependent on potential is in agreement with a sizable body of existing data (see Introduction). As explained by Grygorczyk et al. (1987), a thorough study of the effects of potential on anion exchange requires the use of varying anion gradients to induce different proportions of inward-facing and outward-facing states. We find no effect of

potential in either symmetric Cl^- , a 2-fold inward gradient, or a 100-fold outward gradient. The membrane potentials themselves were not measured but rather inferred from tracer fluxes of $^{86}\text{Rb}^+$ performed with the same flux solutions, gramicidin stock, hematocrit, and temperature. We believe that these indirect estimates of potential are valid, at least semiquantitatively. With valinomycin such inferences can be misleading because valinomycin is a carrier with differing translocation rates of the loaded and unloaded forms (see Bennekou and Christophersen, 1986). With the gramicidin channel, however, the reason that the $^{86}\text{Rb}^+$ efflux is so much slower in the K^+ -free medium must be that the membrane potential is quite negative.

Grygorczyk et al. (1987) found a much larger effect of potential on the flux in the oocyte expression system than we find in red cells; a 100-mV change in potential caused nearly a 1.5-fold increase in efflux. We find less than 10% change over a similar range of potentials. We do not know the reason for the difference, but we believe that it is unlikely to be a species difference. The amino acid sequences of mouse band 3 and human band 3 are highly homologous in the membrane domains (Kopito and Lodish, 1985; Tanner et al., 1988), and the arrangement of the polypeptide in the membrane appears to be very similar in the two species (Raida et al., 1989). Functionally, Cl^- transport is similar in human and mouse red cells (Hanke-Baier et al., 1988), except that the turnover number (ions per protein per second) is higher in mouse (Raida et al., 1989). There have been no estimates of potential dependence of Cl^- exchange in mouse red cells. Although species differences cannot be ruled out at present, the difference between our results and those obtained with the oocyte expression system are more likely a consequence of different processing or insertion, or final lipid environments of the protein in the two systems. In any case, in the native human red blood cell the anion translocation event is very nearly electrically silent. A small potential dependence could have escaped detection, but our data indicate that anion translocation involves the equivalent of <0.1 charges moving through the transmembrane field.

What is the nature of the translocation event? There is still no answer to this fundamental question. There is considerable evidence for a thin-barrier mechanism for the band 3-mediated anion exchange (see below); in a thin-barrier model, most of the transmembrane potential drop takes place over a narrow distance somewhere in the interior of the protein. The present data indicate that the rate-limiting kinetic event must involve very little net charge moved through this region of high electric field. If as little as 0.1 net charge moved all the way through the potential drop (or a full charge moved 10% of the way through the drop), there should have been a detectable effect on the exchange flux (Fig. 2). Beyond this rather limited statement, little else can be said about the nature of the translocation event. The event could involve the moving of gates, as in the lock-carrier (Gunn, 1978), or any of several other mechanisms, including a lateral realignment of two opposing half-channels or a local shift in the configuration of the anion-site complex.

Potential Dependence of Anion Binding

The data in Figs. 8 and 10 demonstrate in two independent ways that, in a medium containing 0.1 mM Cl^- or HCO_3^- and 100 mM SO_4^{2-} , a negative intracellular

potential accelerates monovalent anion exchange. From the data in Figs. 2, 4, and 7, it is unlikely that the voltage effect is related to the translocation event. The simplest explanation of the effect is that the inhibition by SO_4^- of either Cl^- or HCO_3^- influx is voltage dependent; a negative potential lowers the inhibitory potency and accelerates the exchange. Suppose the electrical potential near the extracellular anion binding site is a fraction ϕ of the transmembrane potential. The local concentration of Cl^- near the site should be a Boltzmann factor $e^{\phi FV/RT}$ times the bulk concentration. The local concentration of SO_4^- should be a factor of $e^{2\phi FV/RT}$ times the bulk concentration. Substituting into Eq. 3 gives the following expression for the potential dependence of the influx of Cl^- or HCO_3^- :

$$J = J_o e^{-\phi FV/RT} \quad (4)$$

In Eq. 4, J_o is the flux at zero membrane potential. The potential dependence of the flux, according to this interpretation, is simply a consequence of the fact that the local concentration of the divalent anion is more strongly dependent on potential. The SO_4^- concentration (100 mM) is much higher than the half-maximal inhibitory concentration (4–5 mM) at this temperature and pH (Milanick and Gunn, 1982), so the approximation represented by Eq. 3 should apply. The flux changes by a factor of 1.5–2 for a change of potential of 100 mV, indicating that $\phi = 0.10$ – 0.15 . That is, the incoming SO_4^- experiences ~10–15% of the transmembrane potential drop in moving from the bulk solution to the outward-facing site. This estimate is similar to the estimate of 10% derived from SO_4^- influx measurements at 20°C and pH 6 (Jennings, 1989*a, b*). Neither estimate is claimed to be precise; the important point is that we now have three kinds of evidence (SO_4^- influx; Cl^- - HCO_3^- exchange, and $^{36}\text{Cl}^-$ influx) that extracellular anion binding to the outward-facing transport site is influenced by the membrane potential. We believe that the most reasonable interpretation of these results is that the binding site lies at the base of a diffusional access channel.

In the Na^+ channel from rat skeletal muscle, there is voltage-dependent block by a variety of guanidinium toxins (Moczydlowski et al., 1984). Interestingly, the voltage dependence of the block is not affected by the net charge on the toxin. The charge independence of the block is consistent with the idea that the membrane potential changes toxin affinity by changing the conformation of the protein (Moczydlowski et al., 1984). A voltage-dependent change in conformation is of course a possible explanation of our results as well, but it should be emphasized that the Na^+ channel result is completely different from our finding that only multivalent, and not monovalent, anions give a potential-dependent inhibition of Cl^- or HCO_3^- influx. Moreover, the effect depends on the charge on the anion, not its shape. The inhibition of HCO_3^- influx by SO_4^- or citrate is voltage dependent, but that by CH_3SO_3^- and glucuronate (one experiment, not shown) is not voltage dependent. A voltage-dependent conformational change could conceivably change the SO_4^- and citrate affinity but not the CH_3SO_3^- affinity relative to that of HCO_3^- , but we believe that a simple biophysical mechanism, i.e., an access channel leading to the transport site, is a more likely explanation.

Other Evidence for an Extracellular Access Channel

There are several other indications of a diffusional access channel leading to the outward-facing transport site. Fluorescence resonance energy transfer measurements by Rao et al. (1979) indicate that the distance between the stilbenedisulfonate site and a sulfhydryl group on the cytoplasmic domain is only ~ 40 Å. Macara et al. (1983) later found that eosinmaleimide, bound at a site that overlaps with the stilbenedisulfonate site, can be quenched collisionally by intracellular Cs^+ ; therefore, either intracellular Cs^+ can diffuse most of the way outward before encountering a permeability barrier in the protein, or eosinmaleimide can move most of the way inward, perhaps in concert with a conformational change similar to that which seems to "lock in" stilbenedisulfonates such as DBDS (Verkman et al., 1983; Posner and Dix, 1985). The nuclear magnetic resonance studies of Falke and Chan (1986) showed that two inhibitors of band 3-mediated anion exchange can inhibit ^{35}Cl line broadening without competing for Cl^- binding. This result could be explained if the agents block diffusion of Cl^- through an access channel.

Further evidence for an access channel has been derived from chemical modification studies with Woodward's reagent K and borohydride. Short exposures at 0°C convert a glutamate side chain in band 3 to an alcohol (Jennings and Anderson, 1987). The modified glutamate residue appears to be the residue involved in the H^+ - SO_4^- cotransport that accompanies Cl^- - SO_4^- exchange (Jennings and Al-Rhaiyel, 1988). The modification of the outward-facing carboxyl group has a major effect on the intracellular pH dependence of SO_4^- efflux. The simplest interpretation of this result is that the carboxyl group can cross the permeability barrier (see Jennings and Al-Rhaiyel, 1988); thus, the finding is consistent with a thin-barrier model of the transport pathway. Any model with a single thin barrier requires diffusional access channels.

Asymmetry in the Voltage Dependence of SO_4^- -Cl Competition

There is abundant evidence for both structural and functional asymmetry in band 3 (see Gunn and Fröhlich, 1979; Knauf et al., 1984, 1989; Knauf and Mann, 1984; Passow, 1986), and it is of interest to ask whether the competition between intracellular Cl^- and SO_4^- is voltage dependent. The experiments in Fig. 4 show that, in the presence of a 30-fold inward Cl^- gradient and a 20-fold excess of intracellular SO_4^- over Cl^- , there is little effect of potential of the $^{36}\text{Cl}^-$ - Cl^- exchange flux. There may be very slight inhibition of Cl^- efflux by a negative potential, but certainly the effect of potential is much larger on extracellular SO_4^- - Cl^- competition. The $K_{1/2}$ for intracellular SO_4^- has been estimated to be 35 mM (Schnell and Besl, 1984). Accordingly, at 100 mM intracellular SO_4^- $\sim 75\%$ of the inward-facing transporters are expected to be occupied with SO_4^- . Even though occupancy of the sites by SO_4^- is $<90\%$, the occupancy should be sufficiently high that any voltage dependence of SO_4^- binding, if present, should be detectable. We tentatively conclude that the access pathway to the inward-facing substrate binding site is of lower electrical resistance than that leading to the outward-facing site.

Possible Role of the Modifier Site

The working assumption throughout this work is that Cl^- and SO_4^- compete for an outward-facing transport site, and that the inhibitory effect of SO_4^- on Cl^- or HCO_3^- influbax may be attributed to SO_4^- binding to that site. The evidence in favor of this assumption is that the apparent affinity for extracellular SO_4^- as a substrate is similar to its apparent affinity as an inhibitor of Cl^- transport (Milanick and Gunn, 1982, 1984). Moreover, the inhibitory potency of SO_4^- (Milanick and Gunn, 1982) and its affinity as a substrate (Jennings, 1989*b*) have similar dependences on extracellular pH. Finally, under conditions of full outward recruitment of transporters, high concentrations of SO_4^- are not self-inhibitory for SO_4^- influx (Milanick and Gunn, 1984), indicating that SO_4^- does not bind significantly to any modifier site that may be present on the extracellular side of outward-facing states. Therefore, the inhibitory effect of SO_4^- on Cl^- or HCO_3^- influx is probably caused by competition for the outward-facing transport site rather than some other site. Accordingly, the voltage dependence of that inhibition is evidence for a restricted access channel leading to the outward-facing transport site.

APPENDIX

Estimation of Membrane Potential from Tracer Rubidium Flux

We have used the efflux of $^{86}\text{Rb}^+$ as an estimate of gramicidin-mediated changes in membrane potential. The initial $^{86}\text{Rb}^+$ efflux into a medium containing 150 mM K^+ is ~50 times as large as the initial efflux into a medium containing only NMG^+ (Fig. 1*B*). If the $^{86}\text{Rb}^+$ flux obeys the constant field equation, the algebraic expression for the flux is

$$J_{\text{Rb}} = P_{\text{Rb}}[\text{Rb}^+]_i[FV/RT]/(1 - \exp[-FV/RT]), \quad (1A)$$

where P_{Rb} is the ionic permeability coefficient for Rb^+ , $[\text{Rb}^+]_i$ is the intracellular Rb^+ concentration, and V is the membrane potential. In the 150 mM K^+ medium, J_{Rb} is approximately equal to $P_{\text{Rb}}[\text{Rb}^+]_i$, because the membrane potential is near zero (very small gradients of K^+ and Na^+ across the membrane). In the NMG^+ medium, the initial efflux is 0.023 times that in the 150 mM K^+ medium. Assuming that P_{Rb} is not changed by replacement of extracellular K^+ with NMG^+ (see below), a membrane potential of -130 mV would cause the flux to equal $0.023 P_{\text{Rb}}[\text{Rb}^+]_i$. We estimate, then, that the membrane potential is -130 mV in the all- NMG^+ medium under our usual conditions of gramicidin treatment.

The constant field equation is not a precise description of ion movement through the gramicidin channel. The channel has two binding sites; in the presence of symmetric 150 mM K^+ some of the channels are doubly occupied with K^+ (see Finkelstein and Andersen, 1981). However, even at these concentrations, the majority of channels appear to be singly occupied, because K^+ and Na^+ behave as if they permeate the gramicidin channel independently. The bionic potentials for Na^+ and K^+ across gramicidin-treated planar lipid bilayers are nearly constant (26.9–29.2 mV) at salt concentrations between 1 and 100 mM, but deviate significantly at concentrations of 1 M and above (Urban et al., 1980). At concentrations of 0–150 mM K^+ , ion interactions in the gramicidin channel should not have major effects on the flux of $^{86}\text{Rb}^+$. Therefore, although single-filing effects can take place in the gramicidin channel, these effects do not invalidate the use of $^{86}\text{Rb}^+$ fluxes to estimate the membrane potential.

Our calculation of the membrane potential in the presence of an outward K^+ gradient is based on the slow $^{86}\text{Rb}^+$ flux when NMG^+ has replaced all the extracellular K^+ and Na^+ . If

NMG⁺ caused a block of the channel, the estimate of potential could be seriously in error. However, Fig. 1 A shows that in the presence of 135 mM NMG⁺ and 15 mM the ⁸⁶Rb⁺ efflux is rapid, indicating that NMG⁺ does not impede transport through the gramicidin channel. Therefore, the very slow ⁸⁶Rb⁺ efflux in an all-NMG⁺ medium is a consequence of a very negative membrane potential rather than NMG⁺ block of the gramicidin channel.

It is noteworthy that, in a medium containing 15 mM K⁺, the extracellular ⁸⁶Rb⁺ increases rapidly with time to a value that is only ~90% of the value that would eventually be reached after a long incubation (Fig. 1 A, *solid symbols*). The reason for this biphasic tracer efflux is that, as long as there is an outward K⁺ gradient, the ⁸⁶Rb⁺ will be distributed asymmetrically according to the membrane potential. The hematocrit in these experiments is 2%; cell water is ~1.4% of the total water present. If the membrane potential is -55 mV, as would be true in the presence of a 10-fold outward K⁺ gradient, then the steady-state intracellular ⁸⁶Rb⁺ concentration should be 10 times the extracellular concentration, and ~12% of the total ⁸⁶Rb⁺ should remain inside the cells, exactly as observed. Therefore, in the presence of 15 mM extracellular K⁺, gramicidin clamps the membrane potential at a value very near the K⁺ Nernst potential. In a separate experiment (not shown), the gramicidin-mediated ⁸⁶Rb⁺ efflux into a medium containing 3 mM K⁺ was more dramatically biphasic; after 5 min a steady state was reached in which 45% of the ⁸⁶Rb⁺ was intracellular at a hematocrit of 3%; the ⁸⁶Rb⁺ distribution indicates a membrane potential of about -90 mV, as expected from the K⁺ gradient.

In summary, the above evidence indicates that, at values of extracellular K⁺ between 3 and 150 mM, the membrane potential is equal to the Nernst potential for K⁺. In media that are nominally free of Na⁺ and K⁺, the membrane potential is assumed to be -130 mV, as estimated from the ⁸⁶Rb⁺ efflux under identical conditions. We realize that all these estimates of membrane potential are subject to systematic error, but none of the arguments in this paper require precise measurement of potential.

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