Direct estimate of 1:1 stoichiometry of \( K^+ - Cl^- \) cotransport in rabbit erythrocytes

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Jennings, Michael L., and Mark F. Adame. Direct estimate of 1:1 stoichiometry of \( K^+ - Cl^- \) cotransport in rabbit erythrocytes. *Am J Physiol Cell Physiol* 281: C825–C832, 2001.—This work was undertaken to obtain a direct measure of the stoichiometry of \( Na^+ \)-independent \( K^+ - Cl^- \) cotransport (KCC), with rabbit red blood cells as a model system. To determine whether \( ^{86}Rb^+ \) can be used quantitatively as a tracer for KCC, \( ^{86}Rb^+ \) and \( K^+ \) effluxes were measured in parallel after activation of KCC with N-ethylmaleimide (NEM). The rate constant for NEM-stimulated \( K^+ \) efflux into isosmotic \( NaCl \) was smaller than that for \( ^{86}Rb^+ \) by a factor of 0.68 ± 0.11 (SD, \( n = 5 \)). This correction factor was used in all other experiments to calculate the \( K^+ \) efflux from the measured \( ^{86}Rb^+ \) efflux. To minimize interference from the anion exchanger, extracellular \( Cl^- \) was replaced with \( SO_4^{2-} \), and 4,4′-diiodothiocyanatothiocyanatoethylhydrostibene-2,2′-disulfonic acid was present in the flux media. The membrane potential was clamped near 0 mV with the protonophore 2,4-dinitrophenol. The \( Cl^- \) efflux at 25°C under these conditions is ~100,000-fold smaller than the uninhibited \( Cl^- \) exchange flux and is stimulated ~2-fold by NEM. The NEM-stimulated \( ^{36}Cl^- \) flux is inhibited by okadaic acid and calyculin A, as expected for KCC. The ratio of the NEM-stimulated \( K^+ \) to \( Cl^- \) efflux is 1.12 ± 0.26 (SD, \( n = 5 \)). We conclude that \( K^+ - Cl^- \) cotransport in rabbit red blood cells has a stoichiometry of 1:1.

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driving force for transport and the consequences of KCC for intracellular Cl− would be very different.

There are several kinds of evidence, in addition to the lack of potential dependence of the flux (35), that, in fact, the stoichiometry of KCC is 1:1. Most studies of the K⁺ and Cl⁻ concentration dependence of KCC show no sign of more than one binding site for each ion (3, 11, 19, 38, 52). Most data on the flux reversal point, i.e., the K⁺ (or Rb⁺) and Cl⁻ gradients that result in zero net flux (38, 40), indicate 1:1 cotransport. Finally, the magnitudes of the net K⁺ and Cl⁻ fluxes in osmotically swollen duck red blood cells (44) and the basolateral membrane of Necturus gall bladder (53) are approximately equal.

Although the above data strongly suggest that KCC1 has 1:1 stoichiometry, there is one missing piece of information: a direct measurement of KCC-mediated net K⁺ and Cl⁻ fluxes under conditions in which 1) the membrane potential is clamped to remove the requirement that net K⁺ and Cl⁻ fluxes be equal to preserve global electroneutrality and 2) the KCC-mediated fluxes are clearly distinguished from those through other transport pathways for K⁺ and Cl⁻. To obtain a direct estimate of the stoichiometry of KCC, we have used the rabbit erythrocyte as a model system. The rabbit red blood cell has a comparatively large Cl⁻ dependent K⁺ (or 86Rb⁺) flux that is stimulated by cell swelling or treatment with NEM (2, 58). This flux is very likely mediated by KCC1, although it is possible that KCC3 is also expressed in red blood cells (52). The KCC-mediated 86Rb⁺ flux, although large compared with that in red blood cells of other mammalian species, is still at least four orders of magnitude lower than the 36Cl⁻/Cl⁻ exchange flux mediated by the anion exchanger AE1 (band 3) (4). Nonetheless, we have found that it is possible to inhibit the band 3-mediated flux by a factor of ~10⁵ by a combination of H₂DIDS and removal of extracellular Cl⁻ and HCO₃⁻. With band 3 inhibited, it is possible to measure an NEM-stimulated 36Cl⁻ efflux, which is inhibited by okadaic acid and calyculin A, as expected for KCC (32, 34, 57). The NEM-stimulated K⁺ (or 86Rb⁺) and 36Cl⁻ effluxes indicate that the stoichiometry of KCC in rabbit red blood cells is 1:1.

MATERIALS AND METHODS

**Materials.** Blood was drawn from an ear vein of a healthy New Zealand White rabbit into heparin and stored for ≤4 days at 4°C as whole blood. Radionuclides (86Rb⁺ as RbCl and 36Cl⁻ as NaCl) were obtained from DuPont NEN (Boston, MA). H₂DIDS was synthesized from 4,4’ diaminostilbene-2,2’-disulfonic acid (DADS) by a modification of the method used by Cabanthik and Rothstein (8) as described previously (30). Okadaic acid and calyculin A were purchased from Calbiochem. All other buffers, salts, and reagents were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

**Cell preparation and NEM treatment.** Cells were washed three times and incubated for 90 min at 37°C in HEPES-buffered physiological saline (HPS: 150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM sodium phosphate, 10 mM HEPES, pH 7.4) plus 10 mM glucose to try to establish a reproducible steady state. For 86Rb⁺ efflux measurements, the incubation in HPS included 1 μCi/ml 86RbCl. After the incubation in HPS, cells were washed once in HPS, suspended at 5% hematocrit in HPS, and chilled on ice before treatment with 2 mM NEM as described previously (28). The NEM treatment was at 0°C; treatment at low temperature avoids the inhibitory side reactions that take place during exposure of cells to high concentrations of NEM at 37°C (39). The suspensions were incubated for 15 min on ice and washed once, and the treatment was repeated. Finally, the cells were washed once in HPS, and the pellet was incubated for 20 min at 37°C to activate KCC; the details of the kinetics of activation of KCC after NEM pretreatment are described elsewhere (28). Calyculin A, if present, was added to a final concentration of 50 nM immediately before the 37°C activation incubation. After the 20-min incubation at 37°C, KCC is fully activated. The flux itself was measured at 25°C, because the KCC flux (when fully activated with NEM) has a relatively low temperature dependence at 25–37°C (31); therefore, the KCC flux is larger at 25°C relative to other transport pathways than it is at 37°C.

**Comparison of 86Rb⁺ and K⁺ efflux.** To evaluate 86Rb⁺ as a quantitative tracer for K⁺, we measured net 86Rb⁺ efflux and K⁺ efflux simultaneously in parallel suspensions of cells from the same preparation. Cells were prepared exactly as described above (with or without NEM treatment) and were suspended in 160 mM NaCl, 10 mM HEPES hemisodium (a 1:1 mixture of HEPES free acid and Na⁺-HEPES, resulting in a pH equal to the pK of HEPES), pH 7.45, and 10⁻⁴ M ouabain at 25°C. The extracellular K⁺ concentration was measured at various time points by immersion of a K⁺-selective electrode (Fisher Scientific) in the suspension immediately after calibration of the electrode in 160 mM NaCl, 10 mM HEPES, pH 7.45, and 0.01–10 mM KCl. Total K⁺ in the suspension was measured by the same method after lysis by repeated freezing and thawing. To allow calculation of the volume of cells per volume of suspension, cells were added to the medium from a loosely packed pellet with a positive displacement pipette, and the hematocrit of an aliquot of the stock packed suspension was measured. The intracellular K⁺ content determined in this manner was 111 ± 6.4 (SD) meq/l cells (n = 4 preparations), similar to published values (10).

86Rb⁺ and 36Cl⁻ efflux measurements. To compare NEM-stimulated 86Rb⁺ and 36Cl⁻ effluxes, cells were pretreated with NEM as described above, and the efflux of 86Rb⁺ or 36Cl⁻ was measured at 25°C in a medium consisting of 120 mM Na₂SO₄, 20 mM MOPS, pH 7.1, 20 μM H₂DIDS, 40 μM 2,4-dinitrophenol (2,4-DNP), and 100 μM ouabain. All NEM pretreatments and subsequent washes can be performed on 86Rb⁺-loaded cells with negligible losses of tracer. For 36Cl⁻ efflux measurements, cells were preincubated exactly as described above (but without 86Rb⁺). After the 20-min incubation at 37°C to activate KCC, cells were loaded with 86Cl⁻ by incubating a 50% suspension in HPS containing 1 μCi/ml NaCl for 5 min at 25°C. Cells were then washed twice in chilled 120 mM Na₂SO₄, 20 mM MOPS hemisodium, pH 7.1, and 10 μM H₂DIDS and immediately resuspended in flux medium to start the efflux measurement. The medium had been bubbled with N₂ to minimize the HCO₃⁻ concentration. The H₂DIDS was present to inhibit AE1-mediated exchange of Cl⁻ for SO₄²⁻ or residual HCO₃⁻. The 2,4-DNP was added to clamp the membrane potential at the equilibrium potential for H⁺, which is close to 0 mV in this medium. Ouabain was added as a precaution to prevent any 86Rb⁺ flux from a reversed mode of the Na⁺-K⁺-ATPase, although we do not have any evidence that such a flux is significant under these conditions.
The flux suspensions (2–4% hematocrit) were incubated at 25°C with gentle stirring. Aliquots (0.6 ml) were removed at various intervals and centrifuged for 1 min in a microfuge, and the radioactivity in 0.2 ml of supernatant was determined by liquid scintillation counting (Scintisafe Econo 2, Fisher Scientific). For each efflux measurement, four time points were taken, as well as duplicate samples lysed in 10% trichloroacetic acid for determination of the total counts per minute in the suspension (time point at infinity). The rate constants (h⁻¹) for tracer efflux were determined by fitting the data to a single exponential function using SigmaPlot software (Jandel Scientific).

**RESULTS**

**Comparison of NEM-stimulated K⁺ and Rb⁺ efflux.** These experiments were designed to measure NEM-stimulated net K⁺ and Cl⁻ effuxes under conditions in which the membrane potential was clamped at ~0 mV to remove electrical constraints on the K⁺ and Cl⁻ fluxes. In our experience, the most accurate way to measure solute transport is with radioactive tracers, and ⁸⁶Rb⁺ is commonly used as a tracer for K⁺ in the study of KCC (14, 38). To determine whether ⁸⁶Rb⁺ is a quantitative tracer for K⁺ with regard to rabbit red cell KCC, cells were washed and loaded with ⁸⁶Rb⁺, and KCC was activated by pretreatment with NEM. Cells were then washed and resuspended in 160 mM NaCl-10 mM HEPES, pH 7.5, at 25°C for measurement of efflux of ⁸⁶Rb⁺ and K⁺ as described in MATERIALS AND METHODS.

As shown in Fig. 1, NEM stimulates both K⁺ and ⁸⁶Rb⁺ fluxes, but the rate constant (h⁻¹) for both control and NEM-stimulated K⁺ efflux is lower than that for ⁸⁶Rb⁺ efflux. In five experiments, the NEM-stimulated K⁺ efflux rate constant (difference between control and NEM-treated cells) was lower than that for ⁸⁶Rb⁺ by a factor of 0.68 (range 0.54–0.76), indicating that ⁸⁶Rb⁺ is not a perfect tracer for K⁺ efflux under these conditions. Nonetheless, the accuracy and convenience of using ⁸⁶Rb⁺ make it superior to ion-selective electrodes or the short-lived radionuclide ⁴²K⁺. Accordingly, the remaining cation efflux measurements in this study (see Fig. 5) were performed with ⁸⁶Rb⁺, and the K⁺ efflux was calculated by assuming that the NEM-stimulated K⁺ efflux rate constant is 0.68 times that for ⁸⁶Rb⁺.

**Clamping the membrane potential with 2,4-DNP.** The goal of these experiments is to measure NEM-stimulated K⁺ (or ⁸⁶Rb⁺) and Cl⁻ effluxes under conditions of maximum inhibition of other transport pathways for these ions. However, if K⁺ and Cl⁻ are the main permeant ions, the constraint of electroneutrality would require that net K⁺ and Cl⁻ effuxes be equal, even if the actual cotransport itself were an electrogenic 1:2 or 2:1 process. To remove the electrical constraint on the K⁺ and Cl⁻ fluxes, the protonophore 2,4-DNP was added to the flux medium to provide a conductive pathway for H⁺. Macey et al. (45) showed that protonophores can increase the H⁺ permeability of the human red cell membrane sufficiently to make the membrane potential close to the Nernst potential for H⁺. This finding is in agreement with the earlier observation of Harris and Pressman (23) that protonophores increase the rate of valinomycin-mediated K⁺ efflux in human red blood cells.

We showed several years ago that, in rabbit red blood cells, 2,4-DNP induces the expected H⁺ influx in the presence of valinomycin and an outward K⁺ gradient (29). We recently verified that, under the conditions of the present experiments (rabbit red blood cells, 25°C, pH 7.1), 40 μM 2,4-DNP accelerates the valinomycin-mediated ⁸⁶Rb⁺ efflux from rabbit red blood cells suspended in a K⁺-free medium (2 experiments, not shown). Therefore, the conductive proton permeability in the presence of 2,4-DNP is sufficiently high to balance a valinomycin-mediated conductive K⁺ flux, which is much higher than the K⁺ fluxes observed in the absence of valinomycin. If there were a charge imbalance in the efflux of K⁺ and Cl⁻ through KCC in red blood cells, the proton conductance mediated by 2,4-DNP would be high enough to allow the efflux of different amounts of K⁺ and Cl⁻. Control experiments also showed that 40 μM 2,4-DNP does not affect the basal or NEM-stimulated ⁸⁶Rb⁺ efflux, measured as in Fig. 1. All further experiments were carried out in the presence of 40 μM 2,4-DNP, which has the added...
Inhibition of Cl\(^{-}\) exchange by H\(_2\)DIDS and removal of extracellular Cl\(^{-}\). It is so difficult to measure a Cl\(^{-}\)-tracer flux associated with red cell KCC because red blood cells have a very large Cl\(^{-}/\)Cl\(^{-}\) exchange flux mediated by band 3 (AE1). In a medium containing 150 mM Cl\(^{-}\) at pH 7.4, the rate constant for 36Cl\(^{-}\)/Cl\(^{-}\) exchange in rabbit red blood cells at 0°C is ~3/min (unpublished data), which is similar to that in human red blood cells under comparable conditions (20). With the assumption that the temperature dependence of red cell Cl\(^{-}/\)Cl\(^{-}\) exchange is the same in rabbits and humans (4), the rate constant for Cl\(^{-}/\)Cl\(^{-}\) exchange at 25°C in rabbit red blood cells should be ~180/min. The rate constant for NEM-stimulated 86Rb\(^{+}\) efflux in rabbit red blood cells at 25°C is ~0.0015/min (see below), which is a factor of 100,000 slower than the AE1-mediated Cl\(^{-}/\)Cl\(^{-}\) exchange flux. Accordingly, it is necessary to inhibit the AE1-mediated flux by a very large factor to have a chance of detecting a Cl\(^{-}\) flux associated with K\(^{+}/\)Cl\(^{-}\) cotransport.

The large 36Cl\(^{-}\) flux mediated by AE1 would initially appear to preclude the possibility of detecting a 36Cl\(^{-}\) flux through KCC in red blood cells. Powerful inhibitors of anion exchange such as DIDS, H\(_2\)DIDS, and related agents are well known (7, 8, 36, 56), but the prospect of using inhibitors to reduce the 36Cl\(^{-}\) efflux by a factor of 100,000 is discouraging. The maximum irreversible inhibition of human AE1 by DIDS is a factor of only ~500 (15). Another approach is to include enough inhibitor in the flux medium at 25°C to inhibit the band 3-mediated Cl\(^{-}\) efflux reversibly by a factor of >100,000. However, high concentrations of DIDS cause reversible inhibition of KCC (12). Accordingly, it is unrealistic to expect that DIDS, H\(_2\)DIDS, or any other agent, by itself, can inhibit AE1-mediated Cl\(^{-}/\)Cl\(^{-}\) exchange sufficiently to allow detection of a KCC-mediated tracer flux.

It is well known that replacement of external Cl\(^{-}\) with slowly permeating anions can strongly reduce the tracer Cl\(^{-}\) efflux from red blood cells (18). For example, the half time of 36Cl\(^{-}\) efflux from human red blood cells in an Na\(_2\)SO\(_4\) medium (treated with N\(_2\) to lower HCO\(_3\)\(^{-}\)) is ~20 min at 23°C (27), which is a factor of ~5,000 slower than Cl\(^{-}/\)Cl\(^{-}\) exchange (4). We therefore tried to minimize the band 3-mediated Cl\(^{-}\) efflux by the combined effects of replacing extracellular Cl\(^{-}\) with SO\(_4\)\(^{2-}\), adding 20 \(\mu\)M H\(_2\)DIDS to the flux medium, and pretreating the medium with N\(_2\) to lower the CO\(_2\)/HCO\(_3\)\(^{-}\) exchange. These conditions lower the rate constant for 36Cl\(^{-}\) efflux by a factor of ~100,000, from ~180/min to 0.002/min (see below), which is the same order of magnitude as the NEM-stimulated K\(^{+}\) efflux. Therefore, we attempted to detect a KCC-mediated (NEM stimulated) 36Cl\(^{-}\) efflux in a medium consisting of 120 mM Na\(_2\)SO\(_4\), 20 mM MOPS, pH 7.1, 20 \(\mu\)M H\(_2\)DIDS, and 40 \(\mu\)M 2,4-DNP. The extracellular and intracellular pH are nearly equal in this medium; therefore, the membrane potential is near 0 in the presence of 2,4-DNP. Also, there are no possible KCC-mediated exchanges (Rb\(^{+}/\)K\(^{+}\) or Cl\(^{-}/\)Cl\(^{-}\) exchange), because the medium contains no extracellular K\(^{+}\) or Cl\(^{-}\).

Lack of effect of traces of CO\(_2\). To minimize residual Cl\(^{-}/\)HCO\(_3\)\(^{-}\) exchange, media were always bubbled with N\(_2\) before the flux experiment. In early experiments, we maintained an N\(_2\) atmosphere throughout the flux experiments but found no detectable effect of allowing contact with an air atmosphere during the efflux measurement. Most of the experiments, therefore, were carried out in an air atmosphere with flux solutions that had been pretreated with N\(_2\). Apparently, there was sufficient inhibition of Cl\(^{-}/\)HCO\(_3\)\(^{-}\) exchange by H\(_2\)DIDS, 2,4-DNP, and SO\(_4\)\(^{2-}\) that traces of HCO\(_3\)\(^{-}\) entering the solution did not stimulate Cl\(^{-}\) efflux significantly.

Lack of effect of 20 \(\mu\)M H\(_2\)DIDS on NEM-stimulated 86Rb\(^{+}\) efflux. To use H\(_2\)DIDS as an inhibitor of band 3 in these experiments, it is necessary to determine whether H\(_2\)DIDS affects KCC under these conditions. In human red blood cells at 0°C, H\(_2\)DIDS inhibits Cl\(^{-}/\)Cl\(^{-}\) exchange half-maximally at 0.05 nM (56); 20 \(\mu\)M should therefore produce ~99% inhibition of anion exchange. We found that 20 \(\mu\)M H\(_2\)DIDS in the flux medium does not have a detectable effect on either the basal or the NEM-stimulated 36Rb\(^{+}\) efflux in red blood cells (Fig. 2).

36Cl\(^{-}\) efflux. Figure 3 shows that pretreatment of cells with 2 mM NEM stimulates 36Cl\(^{-}\) efflux into an Na\(_2\)SO\(_4\) medium containing 2,4-DNP and H\(_2\)DIDS. The baseline flux is very consistent among different cell preparations; we do not know the nature of this Cl\(^{-}\) transport pathway. We found a clear stimulation of the 36Cl\(^{-}\) efflux by NEM in eight of eight experiments. The NEM-stimulated flux is inhibited by the protein phosphatase inhibitors calyculin A (Fig. 4) and okadaic acid (not shown), which are known inhibitors of activation of K\(^{+}/\)Cl\(^{-}\) cotransport (32, 34).

Fig. 2. Lack of effect of 20 \(\mu\)M H\(_2\)DIDS in the flux medium on the efflux of 86Rb\(^{+}\) from rabbit red blood cells. Conditions are described in Fig. 1 legend. Cells had been pretreated with or without 2 mM NEM, and the efflux was carried out in 160 mM NaCl-10 mM HEPES, pH 7.5, at 25°C. Error bars represent the range of 2 fluxes, each determined from a single exponential fit of 4 time points.
is no measurable effect of calyculin A on the $^{36}\text{Cl}^-$ in the absence of NEM treatment, but a small KCC-mediated Cl$^-$ flux could be present and not detected.

**Stoichiometry of NEM-stimulated K$^+$ (or $^{86}\text{Rb}^+$) and Cl$^-$ efflux.** In five cell preparations, the tracer effluxes of $^{86}\text{Rb}^+$ and $^{36}\text{Cl}^-$ were measured in parallel in the same flux solution used in Figs. 3 and 4, with or without pretreatment of the cells with NEM. The pretreatments were the same for the $^{86}\text{Rb}^+$ and $^{36}\text{Cl}^-$ efflux measurements; the only difference was the radionuclide. The ratio of the NEM-stimulated K$^+$ efflux to Cl$^-$ efflux ranged from 0.79 to 1.45 (Fig. 5), with an average of 1.12. The variations among different experiments are considerable, because experiments require measurement of differences. It is clear, however, that the NEM-stimulated effluxes of K$^+$ and Cl$^-$ are very nearly equimolar.

**DISCUSSION**

The experiments described above have provided the most direct evidence to date that the red blood cell K$^+$-Cl$^-$ cotransporter (most likely KCC1) mediates a Cl$^-$ flux and that the cotransport has a 1:1 stoichiometry of K$^+$-Cl$^-$ cotransport. The evidence for 1:1 stoichiometry is that NEM stimulates a $^{36}\text{Cl}^-$ efflux that is very nearly the same as the NEM-stimulated K$^+$ efflux (Fig. 5). The fact that K$^+$ and Cl$^-$ effuxes are equal is not a consequence of the requirement for global electroneutrality, because the protonophore 2,4-DNP was present to allow H$^+$ flux to neutralize any charge imbalance associated with KCC. These results are discussed below in the context of previous work related to the stoichiometry of KCC.

In most studies (3, 11, 19, 38, 52), the KCC-mediated flux of K$^+$ (or Rb$^+$) has a simple hyperbolic (Michaelis-Menten) dependence on the K$^+$ and Cl$^-$ concentrations. Some data suggest a sigmoidal Cl$^-$ dependence of the KCC-mediated K$^+$ or Rb$^+$ flux (5, 14, 46), but the apparent sigmoidicity could be a consequence of the fact that the fluxes are more difficult to measure at low Cl$^-$ concentration, and the relative errors are larger. The apparent sigmoidicity in the Cl$^-$ dependence of KCC in these studies is less pronounced than that exhibited by the Na$^+-K^+\cdot2\text{Cl}^-$ cotransporter (26), which clearly transports two Cl$^-$ ions. Certainly the bulk of the kinetic data on KCC is consistent with a 1:1 mechanism.

Another experimental approach to determine stoichiometry is to measure the flux reversal point, i.e., the
combination of K\(^+\) (or Rb\(^+\)) and Cl\(^-\) gradients at which the net flux is zero. Lauf\(^{38}\) originally showed that the flux reversal point inLK sheep red blood cells is very close to that expected for 1:1 K\(^+-\)Cl\(^-\) cotransport. Subsequently, Delpire and Lauf\(^{11}\) found that, at 37°C, LK sheep red blood cells exhibit a net K\(^+\) flux under conditions in which a 1:1 mechanism would predict no net flux; this result was not in agreement with a 1:1 stoichiometry. More recently, Lauf and Adragna\(^{40}\) clarified this issue by determining the flux reversal point of KCC in pH-clamped LK sheep red blood cells in the presence of various Cl\(^-\) gradients; the results are entirely consistent with 1:1 electroneutral K\(^+-\)Cl\(^-\) cotransport. This work also provides evidence against H\(^+\) or OH\(^-\) cotransport associated with KCC, because the flux reversal point is not dependent on extracellular pH over a wide range.

Zeuthen\(^{60}\) has estimated the stoichiometry of K\(^+-\)Cl\(^-\) cotransport through the ventricular membrane ofNecturusgall blader. The uncertainties in the measurements did not allow calculation of a stoichiometry, but the data are consistent with a 1:1 mechanism. In contrast, Larson and Spring\(^{37}\) presented evidence that the stoichiometry of K\(^+-\)Cl\(^-\) cotransport is 3:2 in Necturus gall blader, although the authors pointed out that parallel transport processes could affect the calculated stoichiometry. Vascular smooth muscle cells exhibit a K\(^+-\)Cl\(^-\) cotransport process\(^1\) that has an apparent stoichiometry of as many as 25 Cl\(^-\) ions per K\(^+\) ion, but this unusual stoichiometry may be related to exchange processes\(^{55}\).

To our knowledge, there has been only one report of a [superscript 36]Cl\(^-\) flux associated with KCC in any cell. Lytle and McManus\(^{43}\) showed that, in duck red blood cells treated with DIDS, there is a [superscript 36]Cl\(^-\) efflux that is stimulated by cell swelling and is dependent on intracellular K\(^+\). This work provided excellent evidence that KCC does, in fact, mediate a Cl\(^-\) flux. Lytle and McManus did not report the stoichiometry of K\(^+\) (or Rb\(^+\)) and [superscript 36]Cl\(^-\) effluxes. In related work, Lytle\(^{44}\) demonstrated very clearly that the net efflux of K\(^+\) and Cl\(^-\) from swollen duck red blood cells (with band 3 inhibited) has a stoichiometry that is indistinguishable from 1:1, as does the net uptake of Rb\(^+\) and Cl\(^-\) into swollen, K\(^+\)-free cells. The main differences between the present results and those of Lytle, other than the species difference (rabbit vs. duck), are that KCC was activated by NEM, rather than cell swelling, and a protonophore was included in our experiments. Lytle’s work on duck red blood cells is completely consistent with our experiments with rabbit red blood cells.

Possible role of parallel pathways. In these experiments, we have assumed that the NEM-stimulated Rb\(^+\) and Cl\(^-\) fluxes are attributable to KCC. This assumption is based on the fact that no other transporter for K\(^+\) or Cl\(^-\) in red blood cells is known to be stimulated by NEM. Moreover, the NEM-stimulated Cl\(^-\) flux (Fig. 4) is inhibited by calyculin A, as was previously known for KCC-mediated Rb\(^+\) fluxes\(^{32, 34}\). Nonetheless, it is useful to consider the possibility that stimulation of KCC by NEM also causes stimulation of either a K\(^+\) or Cl\(^-\) flux through a parallel pathway and thereby introduces error in the stoichiometry estimate.

For example, if KCC were actually an electrogenic 1:2 K\(^+-\)Cl\(^-\) cotransport mechanism, the charge imbalance would tend to drive the membrane potential in the positive direction and cause an efflux of acid equivalents mediated by 2,4-DNP. If the pH change associated with this 2,4-DNP-mediated proton flux caused a stimulation of K\(^+\) efflux through a pathway unrelated to KCC, the observed stoichiometry could be close to 1:1, even though the actual cotransport process is 1:2. Because of the large buffer power of red cell cytoplasm\(^{22}\), it is unlikely that significant intracellular (or extracellular) pH changes take place during the fluxes measured here. In any case, if the KCC flux somehow produced a progressive pH change that induces a K\(^+\) or Cl\(^-\) flux through a separate pathway, the flux should change progressively with time. We observe no indication of a time-dependent flux; the time courses of the effluxes of both [superscript 86]Rb\(^+\) and [superscript 36]Cl\(^-\) are indistinguishable from single exponentials. It is therefore unlikely that the estimate of stoichiometry is distorted by fluxes through parallel transport pathways.

[superscript 86]Rb\(^+\) as tracer for K\(^+\). Our calculation of the K\(^+\) flux from the [superscript 86]Rb\(^+\) tracer flux depends on the assumption that the KCC-mediated (NEM stimulated) K\(^+\) efflux rate constant is 0.68 times the [superscript 86]Rb\(^+\) efflux rate constant. This ratio was determined in five experiments at the same temperature and pH as the other experiments (Fig. 1), but the Rb\(^+\)-to-K\(^+\) comparisons were performed in an NaCl, rather than an Na\(_2\)SO\(_4\), medium. The reason for using an NaCl medium is that the K\(^+\) efflux measurements with microelectrodes require longer times for reasonable accuracy (~100 min vs. 30 min for tracer efflux); at such long times, the intracellular Cl\(^-\) concentration would have been lowered significantly in the Na\(_2\)SO\(_4\) medium. The initial intracellular conditions are identical in all experiments (whether in NaCl or Na\(_2\)SO\(_4\)), and we do not see any reason to suspect that the ratio of [superscript 86]Rb\(^+\) to K\(^+\) efflux rate constants would be any different in Na\(_2\)SO\(_4\) vs. NaCl medium. Nonetheless, our calculation of stoichi-
ometry does depend on the relative K\textsuperscript{+} and Rb\textsuperscript{+} efflux rate constants measured in an NaCl medium.

Other comparisons of Rb\textsuperscript{+} and K\textsuperscript{+} with regard to KCC have been published. Brugnara (5) measured KCC-mediated Rb\textsuperscript{+} and K\textsuperscript{+} efflux from human hemoglobin CC red blood cells and found that K\textsuperscript{+} and Rb\textsuperscript{+} effluxes were similar. It is possible that a minor difference could have been overlooked. Lauf (38) found that, in NEM-treated LK sheep red blood cells, K\textsuperscript{+} and Rb\textsuperscript{+} effluxes through KCC are very similar, although the extrapolated maximal velocity (V\textsubscript{max}) measured with \textsuperscript{42}K\textsuperscript{+} was slightly slower (factor of 0.77) than that measured with Rb\textsuperscript{+}; this result is in qualitative agreement with our finding that \textsuperscript{86}Rb\textsuperscript{+} efflux is more rapid than K\textsuperscript{+} efflux. Dunham and Ellory (14), using swollen LK sheep red blood cells, found a larger difference between the V\textsubscript{max} for \textsuperscript{86}Rb\textsuperscript{+} and K\textsuperscript{+} influx: the V\textsubscript{max} for K\textsuperscript{+} was only \sim 50\% of that for \textsuperscript{86}Rb\textsuperscript{+}. Part, but not all, of the difference was attributable to the fact that the cells were slightly more swollen in the \textsuperscript{86}Rb\textsuperscript{+} experiments. The data of Lauf and Dunham and Ellory are consistent with the idea that the maximal rate of Rb\textsuperscript{+} transport through KCC is slightly greater than that of K\textsuperscript{+}, as we have found for efflux in the present experiments.

The finding that NEM-stimulated effluxes of \textsuperscript{86}Rb\textsuperscript{+} and K\textsuperscript{+} are slightly different certainly does not negate the value of \textsuperscript{86}Rb\textsuperscript{+} (and nonradioactive Rb\textsuperscript{+}) for the study of KCC. The interpretation of most kinetic and regulatory studies of KCC would not be significantly altered by a \sim 30\% difference in the maximal flux of Rb\textsuperscript{+} vs. K\textsuperscript{+}, because most such studies rely mainly on comparisons of the flux of Rb\textsuperscript{+} under different conditions (e.g., different ionic media, cell volume, pH, Mg\textsuperscript{2+}). In fact, the present work on K\textsuperscript{+}-Cl\textsuperscript{−} stoichiometry is one of a relatively few examples of an experiment in which it definitely matters whether Rb\textsuperscript{+} and K\textsuperscript{+} are kinetically identical. Also, we have shown only that K\textsuperscript{+} and Rb\textsuperscript{+} are not identical for efflux from rabbit red blood cells at 25°C; at other temperatures, the kinetic differences could be smaller, and the difference for influx may be smaller than that for efflux. It is worth pointing out that, in some tissues, there are major differences in the characteristics of \textsuperscript{86}Rb\textsuperscript{+} and \textsuperscript{42}K\textsuperscript{+} transport (13). In red blood cells, however, \textsuperscript{86}Rb\textsuperscript{+} is an adequate tracer for K\textsuperscript{+} for nearly all purposes; the differences we find here for KCC are relatively minor.

Role of O\textsubscript{2}. In these experiments, the flux media were bubbled with N\textsubscript{2} to minimize CO\textsubscript{2} and HCO\textsubscript{3}\textsuperscript{−}. The N\textsubscript{2} bubbling caused deoxygenation of hemoglobin, but the O\textsubscript{2} partial pressure in the suspensions was not well controlled. It is known that red cell KCC is influenced by the O\textsubscript{2} tension (17, 33). However, the effects of O\textsubscript{2} are on the regulation of KCC; after maximal stimulation with NEM, as in the present experiments, there is no longer an effect of O\textsubscript{2} on KCC in red blood cells (9). Therefore, even though O\textsubscript{2} partial pressure can have important effects on KCC, these effects were not significant under the conditions of our experiments.

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AJP-Cell Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpcell.org
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