Membrane sidedness and the interaction of H^+ and K^+ on Ca^{2+} -activated K^+ transport in human red blood cells

(pH gradient/potassium channels/intracellular calcium/erythrocyte)

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Contributed by Joseph F. Hoffman, December 6, 1989

ABSTRACT The sided effects of H^+ on Ca^{2+} -stimulated K^+ transport (the Gardos channel) were studied in human red blood cells. Cells were loaded with Ca^{2+} during energy depletion with the internal pH adjusted to desired levels prior to treatment with the anion-exchange inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), which inhibits pH equilibration across the membrane. This treatment provides a "pH clamp" whereby the internal and external H^+ (H_i^+ and H_o^+) concentrations can be varied separately. Channel activity was evaluated by measuring either net K^+ loss or unidirectional $^{42}K^+$ efflux from cells where SO_4^{2-} replaced Cl^- on both sides of the membrane. When pH_i was set at 7.4, decreasing pH_o from values of 8.0 to 5.0 inhibited K^+ efflux. This effect of H_o^+ could be overcome by increasing K_o^+ at all values of pH_o . In addition, this effect of K_o^+ could be separated from its effects on altering the membrane potential, indicating an interaction between K_o^+ and H_o^+ on the channel. A similar interaction was shown to occur between H_i^+ and K_i^+ . K_o^+ is known to be required for activation of Ca^{2+} -stimulated K^+ transport, since the channel in cells preincubated in the absence of K_o^+ (prior to exposure to Ca_i^{2+}) becomes refractory to subsequent activation by Ca_i^{2+} and K_o^+ . We found that H_o^+ would not substitute for K_o^+ in this regard nor would H_o^+ inhibit the protective effect of K_o^+ ; in addition, H^+ was not transported inward in exchange for K_i^+ . Thus it would appear that there are two external sites where K_o^+ interacts with the channel. One site is antagonized by H_o^+ , whereas the second site is required for channel activation independent of H^+ in the range studied. The inside of the channel would have, by an analogous argument, at least one site where K_i^+ and H_i^+ interact.

Since Ca^{2+} -activated K^+ channels in human red blood cells were first described by Gardos (1), much has been learned about the properties of these channels, including the dependencies on intracellular Ca^{2+} (Ca_i^{2+}) and on K^+ on both sides of the membrane (K_i^+ and K_o^+) (2–4). Analysis of the channel's conductance by use of the patch-clamp technique (5–8) and indirect methods (9, 10) has helped to define various channel characteristics, including that the channel operates as an inward rectifier and that K^+ diffuses through it by a single-file mechanism.

Previous work (11, 12) on the effects of H^+ on the Gardos channel has been limited because, until the emergence of anion-exchange inhibitors such as 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (13), it was not possible to separately control inside and outside variations in pH. Nevertheless, Porzig (11) found that the channel was inhibited by H^+ and that increasing Ca_i^{2+} could overcome the inhibition. It was also observed (12, 14) that the inhibitory properties of H^+ were antagonized by K_o^+ . Clearly, in order to sort out the sided effects of H^+ , as well as K^+ , it was necessary to inhibit

pH equilibration across the cell membrane. Thus, following the lead of Wieth *et al.* (15), the use of DIDS provided a way to pH-clamp the cell and to study the separate effects of variations in the H_i^+ and H_o^+ concentrations (16–18). This type of pH clamp (also used in ref. 19) is applied in the present work to explore the sided effects of H^+ on the Gardos channel. A preliminary report of this work has appeared (18).

MATERIALS AND METHODS

Preparation of Cells. Fresh heparinized human blood was obtained from healthy donors. To study Ca^{2+} -stimulated K^+ transport, depletion of the cells' ATP is necessary so that whatever Ca^{2+} taken up will be retained for a period of time sufficient to measure K^+ efflux. Thus, the red cells were washed three times by centrifugation ($15,000 \times g$) at $4^\circ C$, with 10 volumes of the incubation medium that was also used for concomitant loading with Ca^{2+} and depletion of their endogenous energy sources. Ca^{2+} loading was carried out in two ways. The first way used the salicylate method (20) where the cells were also energy-depleted (21) by incubation for 2.5 hr at $37^\circ C$ in a medium that contained 5 mM inosine and 5 mM iodoacetamide in addition to 5 mM $CaCl_2$, 60 mM KCl, 15 mM Pipes, and 75 mM salicylic acid, brought to pH 7.5 with KOH. The second way was by incubation of the cells at $37^\circ C$ in the presence of either $CaSO_4$ or $CaCl_2$ (up to 5 mM) in SO_4^{2-} or Cl^- solutions that contained either 50 mM K_2SO_4 plus 40 mM Na_2SO_4 or 140 mM KCl plus 10 mM NaCl, each being buffered with 30 mM Mops or Hepes at pH 7.5 and containing 5 mM inosine plus 5 mM iodoacetamide for energy depletion.

The cells were suspended at $\approx 10\%$ hematocrit and incubated at $37^\circ C$ for 2.5–4 hr. Afterwards the cells in each case were washed three or four times with 20 volumes of the medium ($4^\circ C$) into which the efflux was to be measured. The composition of the relevant flux media is specified in the legends.

In some experiments Ca^{2+} -induced K^+ transport was studied in reconstituted ghosts (22) made to contain different concentrations of free Ca^{2+} by the incorporation of Ca^{2+} buffers [i.e., either 2–4 mM $MgSO_4$ /1 mM EDTA or (as used in ref. 11) 4 mM N' -(2-hydroxyethyl)ethylenediamine- N,N,N' -triacetic acid]. Free Ca^{2+} concentration was calculated as in ref. 23, utilizing stability constants taken from Durham (24).

Alteration of K_i^+ Concentration. K_i^+ was adjusted to desired concentrations (see Fig. 3C and Table 1) by using a modified nystatin technique (25). Fresh red cells were washed three

Abbreviations: DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; E_m , membrane potential; subscript i and o, inside and outside, respectively.

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times with 10 volumes of the medium in which they were to be exposed to nystatin (Sigma). Thus, cells were washed in media containing 90 mM X_2SO_4 (where X is the sum of Na plus K), 30 mM Mops (pH 7.4), and 30 mM sucrose (to control cell swelling). The cells were then suspended at 5% hematocrit, in the solution of the desired composition, at 0°C for 30 min in the presence of nystatin (40 μ g/ml). The cells were then washed three times with 20 volumes of medium of the same composition, except that it contained 0.1% bovine serum albumin but lacked nystatin, before final suspension in the presence of 5 mM inosine, 5 mM iodoacetamide, 1 mM $CaSO_4$, and $^{42}K^+$ for energy depletion and Ca^{2+} loading as described above. Subsequent adjustments of pH_i and pH_o and treatment with DIDS are described below and in the relevant legends.

pH_i and Stability of pH Gradients. pH_i was adjusted, usually after energy depletion, by titration of a suspension of cells to the desired equilibrated values of pH_o in an unbuffered medium. Afterwards the cells were treated with 50 μ M DIDS and were exposed to this concentration of DIDS for the remainder of the experiment (see legends). pH_i was measured under various conditions by immersing a pH electrode directly into a packed red cell mass (hematocrit >97%) that had been hemolyzed either with the detergent Cutsicum (Fisher) or by freeze-thawing.

The relative stability of various pH gradients was evaluated by estimating pH_i and pH_o at the beginning and end of a 20-min incubation period at 37°C for all of the conditions reported in this paper. The results that follow are typical of two different experiments of each type in which the differences in initial and final values of pH_i were ± 0.1 pH unit. Thus, where SO_4^{2-} was the major anion, cells whose initial pH_i was 7.3 were suspended (at 3–4% hematocrit) in buffered media of varying pH_o . After 20 min, pH_i and pH_o were determined again with the following results (pH_i/pH_o): 6.9/5.0, 7.1/6.0, 7.1/6.5, 7.2/7.0, 7.3/7.5, and 7.8/8.5. While pH_o remained stable during this 20-min interval, there was a change in pH_i and this change was more marked the more acid the initial pH_o was made, especially at pH_o 5.0. Because of these changes, the period of incubation over which the K^+ fluxes were measured was limited to 10 min. This reduced the effective changes in pH_i to ± 0.1 pH unit for initial values of pH_o 5.5 and above. Comparable measurements were made where Cl^- was the major anion: where the initial pH_i was 6.2, pH_i/pH_o values after 20 min were 6.5/7.5 and 6.0/6.0; where the initial pH_i was 7.3, pH_i/pH_o values after 20 min were 7.3/7.5 and 6.7/6.0. While different buffers were used for the different pH ranges (namely, Pipes, Mops, and Hepes, with pK values 6.8, 7.2, and 7.5, respectively), no differences in results were observed when the same buffer was used in experiments with overlapping values of pH.

Measurement of Net K^+ Loss and $^{42}K^+$ Efflux. Suspensions of cells at low hematocrit were incubated under various conditions (see legends), with aliquots removed at different times for analysis. For measurement of net K^+ loss, samples were centrifuged at $15,000 \times g$ for 1 min at 4°C and the cells were washed twice with ice-cold 160 mM NaCl/15 mM Pipes, pH 7.2. The supernatant was removed and a measured volume of packed cells was hemolyzed with 5% (wt/vol) trichloroacetic acid and analyzed for K^+ by flame photometry. K_i^+ was normalized to mmol of K^+ per liter of original cells on the basis of the K_i^+ content of packed cells before suspension. In some instances fractional cell water was determined from the difference in wet minus dry weight (overnight drying at 90°C) of packed cells.

For measurement of unidirectional K^+ efflux, the suspensions consisted of washed cells that had been loaded with ^{42}KCl (New England Nuclear) during the period of energy depletion and Ca^{2+} loading as described above. Efflux was measured over a 10-min interval, during which samples were

taken at various times and centrifuged; the ^{42}K content of the supernatants, as well as of the suspension itself, was measured by scintillation counting. The efflux rate constant (ok_K) in hr^{-1} was estimated from the fractional loss of ^{42}K with time by using linear regression (see ref. 26), where the correlation coefficients were better than 0.9. Efflux of K^+ in mmol per liter of cells per hour was calculated from the product of ok_K times K_i^+ .

RESULTS

Inhibition by H_o^+ . The starting point for the present experiments was to test the extent to which the inhibition by H^+ of Ca^{2+} -stimulated K^+ transport was sided with regard to its membrane locus of action. We treated the cells with DIDS in order to markedly retard the rate of pH equilibration across the membrane, allowing pH_i and pH_o to be varied separately (16–18). Where pH_i was initially fixed at 6.1 (as in Fig. 1A), the rate of Ca^{2+} -stimulated K^+ loss was almost completely inhibited by reducing pH_o from 7.4 to 6.1. The inhibition by quinine indicates that the K^+ loss at pH 7.4 was through the Gardos channel (27). These results are to be compared to those presented in Fig. 1B, where the initial pH_i was set at 7.4. Here, reduction of pH_o to 6.2 also resulted in inhibition of Ca^{2+} -stimulated K^+ loss but to a lesser extent than when pH_i was 6.1, even though the rate of net K^+ loss at pH_o 7.4 was the same in both cases. These results imply that external rather than internal H^+ is primary in inhibiting the Gardos channel.

Unidirectional K^+ Efflux and Ca_o^{2+} . To study the sided effects of H^+ on the Gardos channel in more detail, it was desirable to measure unidirectional efflux of K^+ (with $^{42}K^+$), rather than net K^+ loss, since $^{42}K^+$ effluxes could be evaluated in a sufficiently short time (10 min) so that subsequent changes in the pH gradient across the membrane would be minimized (see *Materials and Methods*). Therefore a preparation was developed that

FIG. 1. The sided effects of H^+ on Ca^{2+} -stimulated K^+ loss from energy-depleted red cells preloaded with Ca_i^{2+} by the salicylate method. pH_i was adjusted to desired values prior to exposure to DIDS, and pH_o to desired values afterwards. The pH values indicated are approximate and refer to initial preset values. K_i^+ was ≈ 70 mmol per liter of cells. Cells were suspended in 2.5 mM KCl/140 mM NaCl/20 mM Tris/100 μ M ouabain/50 μ M DIDS. Quinine, when present, was 250 μ M. Similar results were obtained in two other experiments.

contained sufficient Ca_i^{2+} to maximize $^{42}\text{K}^+$ efflux during subsequent incubation. Cells were energy-depleted, loaded with $^{42}\text{K}^+$, and loaded with various concentrations of Ca_i^{2+} by preexposure to various concentrations of Ca_o during energy depletion in a SO_4^{2-} medium (30 mM Pipes, pH 7.4) according to the second method described in *Materials and Methods*. The use of SO_4^{2-} in this procedure to replace all Cl^- helps to preserve the pH gradient, since the membrane conductance of SO_4^{2-} is less than that of Cl^- (28–31). Also, pH equilibrates across the cells such that at pH_o 7.4, pH_i lies between 7.2 and 7.3. In this procedure cells were pretreated with 50 μM DIDS and washed prior to suspension (at 3–4% hematocrit) in a flux medium that contained 90 mM Na_2SO_4 , 5 mM K_2SO_4 , 50 μM DIDS, 100 μM ouabain, and 30 mM Pipes (pH 7.4). It was found that for cells preexposed to 0.1, 0.6, 1.0, 2.0, and 3.0 mM CaSO_4 , the $^{42}\text{K}^+$ efflux was, respectively, 65, 220, 330, 280, and 300 mmol per liter of cells per hr (similar results were obtained in two other experiments). It was evident that 1 mM Ca_i^{2+} was sufficient to maximize Ca^{2+} -stimulated K^+ efflux; therefore this concentration of Ca_i^{2+} was the standard used in subsequent experiments.

Interaction of H_o^+ with K_o^+ . With the foregoing technique, we studied, at a fixed value of pH_i (7.4), the effects of varying pH_o on Ca^{2+} -dependent K^+ efflux from Ca^{2+} -loaded red cells (Fig. 2). It is evident for all three curves that increasing H^+ results in a marked inhibition of the K^+ efflux. It is unlikely that alterations in Ca^{2+} could be responsible in eliciting the effects of pH_o as seen, for instance, in curve B, since initially the cells all contained the same Ca_i^{2+} and since the same results were obtained when 5 mM rather than 1 mM Ca_i^{2+} was used in the preloading incubation condition. In addition the

FIG. 2. The influence of H_o^+ and its interaction with K_o^+ on Ca^{2+} -stimulated $^{42}\text{K}^+$ efflux from SO_4^{2-} -loaded, energy-depleted red cells. Cells used in experiments A and B contained the indicated approximate concentrations of K_i^+ (mmol of K_i^+ per liter of cell water) after energy depletion, during which time they were also loaded with $^{42}\text{K}^+$, SO_4^{2-} , and Ca^{2+} . The loading concentration of Ca_i^{2+} was 1 mM (see above). Cells used in experiment C were treated with nystatin to replace K_i^+ with Na_i^+ prior to loading with $^{42}\text{K}^+$, SO_4^{2-} , and Ca^{2+} during energy depletion. The cells were suspended in flux media containing 1 mM CaSO_4 , 90 mM X_2SO_4 (where X is Na plus K at the indicated concentrations), 100 μM ouabain, and 50 μM DIDS, with pH_o (buffered with 30 mM Pipes, Mops, or Hepes) as indicated. Cell water was $\approx 65\%$ (wt/wt) in all cases. pH_i was set before treatment with DIDS; the cells were then washed and suspended, at 3–4% hematocrit, in flux media at the indicated values of pH_o . Curves A, B, and C represent the results of separate experiments carried out with different bloods. Similar results were obtained in at least two other experiments of each type.

same overt inhibitory pattern by pH_o (between pH_o 6.0 and 7.5) on K^+ efflux was also observed for the curve B situation in ghosts resealed with a Ca^{2+} -buffer system to contain either 5 or 17 μM free Ca_i^{2+} , where the K^+ effluxes from the two sets of ghosts were indistinguishable from each other. The results represented by the three curves in Fig. 2 also indicate that the effectiveness of H^+ in inhibiting the K^+ efflux is influenced by the concentration of K_o^+ . Thus when K_o^+ is low (10 mM, curve B), inhibition of K^+ efflux is almost complete at pH_o 6.0, while at high K_o^+ (100 mM, curve A), the efflux is only slightly affected at this pH_o . This effect of K_o^+ in shifting the pH_o sensitivity to the left could be due either to an effect of K_o^+ on the membrane potential (E_m) or to an interaction of K_o^+ with H_o^+ or to a composite of the two. It is known that under Ca^{2+} -activated K^+ transport conditions, the cell's E_m approaches that of a K^+ equilibrium potential (9, 10, 12, 31–33). Therefore for the situations where K_o^+ is low (10 mM, curve B) relative to K_i^+ , the cell is hyperpolarized, in contrast to high K_o^+ (100 mM, curve A), where E_m is near zero. It was previously shown (31) that when the cell is hyperpolarized, K^+ efflux is inhibited and K^+ influx is enhanced. Therefore the relief of the inhibition seen in curve B at, say, pH_o 6.0 by raising K_o^+ (curve A) is consistent with a reduction in E_m . On the other hand, if anion (SO_4^{2-}) conductance in DIDS-treated cells is unaffected by changes in pH_o , the calculated change in E_m is insufficient alone to account for the differences in efflux rates. Because the characteristics of activation of Ca^{2+} -stimulated K^+ efflux by various concentrations of K_o^+ are incompletely understood, it was of interest to explore the effects of varying pH_o when E_m was also set near zero but where K_o^+ was relatively low (curve C, Fig. 2). Here, while K^+ efflux is much lower [due presumably to low K_i^+ (34) and high Na_i^+ (35)] than those seen in curves A and B, the inhibitory effects of increasing H_o^+ on K^+ efflux are comparable to that seen at high K_o^+ (curve A). These results separate the effects of changes in E_m from the effects of changes in K_o^+ . These results also imply that the major effects of H_o^+ on Ca^{2+} -stimulated K^+ transport occur either by modulation of the effects of K_o^+ or by competition of H_o^+ with K_o^+ activation sites on the outside of the Gardos channel.

FIG. 3. Interactions of K_o^+ and H_o^+ on Ca^{2+} -stimulated $^{42}\text{K}^+$ efflux from SO_4^{2-} -loaded, DIDS-treated, energy-depleted red cells. Cells were made to contain ≈ 75 mmol of K^+ per liter of cell water after energy depletion, during which time they were also loaded with $^{42}\text{K}^+$, SO_4^{2-} , and Ca^{2+} . Treatment of the cells and the flux media used were the same as described in the legend of Fig. 2. Initial cell water was $\approx 65\%$ (wt/wt). Similar results were obtained in at least two other experiments.

Additional results on the interaction of H^+ and K_o^+ on Ca^{2+} -stimulated K^+ efflux are shown in Fig. 3. Similar to the effects shown in Fig. 2, it is apparent that increasing K_o^+ at any pH_o increases the K^+ efflux. Note that the cells in this study contained 75 rather than 100 mM K_i^+ , so that E_m was presumably reversed for the case where K_o^+ was 100 mM. Again, whether E_m was positive (100 mM K_o^+) or negative (≤ 50 mM K_o^+), increasing H_o^+ results in inhibition of K^+ efflux. While K^+ efflux is seen to peak at pH_o 6.0 for the case where K_o^+ is 100 mM in Fig. 3, the basis for the decrease in efflux by increasing pH_o (seen also for the curves presented in Fig. 2) is not known. Nevertheless, the results in Fig. 3 support the conclusion that H_o^+ acts to alter the interaction of K_o^+ with the channel protein.

Interaction of H_i^+ with K_i^+ . Because increasing K_o^+ can to a large extent alter the inhibition of Ca^{2+} -stimulated K^+ efflux observed at low pH_o and K_o^+ , the question arises as to whether this might be the cause for the absence of inhibition when the pH_i is low but K_i^+ is high (Fig. 1A). To see whether this was the case, K_i^+ was lowered to the values shown in Table 1, with pH_i set at the indicated values; pH_o was constant (≈ 7.5) for all situations. These results demonstrate that increasing pH_i at constant values of pH_o and K_o^+ simulates the Ca^{2+} -dependent K^+ efflux. This is true whether K_o^+ is 10 or 100 mM. Therefore these results support the idea that H_i^+ and K_i^+ interact at the inside surface of the Gardos channel in a manner analogous to that found for H_o^+ and K_o^+ for the outside of the channel.

Protons, K_o^+ , and Channel Refractoriness. Another property of the Gardos channel is displayed by its dependence upon K_o^+ (22, 36). Ca^{2+} -stimulated K^+ transport can be completely prevented by preincubation of the cells in the absence of K_o^+ . Addition of K_o^+ to cells so treated fails to activate Ca^{2+} -dependent K^+ transport. This is so whether the cells are exposed to K_o^+ before or after Ca^{2+} is increased. Having established that interaction of H_o^+ and K_o^+ occurs, we wished to test whether H_o^+ would substitute for K_o^+ (during preincubation in the absence of K_o^+) in preventing the refractoriness of the Gardos channel activity. To this end cells were preincubated at high H^+ concentration (pH 5.5) either in the absence of K_o^+ or with 5 mM K_o^+ (Fig. 4). High H_o^+ alone (curve A) will not replace K_o^+ (curve B) in preventing the channel's subsequent activation by Ca^{2+} . The results shown in curve A (Fig. 4), by the way, were the same when the cells had been

FIG. 4. H^+ cannot prevent the inhibition of Ca^{2+} -induced K^+ transport in cells preincubated in the absence of K_o^+ . This experiment utilized energy-depleted cells where the effects of two different pretreatments were evaluated under the same final condition. Thus cells energy-depleted in the absence of Ca^{2+} were preincubated for 30 min at $37^\circ C$ with pH_i set at 7.5. The cells were then washed and exposed to 50 μM DIDS, and pH_o was adjusted to 5.5 in the absence of K_o^+ . The cells were then incubated at pH_o 5.5 for 30 min at $37^\circ C$ in the absence of K_o^+ (curve A) or presence of 5 mM K_o^+ (curve B). The composition of this medium was 150 mM NaCl/20 mM Pipes/5 mM KCl/100 μM ouabain/50 μM DIDS. It is necessary to keep the hematocrit of this incubation $<1\%$ in order to keep K_o^+ below 0.01 mM. [This may be the basis for the failure of Vestergaard-Bogind *et al.* (10) to confirm the refractoriness of cells so treated to subsequent activation by Ca^{2+} and K_o^+ .] Afterwards the cells were washed and suspended in media in which the net efflux of K^+ from each group of cells was measured as indicated. The flux medium was 150 mM NaCl/5 mM KCl/20 mM Pipes, pH_o 7.5/10 mM $CaCl_2$ /100 μM ouabain/50 μM DIDS. Ca^{2+} -stimulated K^+ flux was initiated by the addition of Ca^{2+} ionophore A23187 (final concentration, 10 μM) to the cells suspended in the flux medium. The initial concentration of K_i^+ for both sets of cells was ≈ 100 mmol per liter of cell water.

preincubated at pH_o 7.5 rather than at pH 5.5 (22). It is also unlikely that the effect of K_o^+ in protecting the channel during preincubation represents an interaction with H_o^+ , since in this circumstance K^+ efflux is nominally zero at a pH_o of 5.5 (see Fig. 3). Thus, inactivation of the Gardos channel can evidently result from a lack of K_o^+ or be induced by H_o^+ .

DISCUSSION

The primary focus of this study is the mechanism by which protons inhibit Ca^{2+} -dependent K^+ transport (the Gardos channel) in human red blood cells. The main conclusion to be drawn is that not only are the inhibitory effects of H^+ sided but that their effects are antagonized by K^+ . This antagonism between H^+ and K^+ occurs on both sides of the membrane. Thus the inhibitory action of H_o^+ on K^+ efflux through the channel seen at low values of K_o^+ (Figs. 2 and 3) can be prevented by increasing K_o^+ , and inhibition of the channel by H_i^+ is antagonized by K_i^+ . This conclusion is based on the use of high- K_i^+ cells, as shown in Fig. 1 A and B, where net K^+ efflux was the same at pH_o 7.4 whether pH_i was low (6.1) or high (7.4), together with the results presented in Table 1. Important in this analysis of sidedness of action, particularly for the interaction of H_o^+ and K_o^+ , was separation of the effects of E_m from the effects of K_o^+ . Activation of the Gardos channel by Ca^{2+} is known to shift (because of the marked change in the ratio of K^+ conductance to anion conductance) the E_m from its normal Donnan potential toward a K^+ equilibrium potential (E_K). Therefore changes in K_o^+ (at constant K_i^+) result in changes in E_m that can alter K^+ efflux. Thus E_m can be large (inside negative) at low values of K_o^+ and inhibit K^+ efflux (see ref. 31), and the inhibition can be relieved by raising the concentration of K_o^+ with a concomitant decrease in E_m . For the situations presented in Figs. 2 and 3 the effects of changes of E_m were separated from the effects of K_o^+ , indicating a direct interaction of K_o^+ with H_o^+ .

Table 1. Interaction of H_i^+ and K_i^+ on Ca^{2+} -stimulated $^{42}K^+$ efflux from energy-depleted cells

K_i^+ , mM*	K_o^+ , mM	pH_i	$\%k_K$, hr $^{-1}$
20	20	6.5	0.45 $\pm$
	20	7.1	1.93 $\pm$
	20	7.4	0.18 $\pm$
28	10	6.6	0.13
	10	7.4	0.33 $\pm$
	100	7.4	0.35
33	10	6.5	0.30 $\pm$
	100	6.5	0.66
	10	7.3	0.73
	100	7.3	0.92

K_i^+ was varied reciprocally with Na^+ by the nystatin technique prior to loading with Ca^{2+} , $^{42}K^+$, and SO_4^{2-} during energy depletion. pH_i was adjusted to the indicated levels prior to treatment with 50 μM DIDS. After washing, $^{42}K^+$ efflux was measured in all cases at pH_o 7.5 (hematocrit 3–4%) in media that contained the indicated concentrations of K_o^+ (such that the sum K_2SO_4 plus Na_2SO_4 was 90 mM), 1 mM $CaSO_4$, 30 mM Pipes or Mops, 50 μM DIDS, 100 μM ouabain, and, when present, 250 μM quinine. Each efflux measurement ($\%k_K$, in hr $^{-1}$) was carried out in triplicate with a range of variation $\pm 5\%$.

*Concentration based on cell water.

$\pm$ Quinine reduced $\%k_K$ to approximately 0.1 and 0.2, respectively.

From the sidedness of action of H^+ and K^+ as defined by the present work, it appears that the effects of H^+ on Ca^{2+} -stimulated K^+ transport in a preparation where H_i^+ and H_o^+ were not separately controlled (figure 3 in ref. 12) can now be ascribed to the effects of H_o^+ .

Two previous studies (11, 19) have explored the effects of varying H^+ on Ca^{2+} -dependent K^+ transport in red cells. Porzig (11) used resealed ghosts containing different concentrations of Ca_i^{2+} , defined by a Ca^{2+} -buffering system, high K_i^+ , and Cl^- as the principal anion. The ghosts were suspended in a low- K_o^+ medium (5 mM), in the absence of inhibitors of anion transport, such as DIDS, and the rate of net K_i^+ loss was determined at pH values ranging from 6.0 to 8.0 (where pH_o presumably equaled pH_i). He found an interaction between H^+ and Ca_i^{2+} in the sense that inhibition of K^+ loss by H^+ could be counteracted by raising Ca_i^{2+} . While, as mentioned earlier, we found no difference in the inhibitory effects of pH_o on K^+ efflux (as in Fig. 2) when Ca_i^{2+} was varied from 5 to 17 μM (we did not vary pH_i in this context), it is not clear that the two assay systems are comparable. Even so, it is reasonable to assume that the Gardos channel's activity (K^+ conductance) is modulated primarily by Ca_i^{2+} and secondarily by H^+ and K^+ on both sides of the membrane.

Stampe and Vestergaard-Bogind (19) studied, at saturating values of Ca_i^{2+} , the effects on the Gardos channel of pH gradients across the membrane in energy-depleted red cells that had been treated with DIDS, as in our work. In contrast to our results, they observed that when pH_i was 5.8 the rate of net K^+ loss was maximal and unaffected by changes in pH_o that ranged from 6.3 to 7.9. We found that when pH_i was 6.1, net K^+ efflux was inhibited at pH_o 6.1 but not at pH_o 7.4 (Fig. 1A). The basis for this disparity is not clear but may lie in the different values of pH_i employed or in their use of NO_3^- instead of Cl^- , because the membrane conductance for NO_3^- is higher than that for Cl^- . Hence the cells in NO_3^- would presumably be depolarized relative to the cells in Cl^- and this could reduce the relative drag on the movement of K^+ (cf. ref. 31). On the other hand, this consideration does not take into account the relative effects of interactions between H_o^+ and K_o^+ , as discussed before, on E_m . Nevertheless, differences in the relative membrane conductance of Cl^- versus SO_4^{2-} have been shown to alter K^+ efflux through the Gardos channel in a manner expected from the differences in the resultant values of E_m (31).

Another possible consequence of E_m effects on Gardos channel activity concerns the relationship between K^+ efflux and K_o^+ . It has been reported (12, 36–39) that activation of Ca^{2+} -dependent K^+ transport is biphasic, reaching peak activity at 2–3 mM K_o^+ and then falling off as K_o^+ is increased further. This is the situation in the absence of DIDS whether the principal anion is Cl^- or SO_4^{2-} . But when DIDS is used the response to K_o^+ is different, as seen in Fig. 3, where increasing K_o^+ , at any pH_o , increases K^+ efflux. While DIDS, by decreasing anion conductance, brings E_m closer to E_K , with the magnitude of the driving force for K^+ efflux being affected by changes in K_o^+ , it seems that, in the absence of DIDS, K_o^+ has an additional effect to that of E_m in modulating K^+ efflux through the channel. More work is needed to clarify this additional role of K_o^+ .

In considering the effects of pH_o and K_o^+ on the Gardos channel as described in this paper, it appears that two sites at the outside of the channel are needed to explain the requirement for K_o^+ and its interaction with H_o^+ . One site, when occupied by K^+ , keeps the channel open and may also be a transport site. When K^+ is absent from this site the channel is inactivated with a long relaxation time or becomes refractory to subsequent activation by K^+ (Fig. 4, curve A). Since this action of K_o^+ is not altered by H_o^+ (Fig. 4, curve B), this site must be different from the one that is needed to account for the

interaction between K_o^+ and H_o^+ as already discussed (e.g., Fig. 2). (This is so whether or not H_o^+ acts at the first site to promote closure or prevent opening of the channel.) It is not clear whether protons at this second outside site act directly or allosterically to alter the channel's conductance to K^+ . By an analogous argument, a site similar to the second outside site must also be present at the inside of the channel in order to account for the interaction between H_i^+ and K_i^+ as discussed in connection with Table 1. It is possible, of course, that this site may also participate in the interaction between H^+ and Ca^{2+} as characterized by Porzig (11).

This work was supported in part by National Institutes of Health Grants HL09906 and AM17433.

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