Ca-induced K Transport in Human Red Blood Cell Ghosts Containing Arsenazo III

Transmembrane Interactions of Na, K, and Ca and the Relationship to the Functioning Na-K Pump

DOUGLAS R. YINGST and JOSEPH F. HOFFMAN

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT Increasing free intracellular Ca (Ca_i) from <0.1 μM to 10 μM by means of A23187 activated Ca-stimulated K transport and inhibited the Na-K pump in resealed human red cell ghosts. These ghosts contained 2 mM ATP, which was maintained by a regenerating system, and arsenazo III to measure Ca_i. Ca-stimulated K transport was activated 50% at 2–3 μM free Ca_i and the Na-K pump was inhibited 50% by 5–10 μM free Ca_i. Free Ca_i from 1 to 8 μM stimulated K efflux before it inhibited the Na-K pump, dissociating the effect of Ca on the two systems. 3 μM trifluoperazine inhibited Ca-stimulated K efflux and 0.5 mM quinidine reduced Na-K pumping by 50%. In other studies, incubating fresh intact cells in solutions containing Ca and 0.5 μM A23187 caused the cells to lose K heterogeneously. Under the same conditions, increasing A23187 to 10 μM initiated a homogeneous loss of K. In ATP-deficient ghosts containing Ca_i equilibrated with A23187, K transport was activated at the same free Ca_i as in the ghosts containing 2 mM ATP. Neither Ca_o nor the presence of an inward Ca gradient altered the effect of free Ca_i on the permeability to K. In these ghosts, transmembrane interactions of Na and K influenced the rate of Ca-stimulated K efflux independent of Na- and K-induced changes in free Ca_i or sensitivity to Ca_i. At constant free Ca_i, increasing K_o from 0.1 to 3 mM stimulated K efflux, whereas further increasing K_o inhibited it. Increasing Na_i at constant K_i and free Ca_i markedly decreased the rate of efflux at 2 mM K_o, but had no effect when K_o was ≥20 mM. These transmembrane interactions indicate that the mechanism underlying Ca-stimulated K transport is mediated. Since these interactions from either side of the membrane are independent of free Ca_i, activation of the transport mechanism by Ca_i must be at a site that is independent of those responsible for the interaction of Na and K. In the presence of A23187, this activating site is half-maximally stimulated by ~2 μM free Ca and is not influenced by the concentration of ATP. The

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partial inhibition of Ca-stimulated K efflux by trifluoperazine in ghosts containing ATP suggests that calmodulin could be involved in the activation of K transport by Ca_i. Part of the Na-K pump apparatus could be the mechanism that underlies Ca-stimulated K transport because both processes are inhibited by the same drugs, including quinidine, and the pump could account for the transmembrane interactions of Na and K described above. Nevertheless, functioning Na-K pumps are not altered by Ca_i to transport K selectively, because K transport is activated by Ca_i before the Na-K pump becomes inhibited.

INTRODUCTION

Intracellular Ca (Ca_i) provokes a specific and marked increase in the K permeability of human red blood cells (Gardos, 1958), altering both cell volume (Passow, 1963) and membrane potential (Glynn and Warner, 1972; Hoffman and Knauf, 1973). The responsible transport mechanism is unknown, but a number of interactions of Na and K suggest that the transport of K is mediated. For instance, when cells or resealed ghosts that have been depleted of ATP in order to inactivate the outwardly directed Ca pump are suspended in solutions with sufficient Ca to increase Ca_i, increasing K_o from <0.1 to 2 mM K_o stimulates K efflux, and then as K_o is increased above 2 mM, it inhibits the rate of efflux (Blum and Hoffman, 1971; Knauf et al., 1975). K_o may even be an absolute requirement to obtain any Ca-induced K transport (Knauf et al., 1975; Heinz and Passow, 1980). Other observations are that intracellular Na (Na_i) inhibits K efflux in a manner that depends on K_o, and that Na_i alters the activation and inhibition of K efflux by K_o (Riordan and Passow, 1973; Knauf et al., 1975; Hoffman and Blum, 1977). These results, however, were obtained under conditions where Ca_i was unknown and changing, so that it is uncertain which represent direct transmembrane interactions of K and Na with the transport mechanism and which are secondary to Na- and K-induced alterations in Ca_i. In support of this latter interpretation, Porzig (1977) suggested that the activation of K efflux by low K_o may be due to a stimulation of Ca influx by K_o, because the rate of K efflux in resealed ghosts containing Ca_i buffered by EGTA was unaltered by such changes in K_o. Similarly, the inhibitory effect of high K_o on K efflux has been explained by K_o-induced inhibition of Ca influx (Lew, 1974; Knauf et al., 1975; Hoffman and Blum, 1977). In one study at constant free Ca_i (Simons, 1976), Na_i has been shown to inhibit Ca-activated K:K exchange at low and not at high K_o, but it was not established whether this was due to an effect of K_o on competition between Na_i and K_i or to K_o-induced alterations in the activation of K efflux by Ca_i. Therefore, to determine whether these transmembrane effects of Na and K on the rate of Ca-stimulated K efflux could be due to Na- and K-induced changes in either the availability or sensitivity to Ca_i, these interactions were studied in energy-depleted resealed ghosts containing the Ca indicator dye arsenazo III, in which Na_i and K_i could be manipulated and free Ca_i could be measured (Yingst and Hoffman, 1983). In carrying out these studies, we made use of the information obtained

in the preceding paper (Yingst and Hoffman, 1984) that resealed ghosts are heterogeneous in their passive Ca permeability and that the ionophore A23187 can be used to equilibrate Ca and eliminate Ca-induced K heterogeneity.

In addition to stimulating K transport, Ca_i also inhibits the Na-K pump of human red blood cells (Hoffman, 1962; Dunn, 1974; Gardos et al., 1977). This observation, plus the action of certain drugs such as ouabain, oligomycin, furosemide, and quinidine, which inhibit both Ca-stimulated K transport and the Na-K pump, leads to the suggestion that if Ca-stimulated K efflux is a mediated process, then an altered form of the Na-K pump is a possible candidate as the underlying mechanism (Hoffman and Blum, 1977). Since the evidence for this proposal is still equivocal (Hoffman et al., 1980), we have tested the hypothesis that Ca alters functioning Na-K pumps to selectively transport K by comparing the sensitivity of the Na-K pump and Ca-induced K transport to various concentrations of free Ca_i. For instance, if the same free Ca_i inhibited the Na-K pump at the same concentration or at the same rate as it stimulated K efflux, this would be evidence that the two processes could be related. In this paper we measure the effect of Ca_i both on the Na-K pump and on Ca-stimulated K efflux under identical conditions using resealed ghosts containing arsenazo III and a regenerating system to maintain ATP at a constant concentration. Since ATP activates the Ca pump, which would lower Ca_i, A23187 was added to the ghosts suspended in solutions buffered with free Ca_o such that the resultant concentration of free Ca_i measured with arsenazo III is a balance between the outwardly directed Ca pump and the ionophore-induced inward leak. Since these ghosts also contained Mg to help run the Na-K pump and since the absorbance of arsenazo III is altered by the presence of Mg, we developed a method to measure both free Ca_i and Mg_i from the absorbance of arsenazo III so that intracellular concentrations of both ions can be monitored simultaneously. In addition to these issues, the claim that A23187 induces a change in the Ca affinity of K transport (Lew and Ferreira, 1976) is examined in terms of Ca and K heterogeneity in energy-replete cells. Finally, the effect of trifluoperazine on the rate of Ca-stimulated K efflux is examined. Preliminary reports of some of these data have been published elsewhere (Yingst and Hoffman, 1979, 1981; Hoffman et al., 1980; Hoffman and Yingst, 1981).

MATERIALS AND METHODS

Preparation of Resealed Ghosts

Energy-depleted ghosts were made as described in the preceding paper (Yingst and Hoffman, 1984). In brief, blood was collected in heparin from healthy donors, washed, depleted of ATP by prolonged incubation at 37°C, hemolyzed and reversed at 0°C, resealed at 37°C for 45 min in the final hemolysis solution, and washed. They were then centrifuged on a 10% sucrose cushion, which separates ghosts with a lower passive permeability to ions (type II) from those with a higher permeability (type III) (Bodemann and Passow, 1972; Yingst and Hoffman, 1983). The tight ghosts (type II) were selected

and used for the experiments described in this paper. The contents of these ghosts were varied depending on the experiment and are specified in the legends to Tables I and III and Figs. 5–7.

Ghosts in which the Na-K pump was studied were made from fresh cells without prior energy depletion and contained an ATP-regenerating system consisting of phosphocreatine and creatine phosphokinase to maintain the ATP at a constant level throughout the flux measurements (Glynn and Hoffman, 1971). Fresh cells from healthy donors were washed three times at 0–4°C in 150 mM NaCl, 20 mM Hepes, and 0.1 mM EDTA after removing the buffy coat. The cells were hemolyzed following the same procedure as the ATP-deficient ghosts (Yingst and Hoffman, 1984), except that the hemolyzing solution contained the ATP-regenerating system and a higher concentration of total Mg. The final concentrations of intracellular constituents are given in the legends to Tables IV and V and Figs. 2–4.

Flux Measurements

The procedures for measuring Na and K effluxes are detailed in the appropriate figure legends because the protocol varied. All fluxes were carried out in the presence of A23187 to equilibrate Ca (Yingst and Hoffman, 1984) at 1–1.67% hematocrit in 25- or 50-ml Erlenmeyer flasks, which were gently shaken in a water bath at 37°C. The rate constant for unidirectional K or Na efflux (in units of reciprocal hours) was calculated from the fractional loss of ^{42}K or ^{24}Na (Hoffman, 1958). The results presented are representative of at least three similar experiments.

Measurements of Free Ca_i and Mg_i

Free Ca_i was calculated from Ca-sensitive changes in the absorbance of entrapped arsenazo III with a known total dye concentration, hematocrit, and dissociation constants and molar extinction coefficients for 1:1 (CaD) and 2:2 (Ca_2D_2) complexes of Ca and dye (Yingst and Hoffman, 1983). All measurements were carried out in the presence of A23187 to equilibrate Ca following the procedures established in the initial calibration of the dye in resealed ghosts (Yingst and Hoffman, 1983).

In experiments in which free Mg_i was <50 μM Mg (Tables I–III and Figs. 5–7), free Ca_i was calculated by method A from the change in absorbance at 655 nm (ΔA_{655})[1] measured in a DW-2 dual-wavelength spectrophotometer (Aminco-Bowman, Silver Spring, MD) (Yingst and Hoffman, 1983, 1984). Ghosts in these experiments initially contained >50 μM free Mg, but this concentration rapidly declined when A23187 was added to the ghosts suspended in Mg-free solutions. The response of arsenazo III in resealed ghosts as a function of free Ca_i using method A is illustrated in Fig. 1 of the preceding paper (Yingst and Hoffman, 1984).

When free Mg_i was >50 μM (Tables IV and V and Figs. 2–4), both free Ca_i and Mg_i were calculated from changes in the absorbance of arsenazo III at 600, 630, and 655 nm as described here (method C). The change in absorbance at one of these wavelengths, say 655 nm, is equal to

$$\Delta A_{655} = (A_{655} - A_{700})_{\text{Ca and Mg}} - (A_{655} - A_{700}), \quad (1)$$

where $(A_{655} - A_{700})_{\text{Ca and Mg}}$ is the difference in absorbance between 655 nm and the reference wavelength of 700 nm for a sample with Ca and Mg, and $A_{655} - A_{700}$ is the difference in absorbance between the same two wavelengths for an identical sample containing no free Ca or free Mg, i.e., for ghosts containing no free Ca_i or Mg_i above the detection limit of the dye, which is in the range 0.1–0.3 μM free Ca_i or 10–30 μM free

[1] The abbreviation for absorbance (A) is synonymous with "AB" in Yingst and Hoffman, 1983.

Mg_i, depending on the ghost preparation. Free Ca calculated from ΔA is, therefore, a change in free Ca referenced to a condition where free Ca is below the detection limit of the dye.

At each wavelength the ΔA is equal to the sum of changes in absorbance of CaD, Ca_2D_2, and MgD. Thus, at each of the three wavelengths:

$$\Delta A_{600} = \Delta\epsilon_1 \cdot [CaD] + \Delta\epsilon_2 \cdot [Ca_2D_2] + \Delta\epsilon_3 \cdot [MgD]; \quad (2)$$

$$\Delta A_{630} = \Delta\epsilon_4 \cdot [CaD] + \Delta\epsilon_5 \cdot [Ca_2D_2] + \Delta\epsilon_6 \cdot [MgD]; \quad (3)$$

$$\Delta A_{655} = \Delta\epsilon_7 \cdot [CaD] + \Delta\epsilon_8 \cdot [Ca_2D_2] + \Delta\epsilon_9 \cdot [MgD], \quad (4)$$

where $\Delta\epsilon_1$ to $\Delta\epsilon_9$ are the appropriate molar extinction coefficients for a change in absorbance, CaD and Ca_2D_2 are 1:1 and 2:2 complexes of Ca and dye, and MgD is a 1:1 complex of Mg and dye, respectively (Yingst and Hoffman, 1983). From these three equations one can derive separate expressions for CaD, Ca_2D_2, and MgD:

$$[MgD] = (\Delta A_{630} - \Delta\epsilon_4 \cdot [CaD] - \Delta\epsilon_5 \cdot [Ca_2D_2])/\Delta\epsilon_6; \quad (5)$$

$$[CaD] = (\Delta A_{655} - \Delta\epsilon_8 \cdot [Ca_2D_2] - \Delta\epsilon_9 \cdot \Delta A_{630}/\Delta\epsilon_6 \quad (6)$$

$$+ \Delta\epsilon_9 \cdot \Delta\epsilon_5 \cdot [Ca_2D_2]/\Delta\epsilon_6)/x; \quad (7)$$

$$[Ca_2D_2] = (\Delta A_{600} - y \cdot \Delta A_{655}/x + \Delta\epsilon_9 \cdot \Delta A_{630} \cdot y/\Delta\epsilon_6 \cdot x$$

$$- \Delta\epsilon_3 \cdot \Delta A_{630}/\Delta\epsilon_6)/(\Delta\epsilon_9 \cdot \Delta\epsilon_5 \cdot y/\Delta\epsilon_6 \cdot x - \Delta\epsilon_8 \cdot y/x + w),$$

where y is $(\Delta\epsilon_1 - \Delta\epsilon_3 \cdot \Delta\epsilon_4)/\Delta\epsilon_6$, x is $(\Delta\epsilon_7 - \Delta\epsilon_9 \cdot \Delta\epsilon_4)/\Delta\epsilon_6$, and w is $(\Delta\epsilon_2 - \Delta\epsilon_3 \cdot \Delta\epsilon_5/\Delta\epsilon_6)$.

Knowing [MgD], [CaD], $[Ca_2D_2]$, and $[D_T]$, [D] can be calculated from:

$$[D_T] = [D] + [CaD] + 2[Ca_2D_2] + [MgD]. \quad (8)$$

Free Mg and Ca are calculated from the appropriate mass action equation:

$$[\text{free Ca}] = (K_{d,Ca_2D_2} \cdot [Ca_2D_2]/[D]^2)^{0.5}; \quad (9)$$

$$[\text{free Mg}] = K_{d,MgD} \cdot [MgD]/[D], \quad (10)$$

where K_{d,Ca_2D_2} and $K_{d,MgD}$ are the appropriate dissociation constants measured at 37°C and an ionic strength of 0.17 M (Yingst and Hoffman, 1983).

Materials

All chemicals except where noted were reagent grade. EDTA (ethylene-diamine-tetra-acetic acid), and HEDTA (*N*-hydroxyethylethylene-diamine-triacetic acid) were purchased from Sigma Chemical Co. (St. Louis, MO) as disodium salts. A23187 was obtained both from E.I. Lilly, Inc. (Indianapolis, IN) and from Calbiochem-Behring Corp. (La Jolla, CA). The arsenazo III came as a practical grade from Sigma Chemical Co. and was purified as described (Yingst and Hoffman, 1978, 1983). Choline chloride was purchased from Syntex Agri-Business, Inc. (Springfield, MO).

RESULTS

Effects of Ca in Ghosts with Functioning Pumps

The relative effects of Ca_i on activating Ca-stimulated K transport and inhibiting the Na-K pump were measured under very similar conditions in resealed ghosts containing a constant source of ATP (Fig. 1) provided by a regenerating system

based on phosphocreatine and creatine phosphokinase (Glynn and Hoffman, 1971). Free Ca_i was controlled by suspending the ghosts in solutions containing various concentrations of buffered free Ca_o and balancing the efflux of Ca via the Ca pump by an influx of Ca mediated by A23187. The resultant free Ca_i was then measured with entrapped arsenazo III (Fig. 1). In the presence of ATP to run the Ca pump, free Ca_i was 25–40% of free Ca_o, whereas in energy-

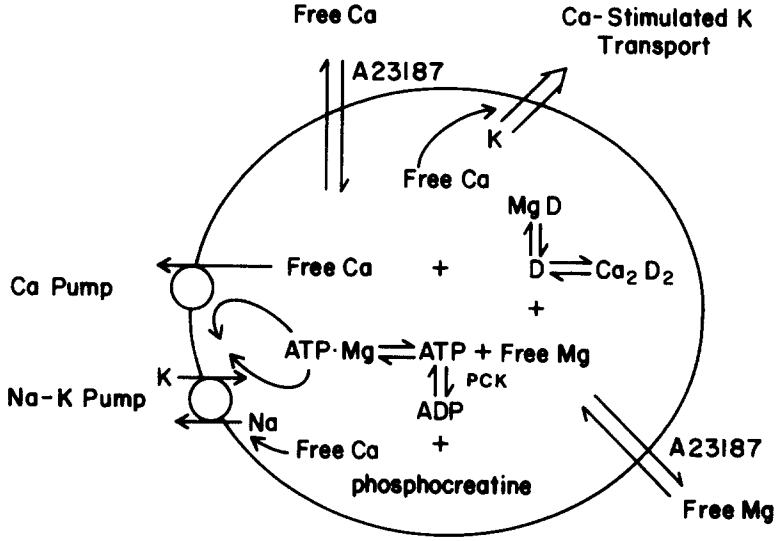

FIGURE 1. A diagram of how free Ca_i is controlled and measured in resealed ghosts containing a functioning Ca pump fueled by ATP. The ghosts are suspended in solutions containing free Ca, free Mg, and A23187, an ionophore for both Ca and Mg. Free Ca_i is the balance between A23187-mediated influx and active transport out of the ghosts via the Ca pump. Free Ca_i is in equilibrium with bound Ca on the inside of the ghosts, including that complexed with the dye arsenazo III (Ca_2D_2). Similarly, arsenazo III forms a complex with Mg (MgD). The concentrations of free Ca_i and free Mg_i are calculated from the concentrations of CaD, Ca_2D_2, and MgD by method C. The concentration of ATP is maintained constant through the use of an ATP-regenerating system based on phosphocreatine and creatine phosphokinase (PCK). The ghosts containing ATP and functioning Ca pumps are used to study the relationship between Ca-induced K efflux and Ca inhibition of the Na-K pump (Figs. 2 and 3) and to determine the Ca sensitivity of K transport in ghosts containing ATP (Fig. 4).

depleted ghosts, free Ca_i was equal to free Ca_o when the membrane potential was close to zero.

The effects of free Ca_i on K transport and ouabain-sensitive ^{24}Na efflux from two sets of ghosts made from the cells of a single donor are first shown as a function of time (Fig. 2). Note that at the first time point of 11 min both 8 μM and <1 μM free Ca_i stimulated the efflux of K, but did not inhibit ouabain-sensitive Na efflux compared with ghosts containing no free Ca_i. Thus, the effect of Ca on K transport can be separated from its effect on the Na-K pump.

Furthermore, stimulation of K efflux is greater at 8 μM than at <1 μM free Ca_i and yet there is no difference in how these two concentrations affect the Na-K pump. At higher Ca concentrations and longer time points, both transport processes are affected (Fig. 2). The results of Fig. 2 are similar to those obtained

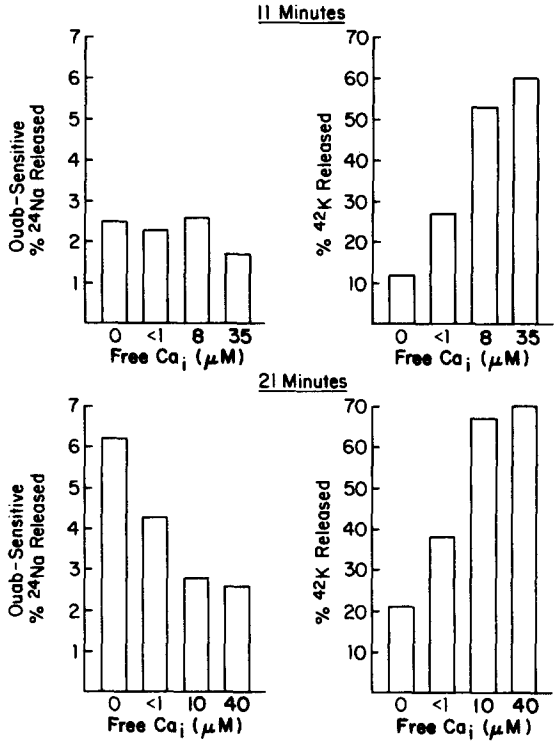

FIGURE 2. The effect of free Ca_i on inhibiting the Na-K pump and stimulating K transport. The measurements were carried out on two sets of ghosts made concurrently from the cells of a single donor, one containing ^{42}K and the other ^{24}Na. In addition, both sets of ghosts contained 30 mM Na, 6 mM K, 121 mM choline, 20 mM Hepes, 2 mM ATP, 2 mM Mg, 10 mM phosphocreatine, 160 μg/ml creatine phosphokinase, 50 μM EGTA, and 100 μM arsenazo III. The procedure for carrying out the fluxes was to suspend the ghosts at 1.67% hematocrit at 0°C in the flux solutions, which contained 147 mM Na, 6 mM K, 20 mM Hepes, 5 mM EDTA, 5 μM A23187, 0.1 mM ouabain where appropriate, and different concentrations of Mg and Ca. The ghosts were then warmed to 37°C and both fluxes were measured over the same period of time. The phosphocreatine and creatine phosphokinase were incorporated to maintain the ATP at a constant value throughout the fluxes (Glynn and Hoffman, 1971). The resultant concentrations of free Ca_i and Mg_i were measured by method C. The free Mg_i was 0.8 mM. The concentration of 6 mM K inside and out was chosen to activate the Na-K pump, limit the inhibitory effect of K_o on Ca-stimulated K transport, prevent net K loss, which could lead to changes in cell volume, and minimize changes in membrane potential. Each of the values given above is the mean of two separate measurements.

in four other experiments and clearly show that stimulation of K efflux can be dissociated from inhibition of the Na-K pump.

To determine the average effect of Ca_i on the Na-K pump and Ca-stimulated K efflux at both time points in experiments like that shown in Fig. 2, the rate constant for either Ca-stimulated ^{42}K efflux or ouabain-sensitive ^{24}Na efflux was calculated for each Ca condition. Then the percent inhibition for the Na-K pump and the percent stimulation for ^{42}K efflux were computed by comparing these rates to those obtained at 0 free Ca. The results of this type of analysis suggest that the sensitivities of the two processes measured over the same period of time are similar (Fig. 3). This conclusion does not, however, imply that Ca_i affects each the same way. In the four experiments shown in Fig. 3, the rate of ouabain-sensitive Na efflux at 0 free Ca could generally be fit well by a single exponential. At values of free Ca_i that inhibited the Na-K pump at the second time and not at the first, there was often less ^{24}Na released at the second time point than would be expected based on a single exponential. At high concentrations of free Ca_i, which inhibited at both time points, such deviations were insignificant. Trends for the K fluxes were clear-cut. In experiments in which the last time point was longer than 14 min, the percent ^{42}K released at the last point was always somewhat lower than expected from a single-exponential fit to earlier time points. The maximum deviations were 20% at the highest Ca concentrations. Such deviations from a single exponential are probably not due to changes in volume or membrane potential caused by K loss, because both the ghosts and the flux solutions were made to contain the same concentration of K. Thus, the effects of Ca_i are complex. Ca_i can inhibit the Na-K pump before it stimulates K efflux (Fig. 2), but the average Ca sensitivities of the two processes are similar (Fig. 3).

In ghosts containing the regenerating system and ~2 mM ATP, Ca-stimulated K transport is half-maximally stimulated by ~2 μM free Ca_i (Fig. 4). This affinity for Ca is very close to the sensitivity to Ca measured under a variety of conditions in ghosts into which no ATP was incorporated (Figs. 5 and 6).

Transmembrane Interactions of Na and K

EFFECT OF K_o To test whether K_o activates and inhibits at constant free Ca_i, Ca-stimulated K transport was measured in resealed ghosts depleted of ATP as a function of K_o at constant free Ca_i monitored with entrapped arsenazo III (Table I). The results confirm the effects of both high and low concentrations of K_o on K efflux observed in earlier studies (Blum and Hoffman, 1971; Kregenow and Hoffman, 1972) and demonstrate that the effects of K_o represent direct interactions of K_o with the transport mechanism and are not due simply to secondary effects of K_o on Ca influx and the availability of Ca_i to stimulate K efflux. This effect of K_o on the permeability of the membrane to K is even greater when changes in the electrochemical driving forces are considered. Table I shows the value of the K permeability (P_K) calculated from the measured rate constant (^ok_K) at each value of K_i/K_o using the constant field equation (Hunter, 1971, 1977). Note that changes in P_K as a function of K_o are larger than changes in the value of the rate constants alone (Table I). The membrane potentials (E_m)

in Table I are also calculated from the constant field equation to fit the measured rate of K efflux at the stated concentration of K and Cl on either side of the membrane.

INTERACTION OF NA_i AND K_i AS A FUNCTION OF FREE CA_i Previous investi-

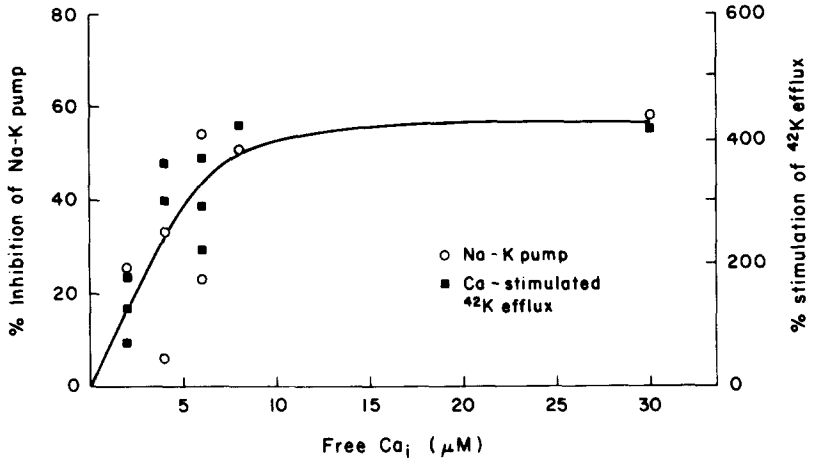

FIGURE 3. Percent inhibition of the Na-K pump and percent stimulation of Ca-induced K transport as a function free Ca_i. The data are from four separate experiments in which both the Na-K pump and the Ca-stimulated K efflux were measured in each of four pairs of ghosts under the same conditions over the same period of time. The activity of the Na-K pump was assayed by measuring the rate of ouabain-sensitive ^{24}Na efflux. The percent inhibition at a given free Ca_i is based on the ouabain-sensitive flux measured in the same experiment at 0 free Ca_i. Similarly, the percent Ca stimulation of K efflux at a given free Ca_i is referenced to the rate of ^{42}K efflux measured at 0 free Ca_i in the same experiment. The procedure for carrying out the fluxes was to suspend the ghosts at 0°C in the flux solutions, which contained 147 mM Na, 6 mM K, 20 mM Hepes, 5 mM EDTA, 5 μM A23187, 0.1 mM ouabain where appropriate, and variable concentrations of Mg and Ca to give 1 mM free Mg and free Ca concentrations of 0.5–30 μM. The ghosts were then warmed to 37°C and the appropriate fluxes were measured over the same period of time, which, depending on the experiment, varied from 14 to 20 min. The initial contents of the ghosts were 30 mM Na, 6 mM K, 121 mM choline, 20 mM Hepes, 2 mM ATP, 2 mM Mg, 10 mM phosphocreatine, 160 μg/ml creatine phosphokinase, 50 μM EGTA, and 100 μM arsenazo III. The phosphocreatine-creatine phosphokinase was included to maintain a constant concentration of ATP. The resultant concentrations of free Ca_i and free Mg_i were calculated from the absorbance of arsenazo III measured by method C. After the first few minutes, the free Ca_i under a given set of conditions remained relatively constant throughout the flux, and average values during this period are shown above. For most of the experiments the free Mg_i was in the range of 0.5–2 mM. At 0 μM free Ca_i the ouabain-sensitive Na efflux was in the range of 3.3–6.5 mmol Na/liter ghosts·h, with an average value to 5.2 mmol Na/liter ghosts·h. The passive loss of ^{42}K at 0 μM free Ca_i varied from 3.6 to 5 mmol K/liter ghosts·h with an average value of 4.6 mmol K/liter ghosts·h.

gations have shown that Na_i inhibits the rate of Ca-stimulated K efflux (Riordan and Passow, 1973; Knauf et al., 1975; Simons, 1976; Hoffman and Blum, 1977), but from these studies it is not clear whether this inhibition was due to a competition of Na_i with K_i or to Na_i-induced changes in the Ca sensitivity of the K transport mechanism. To test these two possibilities, the ratio of Na_i to K_i was varied in ghosts depleted of ATP, and K efflux was measured as a function of free Ca_i as monitored with entrapped arsenazo III. Fig. 5 shows that at low K_i

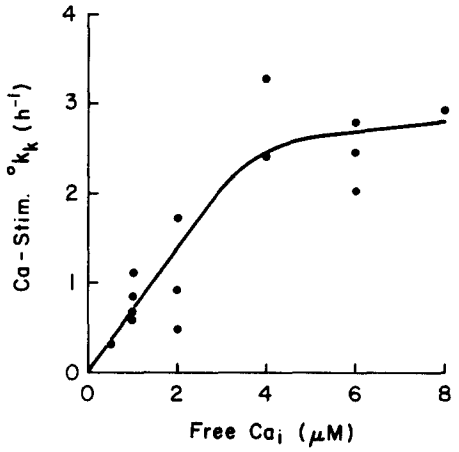

FIGURE 4. The rate constant for Ca-stimulated ^{42}K efflux (Ca-stim. $^{\circ}k_K$) as a function of free Ca_i in ghosts containing 2 mM ATP and functioning Ca pumps. Ghosts were suspended at 1.5% hematocrit at 0°C in the flux solutions containing 147 mM Na, 6 mM K, 20 mM Hepes, 5 mM EDTA, 5 μM A23187, and variable concentrations of Mg and Ca to give 1 mM free Mg and free Ca concentrations of 0.5–10 μM at 37°C. The ghosts were then warmed to 37°C and the efflux of ^{42}K was measured over the next 12–20 min, depending on the experiment. The initial contents of the ghosts were 30 mM Na, 6 mM K, 121 mM choline, 20 mM Hepes, 2 mM ATP, 2 mM Mg, 50 μM EDTA, 100 μM arsenazo III, 10 mM phosphocreatine, and 160 μg/ml creatine phosphokinase to maintain constant ATP. The resultant free Ca_i and free Mg_i were calculated by method C. After the first few minutes the free Ca_i under a given set of conditions remained relatively constant throughout the flux interval; average values during these periods are shown. For most of the experiments the free Mg_i was in the range of 0.5–2 mM. The data are from seven independent experiments, four of which also included in Fig. 3. Each point is the mean of two separate measurements.

increasing Na_i markedly depresses $^{\circ}k_K$ at all values of free Ca_i, whereas at high K_i increasing Na_i only slightly decreases $^{\circ}k_K$. Also, Na_i has little effect on the free Ca_i activating K efflux. Na_i, therefore, inhibits K efflux by competing with K_i, not with Ca_i, or by otherwise changing the sensitivity to Ca_i.

In ghosts with <5 mM Na_i, $^{\circ}k_K$ is significantly lower when K_i is 100 mM as compared with 25 mM K_i (Fig. 5). This reduction is due to changes in the electrochemical driving forces, instead of an effect of K_i on the transport

mechanism, because the calculated values of P_K are the same at the two concentrations of K_i (Table II).

EFFECT OF K_o ON THE COMPETITION OF NA_i WITH K_i In energy-depleted ghosts the competition of Na_i and K_i observed in Fig. 5 is altered by K_o independent of K_o-induced changes in the sensitivity to Ca_i (Fig. 6). Increasing

TABLE I

K_o	Free Ca_i	^ok_K	P_K	E_m
mM	*μM*	h^{-1}	h^{-1}	*mV*
3	<0.1	0.16±0.02	0.17	−1
0.1	5.4	0.55±0.04	0.6	−5
2	6.4	3.58±0.34	6.7	−30
3	5.0	4.02±0.42	8.0	−33
16	6.0	2.53±0.21	3.6	−18
140	5.1	1.26±0.10	1.3	0

The effect of K_o on the measured rate constant for K efflux (^ok_K), and corresponding values for the permeability to K (P_K) expressed as a rate constant, and the membrane potential (E_m). P_K and E_m were calculated from K_i, K_o, Cl_i, and Cl_o by means of the Goldman equation (Hunter, 1971, 1977) to consider changes in the electrochemical driving forces as a function of K_o. A value of 2.8×10^{-8} cm/s was used for the net chloride permeability (P_{Cl}) (Knauf et al., 1977), and it was assumed that P_K and P_{Cl} were much greater than the permeability to Na (P_{Na}). P_K and E_m were calculated from the value of B (Hunter, 1977) determined by iteration to fit the measured rate constant:

$$^ok_K = \frac{B \cdot P_{Cl} \cdot [Cl]_i - P_{Cl} \cdot [Cl]_o}{[K]_i - B \cdot [K]_o} \cdot \frac{1nB}{B - 1},$$

where

$$B = \frac{P_{Cl} \cdot [Cl]_o + P_K \cdot [K]_i}{P_{Cl} \cdot [Cl]_i + P_K \cdot [K]_o}.$$

The first equation was derived by substituting the expression for B into Eq. 3 of Hunter (1977). The flux measurements were carried out by suspending the ghosts at 1% hematocrit in a solution containing 20 mM Hepes, 1.5 mM HEDTA, K as given above, and sufficient Na so that the sum of Na and K was 150 mM. A23187 at a final concentration of 5 μM was added to all solutions and the initial release of ^{42}K was measured. Ca was then added to the solutions and the efflux of ^{42}K was measured over the next 10 min. The rate constant for the unidirectional efflux of ^{42}K (^ok_K) was calculated from the fractional loss of ^{42}K after correcting for a 10–15% loss of ^{42}K before Ca was added. Measurements with entrapped arsenazo III showed that the added Ca equilibrated in <30 s to give the values of free Ca_i listed. The ghosts initially contained 107 mM K, 40 mM Na, 1 mM Mg, 20 μM EGTA, 20 mM Hepes, and 140 μM arsenazo III. All measurements were carried out at 37°C at pH 7.2 and each value of ^ok_K is the mean of three measurements ±1 SD.

Na_i at 2 mM K_o inhibits at all concentrations of free Ca_i, whereas at 20 mM K_o the same increase in Na_i has no effect on ^ok_K at any of the concentrations of free Ca_i tested (Fig. 6). Increasing Na_i or free Ca_i has no effect on K efflux at low (<0.1 mM) K_o, because K_o is now insufficient to activate Ca-stimulated K efflux (Table I).

Effects of Ca_o

An increase in free Ca_i is sufficient to cause an increase in the K permeability of human red blood cells (Blum and Hoffman, 1972). However, it is not known

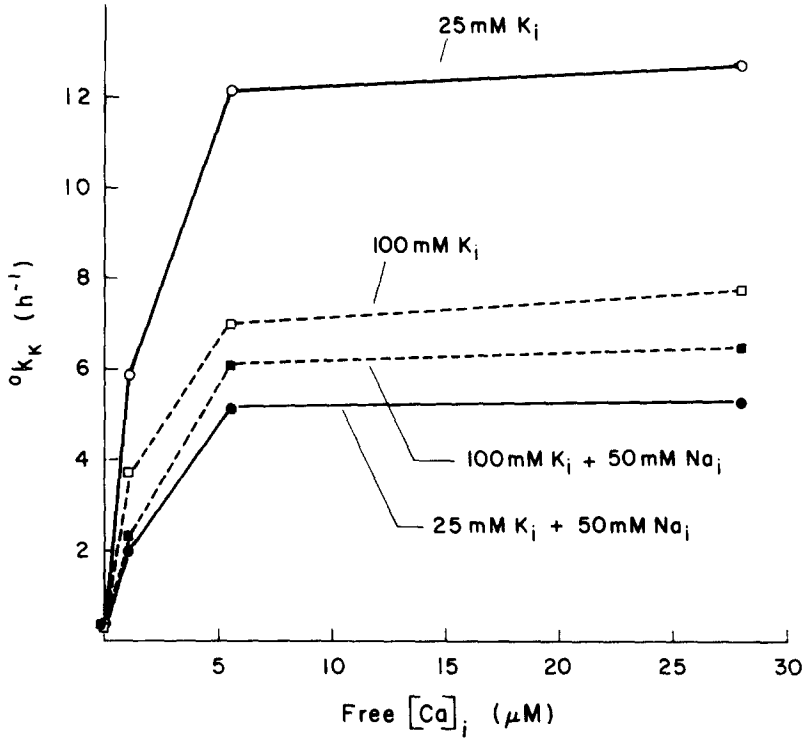

FIGURE 5. The effect of Na_i on ^{42}K efflux at two concentrations of K_i as a function of free Ca_i. The experiment was carried out using four different sets of ghosts, each containing a different combination of Na_i and K_i: (*a*) 25 mM K_i and <5 mM Na_i, (*b*) 25 mM K_i and 50 mM Na_i, (*c*) 100 mM K_i and <5 mM Na_i, and (*d*) 100 mM K_i and 50 mM Na_i. The experiment was conducted by suspending the ghosts at 37°C in the flux solution containing various concentrations of free Ca, adding 5 μM A23187 (final concentration) to equilibrate free Ca and measuring the efflux of ^{42}K over the next 10 min. The rate of K efflux was calculated from the fractional loss of ^{42}K and is presented as the rate constant of unidirectional K efflux ($^{o}k_K$) in units of reciprocal hours. Free Ca_i as measured with entrapped arsenazo III equilibrated in <1 min and remained constant during the experiment. In addition to the indicated concentrations of Na_i and K_i, the ghosts also contained 1 mM Mg, 20 mM Hepes, 20 μM EGTA, 140 μM arsenazo III, and sufficient choline to keep the sum of Na, K, and choline equal to 150 mM. The flux medium contained 2 mM K, 148 choline, 20 mM Hepes, 0.1 mM EGTA, and various concentrations of Ca. The 1 mM Mg originally incorporated into the ghosts decreased to <20 μM when A23187 was added to equilibrate the free Ca, because the flux solution contained no Mg. Each point represents the mean of two separate measurements performed on the same day using four sets of ghosts with various combinations of Na_i and K_i prepared concurrently from the blood of a single donor.

what role, if any, Ca_o or the presence of an inward gradient of Ca plays in altering the effect of Ca_i. To test the effect of Ca_o, resealed ghosts depleted of ATP were made to contain a stable concentration of free Ca_i by incorporating Ca and a high concentration of EGTA. K efflux was then measured in solutions of 0, 0.5, and 1 mM free Ca_o, and the absorbance of arsenazo III was monitored to detect any changes in free Ca_i. Under these conditions the buffering capacity of the EGTA was sufficient to keep free Ca_i constant for 10 min at 0.5 mM Ca_o and for 4 min at 1 mM Ca_o. During this period the rate of K efflux is approximately the same with or without Ca_o (Table III). Thus, neither Ca_o nor an inward Ca gradient influences Ca-stimulated K transport at constant free Ca_i.

Relationship of Changes in Free Ca_i to K Transport

In energy-depleted ghosts containing arsenazo III, and in the presence of A23187 to rapidly equilibrate Ca_i, rapid changes in free Ca_i can readily be manipulated, measured, and related to alterations in K transport. Fig. 7 shows that K transport is stimulated concomitant with an increase in free Ca_i from subthreshold to supramaximal concentrations. Conversely, rapid removal of free Ca_i to below

TABLE II

K_i	Na_i	Ca_i	^ok_K	P_K
mM	*mM*	*μM*	h^{-1}	h^{-1}
25	<5	6	12.19	16.9
100	<5	6	7.06	16.8

Comparison of the rate constants for K efflux (^ok_K) with the calculated K permeability (P_K) at two concentrations of K_i using data from the experiment described in the legend to Fig. 5. P_K was calculated as described in the legend to Table I and considers the changes in the electrochemical driving forces when K_i is altered in energy-depleted ghosts.

threshold immediately inhibits Ca-stimulated K transport (Fig. 7). A23187 has previously been used to equilibrate Ca rapidly in rat red cells to demonstrate that changing Ca_o in a manner consistent with alterations in Ca_i can induce reversible changes in the K permeability (Reed, 1976).

Note that the rate of Ca-stimulated K efflux in Fig. 7 can be fit with a single exponential. This fit is typical of that observed while measuring Ca-stimulated K efflux in energy-depleted ghosts under the various conditions of Figs. 5 and 6 and Tables I and III. Fluxes carried out under the same ionic conditions, but for longer periods of time than used here, were more complex (data not shown).

Drug Effects in Ghosts with Functioning Na-K and Ca Pumps

TRIFLUOPERAZINE Trifluoperazine at a concentration of 3 μM inhibits Ca-induced K transport in ghosts containing ATP (Table IV, A). At higher concentrations, it both inhibits Ca-induced K transport and increases the Ca-independent permeability to K (Table IV, B). The single effect at low concentrations of trifluoperazine was most easily observed when the drug was present in the hemolyzing solution and was trapped inside the ghosts (Table IV, A), whereas the dual effect of higher concentrations on inhibiting Ca-induced K efflux and

increasing the overall permeability of the membrane was evident either when the drug was trapped in the ghosts (data not shown) or when the ghosts were first exposed to the drug during the measurement of K efflux (Table IV, B).

QUINIDINE Quinidine, which has been reported to inhibit Ca-induced K transport in human red blood cells (Lew and Ferreira, 1978), also blocks the Na-K pump in the absence of Ca (Table V), which confirms the results of Lowry et al. (1973). The concentration of 0.5 mM quinidine, which inhibited the Na-K

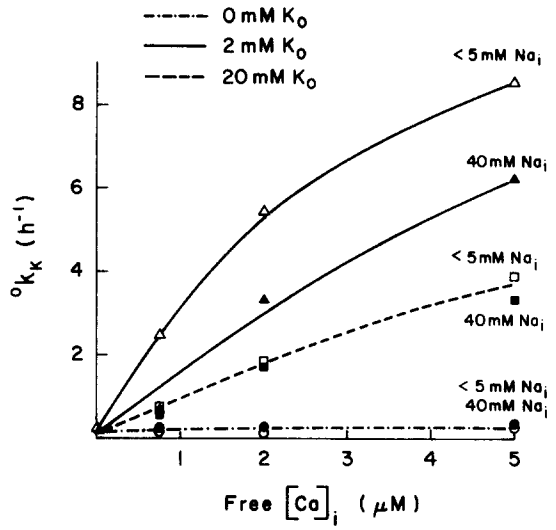

FIGURE 6. The effect of K_o on Na_i inhibition of K efflux as a function of free Ca_i. The experiment was carried out as described in the legend to Fig. 5 using two sets of ghosts, each containing 100 mM K_i and either 40 mM Na_i (solid symbols) or <5 mM Na_i (open symbols). In addition, both sets of ghosts initially contained 1 mM Mg, 20 μM EGTA, 20 mM Hepes, and choline, so that the sum of Na, K, and choline was 150 mM. The extracellular solutions contained either 0, 2, or 20 mM K, 20 mM Hepes, 0.1 mM EGTA, sufficient Ca to give the indicated concentrations of free Ca_i, a final concentration of 5 μM A23187, and the appropriate concentrations of choline, so that the sum of K plus choline was 150 mM. The concentration of Ca_i was measured with entrapped arsenazo III before the measurement of K efflux. Each value of the unidirectional K efflux (^oK_K) is the mean of two separate measurements.

pump by 50% (Table V), is reported to inhibit Ca-induced K transport by 70% (Lew and Ferreira, 1978). The effects of quinidine in the presence of Ca are more difficult to assess because Ca_i alone causes substantial inhibition of the Na-K pump (Table V).

Effect of A23187 on Ca-stimulated K Transport in Intact Cells

In the present study we used A23187 to equilibrate intracellular Ca because we found earlier that resealed ghosts are heterogeneous in their passive permeability to Ca (Yingst and Hoffman, 1984). This heterogeneity led to a variable uptake

of Ca when the ghosts were suspended in a solution containing Ca without A23187. Such variable Ca contents were in turn correlated with a heterogeneous loss of K, which the addition of A23187 effectively removed (Yingst and Hoffman, 1984). However, in using the ionophore to remove heterogeneity, we were concerned about the claim that A23187 itself alters the Ca affinity of Ca-stimulated K transport (Lew and Ferreira, 1976). For instance, it has been contended that in intact cells containing ATP and an outwardly directed Ca pump, high concentrations of ionophore (10 μM) increase the affinity of K transport for Ca, whereas low concentrations of A23187 (0.5 μM) leave the system in its normal low-affinity state (Lew and Ferriera, 1976). Since this conclusion is only warranted if the concentration of the ionophore does not alter the relative amount of ^{42}K lost by cells within the population, the effect of the

TABLE III

Free Ca_o	Free Ca_i	$^{o}k_K$
μM	μM	h^{-1}
<0.1	<0.1	0.29
<0.1	1	1.14
500	1	1.24
1,000	1	1.37

The effect of Ca_o on the rate constant for unidirectional K efflux ($^{o}k_K$) at constant free Ca_i. Energy-depleted resealed ghosts containing 10 mM EGTA, 6.8 mM Ca, and 1 μM free Ca_i, as measured with entrapped arsenazo III, were suspended at 37°C in solutions containing 148 mM Na, 2 mM K, 20 mM Hepes, and 0.1 mM EGTA. The ghosts containing <0.1 μM free Ca were suspended in the same solution without added Ca, which also contained 5 μM A23187. Then either 0, 0.6, or 1.1 mM Ca was added to the extracellular solution and the efflux of K was measured at constant free Ca_i as monitored with entrapped arsenazo III. Because of the presence of EGTA, free Ca_i remained constant for 10 min at 0.5 mM free Ca_o and for 4 min at 1 mM free Ca_o, after which time the buffering capacity of the EGTA was exceeded, the free Ca_i began to increase, and the measurement of K efflux was terminated. In addition to the 10 mM EGTA and 6.8 mM Ca, the ghosts initially contained 135 mM K, 0.5 mM Na, 20 mM Hepes, and 100 μM arsenazo III. All measurements were at 37°C and pH 7.25. Each rate constant is the mean of two independent measurements, all of which were within 12% of each other.

ionophore concentration was examined on the pattern of K loss within a cell population exposed to Ca and ionophore under the same conditions used by Lew and Ferreira (1976) and then separated by subsequent differences in buoyant density in a manner similar to that presented for resealed ghosts (Yingst and Hoffman, 1984). The results of such an experiment are shown in Fig. 8 and demonstrate that the pattern of K loss within the cell population is heterogeneous at the low concentration of ionophore and homogeneous at the higher concentration. At constant Ca_o and a low concentration of ionophore, K loss by individual cells within the cell population varied markedly, whereas at the same Ca_o and at a 20-fold-higher concentration of ionophore, all the cells lost the same K (Fig. 8). In the absence of Ca_o, K is evenly distributed throughout the population of cells and was not affected by the concentration of ionophore (Fig.

8). It is reasonable to assume that the average free Ca_i was greater at 10 than at 0.5 μM A23187, and this difference may account for the greater overall loss of K at the higher ionophore concentration (Fig. 8). Nevertheless, increasing the

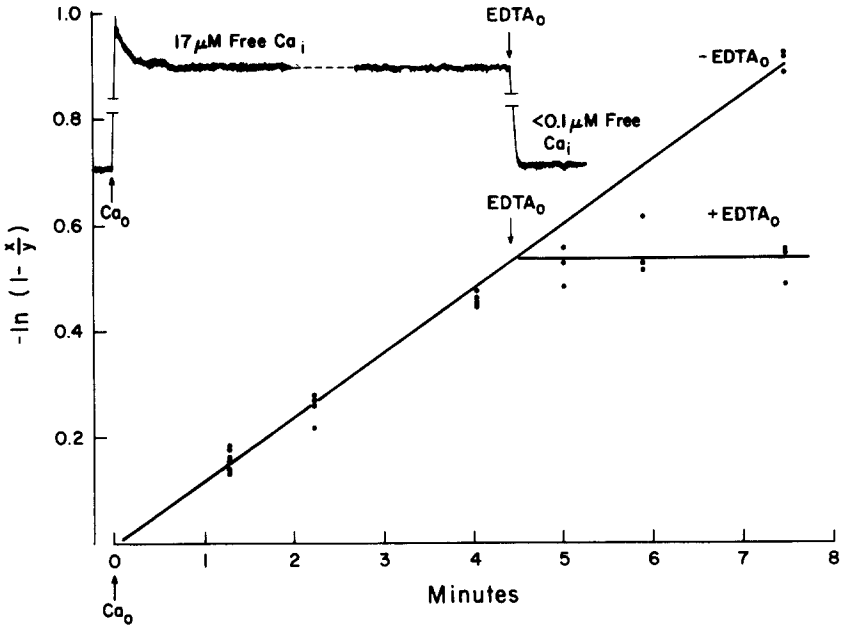

FIGURE 7. The relationship between changes in free Ca_i and Ca-induced K transport. At time zero, Ca_o is added to ghosts that were originally suspended at 1.5% hematocrit in a solution containing 10 μM A23187. The upper trace in the figure is the spectrographic record of the absorbance of arsenazo III inside the ghosts at the dual-wavelength pair of 655–700 nm and shows that the added Ca_o immediately enters the ghosts and is close to its equilibrium value in <5 s. The ordinate is the natural logarithm of the fraction of the initial ^{42}K inside the ghosts, where x is the ^{42}K released from the ghosts and y is the total ^{42}K in the ghosts at the moment Ca was added. 4.5 min after the addition of Ca_o, EDTA was added to the extracellular solution in half of the flasks containing Ca. The change in the absorbance of arsenazo III shows that this addition reduced free Ca_i to the original baseline in <5 s. To determine how quickly Ca induces a change in the K permeability, a least-squares line was drawn through all the points in the ghosts containing 17 μM free Ca_i, and it was found that this line intersects the abscissa 3 s after Ca was added to the first of the six flasks, an operation that originally took a total of 30 s. Similarly, another least-squares lines was calculated for the points from flasks to which EDTA was added. This line intersects the previous least-squares line 4 s after EDTA was added to the first of the three flasks, an operation that took a total of 15 s. The slopes of the two solid lines are equal to the first-order rate constants of Ca-stimulated K efflux. The flux solution contained 150 mM Na, 3 mM K, 20 mM Hepes, 0.1 mM EGTA, and 10 μM A23187; 96 μM Ca was added at time zero. The ghosts were made to contain 147 mM K, 0.5 mM Na, 1.1 mM Mg, 20 mM Hepes, 30 μM EGTA, and 140 μM arsenazo III. Free Mg_i was reduced to <20 μM after the ghosts were added to the flux solution containing A23187.

concentration of the ionophore markedly altered the pattern of K loss within the population of cells. These results do not disprove that the ionophore could change the Ca affinity, but they do suggest that such a conclusion is not yet warranted.

DISCUSSION

We have described the development of a red cell ghost system in which the effects of free Ca_i on the K permeability, the Na-K pump, and the Ca pump can

TABLE IV

Experiment	Free Ca_o	TFP	^oK_K
	μM	μM	h^{-1}
A	<0.1	0	0.43
	5	0	1.37
	<0.1	3	0.39
	5	3	0.74
B	<0.1	0	0.48
	2	0	1.43
	<0.1	50	1.07
	2	50	1.23

The effect of trifluoperazine (TFP) on the rate constant for unidirectional K efflux (^oK_K) in two types of experiments both with ghosts containing the generating system for ATP. In experiment A, 3 μM TFP was in the original hemolysis solution and was incorporated into the ghosts when they were resealed and maintained in all the subsequent wash solutions and in the final flux solutions. In this experiment the ghosts without TFP were prepared at the same time and from the blood of the same donor as those with the incorporated drug. In experiment B, the ghosts were first exposed to TFP during the measurement of K efflux where the drug was present in the flux solutions. In both experiments the ghosts were suspended at 0°C in the flux solution with or without TFP and Ca. The ghosts were then quickly warmed to 37°C and the efflux of K was measured over the next 10 min. Ghosts contained 121 mM choline, 30 mM Na, 6 mM K, 20 mM Hepes, 2 mM ATP, 1 mM Mg, 10 mM phosphocreatine, 160 μg/ml creatine phosphokinase, and 20 μM EGTA. The flux solutions contained 147 mM Na, 6 mM K, 5 mM HEDTA, 20 mM Hepes, 5 μM A23187, and Ca and Mg to give the indicated values of free Ca and Mg. The solution with no free Ca contained 5 mM EGTA and 1 mM Mg. Measurements of free Ca_i in parallel experiments have shown that the ghosts would have contained <0.1 μM free Ca before they were added to the flux solutions containing Ca and A23187 and that the free Ca would have equilibrated in <1 min after the flasks were warmed to 37°C. Rate constants for K efflux are the means of two measurements, which varied by no more than 20%.

be studied simultaneously. The system consists of resealed ghosts containing an ATP-regenerating system and arsenazo III to measure both free Ca_i and free Mg_i. By suspending the ghosts in solutions of various amounts of buffered free Ca_o and adding A23187 to increase the influx of Ca, free Ca_i can be controlled by balancing passive influx and active efflux via the Ca pump and measuring the resultant free Ca_i with arsenazo III. The operation of the Na-K pump and Ca-stimulated K transport at different concentrations of free Ca_i were then studied by measuring the transport of the appropriate isotope. The first observation we

made using this system is that stimulation of K transport by Ca_i can be dissociated from Ca_i inhibition of the Na-K pump. The second is that the sensitivity of the K transport system to Ca_i in the presence of high concentrations of ATP (2 mM) and a functioning Ca pump is the same as in ghosts without ATP. The results presented in this paper also establish that many of the previously described transmembrane effects of Na and K on influencing the rate of Ca-stimulated K transport are primary effects on the K transport system rather than secondary effects caused by Na- and K-induced changes in the availability of Ca_i. The results in this paper also show that the concentration of A23187 used to load energy-replete intact cells with Ca alters the pattern of Ca-stimulated K loss within the cell population. Other observations are that Ca_o does not alter the

TABLE V

Free Ca_o	Quinidine	$^ok_{Na}$	$^ok_{Na}$	Percent inhibition of Na-K pump
μM	mM	h^{-1}	h^{-1}	
		− ouabain	+ ouabain	
0	0	0.292	0.156	0
0	0.5	0.228	0.164	53
6	0	0.191	0.153	72
6	0.5	0.169	0.113	60

The effect of quinidine on the Na-K pump in ghosts containing the generating system for ATP. The flux was carried out by suspending the ghosts at ~1% hematocrit in the appropriate solution at 0°C with or without 0.1 mM ouabain, quinidine, or Ca. The ghosts were then warmed to 37°C and the efflux of ^{24}Na was measured over the next 30 min. Ghosts contained 121 mM choline, 30 mM Na, 6 mM K, 20 mM Hepes, 2 mM ATP, 1 mM Mg, 10 mM phosphocreatine, 160 μg/ml creatine phosphokinase, and 20 μM EGTA. The flux solutions contained 147 mM Na, 6 mM K, 5 mM HEDTA, 20 mM Hepes, 5 μM A23187, and Ca and Mg to give 6 μM free Ca and 0.1 mM free Mg. The solution with no free Ca contained 5 mM EGTA in place of the 5 mM HEDTA and 1 mM Mg. Measurements of free Ca_i in parallel experiments have shown that the ghosts would have contained <0.1 μM free Ca before they were added to the flux solutions containing Ca and A23187 and that the free Ca would have equilibrated in <1 min after the flasks were warmed to 37°C. The rate constant for K efflux (^ok_K) is given in units of reciprocal hours. The values are the mean of three separate measurements in one experiment. All values were within 6% of the mean.

effect of Ca_i on Ca-stimulated K efflux and that transient changes in the K permeability can be directly related to measured alterations in free Ca_i. Finally, it was established that quinidine, an inhibitor of the Ca-induced K transport system, also inhibits the Na-K pump and that trifluoperazine partially blocks Ca-stimulated K transport.

The results of the present study establish that the activation of K efflux by low K_o and the inhibitory effect of high K_o (Blum and Hoffman, 1971; Knauf et al., 1975) are independent of K_o-induced changes in the availability of free Ca_i or sensitivity of the transport mechanism to Ca_i, as had previously been suggested (Lew, 1974; Porzig, 1977). Altering K_o at constant K_i would be expected to change the membrane potential, but as evaluated here (Table I) and elsewhere

(Knauf et al., 1975), a consideration of the membrane potential enhances the magnitude of the activation and inhibition by K_o. K_o, therefore, appears to interact directly with the transport mechanism, which is evidence that Ca-stimulated K efflux does not proceed by simple diffusion. However, in making calculations of the membrane potential, we have assumed that Ca-stimulated K transport is passive and responds directly to the electrochemical gradient as described by the Goldman equation. The results of this study confirm the dependency of Na_i inhibition on K_o (Riordan and Passow, 1973; Knauf et al., 1975; Simons, 1976; Hoffman and Blum, 1977) and establish that this dependency is due to a direct effect of K_o on the interaction of Na_i with K_i and not to changes in Ca_i or to K_o-induced changes in the Ca sensitivity of the transport mechanism. It should also be noted in this context that under other conditions, increasing Na_i can also stimulate Ca-induced K efflux in a pattern that depends on K_o (Hoffman and Blum, 1977).

The existence of the transmembrane effects of Na and K discussed above has led to the suggestion that Ca-induced K transport is a mediated process (Hoffman and Blum, 1977; Hoffman et al., 1980). These observations, plus the findings that the process is inhibited by drugs such as ouabain, oligomycin, and furosemide, which also inhibit the Na-K pump, have led to the suggestion that Ca-stimulated K transport may represent an altered form of the Na-K pump apparatus (Blum and Hoffman, 1971; Hoffman and Blum, 1977). The finding that quinidine, which inhibits Ca-induced K transport (Lew and Ferreira, 1978), also inhibits the Na-K pump means that it is a nonspecific inhibitor and that it can be added to the list of agents that affect both systems. On the other hand, the observation that stimulation of K transport by Ca_i can be dissociated from Ca inhibition of the Na-K pump (Fig. 2) suggests that Ca_i does not alter functioning Na-K pumps to selectively transport K. This is now the second case in which Ca-stimulated K efflux has been shown to be dissociated from the Na-K pump, the first being the observation that mature dog red cells that do not have functioning Na-K pumps have a Ca-stimulated K transport system (Richhardt et al., 1979). None of these observations, however, eliminate the possibility that Ca could alter parts of the pump complex that were not actively involved in normal ouabain-sensitive movements of Na and K in the mature red cell. Dog reticulocytes have a high K content (Parker, 1973) that is lost as the cells mature, and puppy red cells have a much higher number of Na-K pumps than mature cells (Miles and Lee, 1972). These pumps are either lost or inactive at maturity, but could be activated by Ca to selectively transport K. In their activated state they would be sensitive to drugs that inhibit the Na-K pump and show the transmembrane interactions of Na and K that are characteristic of functioning Na-K pumps (Hoffman and Blum, 1977). Previous work has already shown that Ca does not affect all Na-K pumps, because there are red cells from some species of animals that have functioning Na-K pumps, but no apparent Ca-stimulated K transport system (Jenkins and Lew, 1973; Brown et al., 1978).

On the basis of data recorded by means of the patch-clamp technique, the presence of K channels in human red cell membranes has recently been reported (Hamill, 1981). It is not yet clear whether these channels are Ca dependent or

K selective, or whether they display the inhibitor sensitivity and transmembrane effects that characterize Ca-dependent K transport in human red blood cells. Nevertheless, this approach could help settle the issue of whether the mechanism is a channel or a carrier.

The dissociation between the Na-K pump and Ca-induced K transport shown in this paper was primarily based on the observation that K transport was stimulated before the Na-K pump was inhibited (Fig. 2). In fact, the overall sensitivities of the two systems are quite similar, with both being affected by 1–10 μM free Ca_i (Fig. 3). It is clear from Fig. 7 that Ca-stimulated K transport is activated almost as soon as Ca_i is raised above threshold, but this appears not to be the case for the Na-K pump, which only becomes inhibited after Ca_i has been elevated for a number of minutes. One explanation for the delay in Ca inhibition

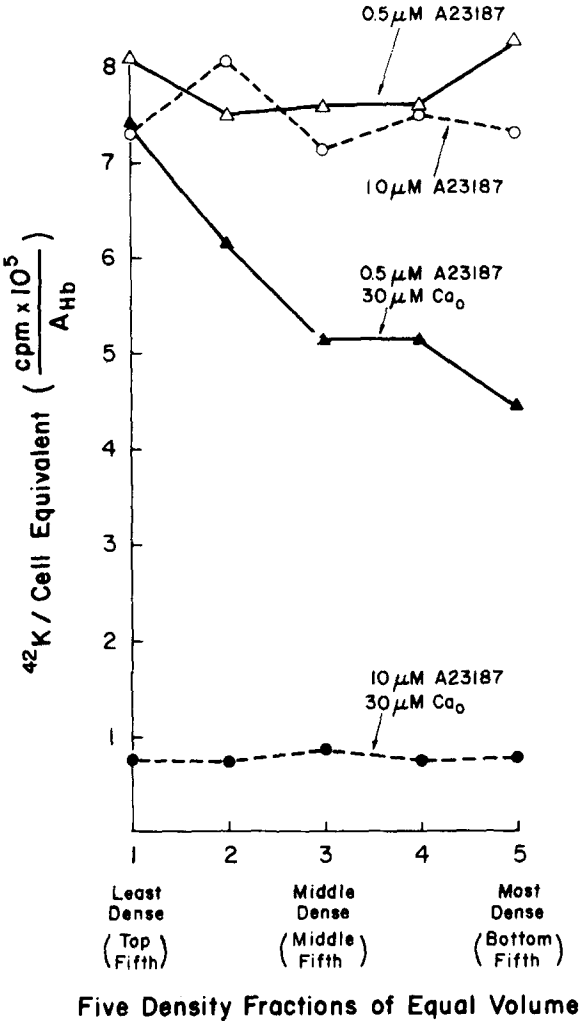

FIGURE 8 (*opposite*). The effect of A23187 on the distribution of K between cells after the cells had been incubated with two concentrations of ionophore with or without Ca_o and then separated by subsequent differences in buoyant density. Freshly drawn blood was washed three times with 10 vol of ice-cold solution containing 75 mM Na, 75 mM K, 10 mM Tris-Cl, 0.1 mM EGTA-Tris, pH 7.5 at 37°C, and four times with the same solution minus EGTA-Tris (Lew and Ferreira, 1976). These cells were then loaded with ^{42}K at 30% hematocrit at 37°C for 2.5 h in a solution containing 10 mM K, 143 mM Na, 20 mM Hepes, 0.3 mM Na_2PO_4, 200 mg% glucose, 25 mg/30 ml adenosine, and 1 mM EGTA. The cells were then washed four times in ice-cold 75 mM Na, 75 mM K, 10 mM Tris-HCl, pH 7.5 (37°C), to remove the extracellular isotope. These cells were then loaded with Ca following the procedure of Lew and Ferreira (1976). 1 ml of packed cells was added to a solution containing 75 mM K, 75 mM Na, 10 mM Tris-HCl, 10 mM inosine, 1 mM Mg, and either 5 mM EGTA or 30 μM Ca, pH 7.5 (37°C), which had been prewarmed to 37°C. Either 5.2×10^{-5} liters of 96 μM A23187 or 5.2×10^{-5} liters of 1.91 mM A23187 were added to the solutions to give final concentrations of 0.5 and 10 μM A23187, respectively. The cells were then incubated in these solutions for 15 min at 37°C. These two concentrations of A23187 were chosen because they represent concentrations that purportedly either do not alter (0.5 μM A23187) or markedly increase (10 μM A23187) the sensitivity of the K transport system to Ca (Lew and Ferreira, 1976). To test whether the Ca loading procedure during the 15-min period had increased the K permeability equally in all ghosts, the K_o in the incubation flasks was diluted from 75 to 10 mM by adding a sufficient volume of a prewarmed (37°C) solution containing 150 mM Na, 10 mM Tris-HCl, 1 mM Mg, 10 mM inosine, and the same concentration of EGTA, Ca, or A23187 to which the cells had been exposed during the first 15 min. This dilution of the K_o without changing the concentration of any other of the constituents in the solution converted the cells with an elevated K permeability caused by accumulated Ca from a K:K exchange mode to conditions favoring a net efflux of KCl that would cause the cells to shrink and increase their relative buoyant density. This dilution of K_o and the procedure that follows is a deviation from the procedure of Lew and Ferreira (1976), who after the initial 15-min incubation added ^{42}K to the Ca loading solution and measured the rate of ^{42}K influx over the next 15 min under conditions where K_i and K_o were the same, clamping the cells at constant volume. In the present experiment, at the end of the second 15-min period, the cells in each flask were centrifuged at 30,000 *g* for 30 s, the supernatant was removed, and the cells were washed one time with 75 mM Na, 75 mM K, 10 mM Tris-HCl, pH 7.5 (37°C). All the cells from each flask were then placed in a 1.5-ml capacity centrifuge tube and spun at 15,600 *g* for 15 min at 2°C in an Eppendorf centrifuge (model 5412; Brinkman Instruments, Westbury, NY). After centrifugation the cells were removed from the centrifuge tube in six successive layers, each of which contained ~150 μl. Each of these samples was in turn added to 1 ml of 75 mM Na, 75 mM K, 10 mM Tris-HCl, pH 7.5 (37°C), and mixed, a sample was removed to determine cyanmethemoglobin with Drabkin's reagent, and the remainder of the sample was counted for ^{42}K. The samples from the entire experiment were counted over a 50-min period and the results are expressed as counts per minute per unit absorbance measured at 540 nm. The top (least dense) layer of cells is on the left and the bottom (densest) is on the right. Each point is the mean of the values at the respective layer of cells in two separate flasks.

of the Na-K pump is that a decrease in pump activity is related to a time-dependent decrease in ATP. There are two reasons, however, to doubt that this is the case. The first is that the ghosts contained a regenerating system for ATP whose capacity to maintain a constant concentration of 2 mM ATP should not have been exceeded during the 20-min flux measurements (Glynn and Hoffman, 1971). Second, the free Ca_i, as monitored with entrapped arsenazo III, remained at 30–40% of free Ca_o throughout the measurement of Na efflux because of the action of the ATP-driven Ca pump. When ATP does drop under these conditions, free Ca_i increases to approximately the same level as free Ca_o. The results given in this paper show that the Na-K pump of human red cells becomes inhibited at a lower concentration of free Ca_i than has previously been reported for intact human red cells (Gardos et al., 1977) and inside-out vesicles made from red cells (Sarkadi et al., 1980). The reasons for this difference in sensitivity to Ca are not presently known.

Experiments in this paper using resealed ghosts containing arsenazo III show that activation of K transport by Ca_i is not significantly altered by ATP. The sensitivity to Ca_i measured in ghosts with and without ATP is quite similar to that using ATP-deficient ghosts containing Ca buffers (Simons, 1976; Porzig, 1977), although the value of 0.4 μM free Ca_i reported by Simons (1976) for half-maximal activation appears to be somewhat lower than would be estimated from the present data. In these experiments, free Ca_i above 5 μM and up to 70 μM free Ca did not appear to inhibit K efflux as observed by Simons (1976), which agrees with Porzig (1977), although higher concentrations of Ca appeared to make the ghosts generally more permeable and this effect may have masked a true inhibition of Ca-stimulated K efflux.

Lew and Ferreira (1976), on the other hand, have reported that the Ca sensitivity of the K transport system of intact cells containing normal levels of ATP is variable and can be altered by the concentration of A23187 used to increase free Ca_i. In contrast, ATP-depleted cells were found to have a high sensitivity to Ca at both low and high concentrations of A23187 (Lew and Ferreira, 1976). Such an effect of A23187 on the Ca affinity may indeed exist, but the results of Fig. 8 suggest that the cells exposed to low concentrations of ionophore lose K heterogeneously. This could occur as a result of underlying heterogeneity in the distribution of Ca at the low concentration of ionophore similar to that found with resealed ghosts (Yingst and Hoffman, 1984). Thus, what appeared to be a low sensitivity to Ca measured at a low concentration of ionophore may in fact represent a heterogeneous response of the cell population. The absence of an effect of the ionophore concentration on the Ca affinity of K transport in energy-depleted cells (Lew and Ferreira, 1976) suggests that even low concentrations of A23187 would be sufficient to overcome any cellular variation in passive permeability and to equilibrate Ca_i in all the cells, whereas in cells containing ATP, it would take a higher concentration of A23187 to overcome cellular variations in both passive permeability and the rate of Ca efflux via the Ca pump. This would explain why the energy-depleted cells show the same high Ca affinity at either low or high concentrations of A23187 (Lew and Ferreira, 1976). Otherwise, it is necessary to propose that reducing the ATP

and changing the ionophore concentration increase the Ca affinity by two independent mechanisms. One would also have to propose that the second ATP-dependent mechanism was only present in cells, because as shown above, the K transport mechanisms in ATP-containing (Fig. 4) and ATP-deficient ghosts (Figs. 5 and 6) have the same sensitivity to Ca.

It should be stressed that the results of Fig. 8 suggest, but do not prove, that cells exposed to low concentrations of A23187 lose K heterogeneously under the conditions used by Lew and Ferreira (1976) to measure Ca-stimulated K transport. In our experiments we followed the exact procedures used by Lew and Ferreira (1976) for the period of time in which the cells were allowed to take up Ca (Fig. 8). During this period the K concentration is the same on both sides of the membrane and therefore any increase in the K permeability should only result in K:K exchange. Then, at the point where Lew and Ferreira would have added ^{42}K to the medium and measured the rate of ^{42}K influx, we reduced K_o so that the cells that had accumulated Ca would lose a net amount of K, shrink, and increase in buoyant density so that they could be separated from the other cells by centrifugation. It is doubtful that we could have detected heterogeneity without reducing K_o, because it was this step that presumably initiated the Ca-induced changes in buoyant density, resulting in cells of different density, which was the basis for separating the cells. There is, however, a question as to whether reducing K_o initiated the heterogeneity or simply allowed it to be expressed. One reason for favoring the latter interpretation is that it is difficult to imagine how reducing K_o per se could have initiated heterogeneity. For instance, reducing K_o in the absence of Ca did not introduce heterogeneity at either concentration of ionophore (Fig. 8). Certainly, cells that had sufficient Ca to increase P_K at the end of the first 15-min period could have hyperpolarized when K_o was reduced and then accumulated more Ca during the second 15-min period. Nevertheless, if all the cells had had the same Ca_i at the point when K_o was reduced, then they all should have accumulated the same Ca and lost the same K during the subsequent period. We therefore suggest that the heterogeneity found at the low concentration of ionophore in experiments such as Fig. 8 is indicative of heterogeneity in the experiments that have been presented as evidence that A23187 changes the Ca affinity of Ca-dependent K transport (Lew and Ferreira, 1976). It may turn out that the ionophore does indeed cause changes in Ca affinity, but before such a conclusion is warranted, the possibility of ionophore-induced changes in heterogeneity has to be eliminated.

The increase in free Ca_i that stimulated K efflux is in the right range to also bind calmodulin, an intracellular protein that has been proposed to mediate many of the effects of Ca_i in a variety of cells (Cheung, 1980). The results presented in Table IV, in which trifluoperazine inhibited the rate of Ca-stimulated K efflux, suggest that calmodulin could be involved in Ca activation of K transport. Trifluoperazine is a phenothiazine and antipsychotic agent that inhibits the actions of calmodulin in other tissues (Weiss and Levin, 1978; Gietzen et al., 1980). It has previously been reported (Plishker et al., 1980) that trifluoperazine causes an increase in Ca-stimulated K transport in intact human red cells, although in this case K efflux was thought to be stimulated by an increase in Ca_i

caused by trifluoperazine-induced inhibition of the Ca pump, which is regulated by calmodulin (Larsen and Vincenzi, 1979). Recently, a number of other phenothiazines and also diphenylbutyl piperidine neuroliptics have been shown to inhibit Ca-stimulated K transport and inhibit calmodulin-stimulated Ca-ATPase with similar affinities (Lackington and Orrego, 1981). On the other hand, the specificity of the phenothiazines for calmodulin has been questioned and the effects of these drugs on K transport could be due to nonspecific hydrophobic interactions (Roufogalis, 1981; Schatzman et al., 1981). Tentative evidence for the role of a cytoplasmic factor, although not necessarily calmodulin, in activating Ca-stimulated K transport has also been reported (Sarkadi et al., 1980).

In summary, this paper shows that Ca-stimulated K transport can be characterized by specific transmembrane interactions of Na and K. Since these interactions from either side of the membrane are independent of free Ca_i, activation of the transport mechanism must be at a site that is independent of those responsible for the interaction of Na and K. In the presence of A23187, this activating site is half-maximally stimulated by ~2 μM free Ca and is not influenced by the concentration of ATP. Partial inhibition of Ca-stimulated K transport by trifluoperazine suggests that calmodulin could be involved in the activation of K transport by Ca_i. Part of the Na-K pump apparatus could underlie Ca-stimulated K transport because both processes are inhibited by the same drugs, including guinidine, and the pump could account for the transmembrane interactions of Na and K. Nevertheless, functioning Na-K pumps are not altered by Ca_i to transport K selectively, because K transport can be activated by Ca_i before Ca_i inhibits the Na-K pump.

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