J. Membrane Biol. 6, 315–328 (1971)

The Membrane Locus of Ca-Stimulated K Transport in Energy Depleted Human Red Blood Cells

RONALD M. BLUM and JOSEPH F. HOFFMAN

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

Received 21 April 1971

Summary. Energy depleted human red cells subsequently exposed to iodoacetate (IAA) develop, upon the addition of Ca, a marked increase in K permeability, while Na permeability is unaltered. The kinetic characteristics of this augmented K permeability indicate that the transport process is membrane mediated. Thus, the inward and outward rate constants for K increase as the concentration of external K is increased reaching maximum values between 2 to 5 mM; further increases in external K results in a partial reduction of the rate constants. In addition, the Ca-stimulated K transport system displays counterflow of ^{42}K during its influx when a large gradient of ^{39}K exists (inside high, outside low). Furthermore, the Ca-induced K transport is inhibited by ouabain. The sensitivity of the Ca-induced system to ouabain parallels the action of ouabain on the Na-K pump. At least part of the increased K transport occurs through a preexisting pathway since ouabain bound to cells before exposure to Ca and IAA results in an inhibition of K outflux. Since ouabain does not alter the affinity of the cells for Ca, it is concluded that at least a portion of the increased K transport results from Ca acting to increase the turnover rate of the same system which serves as the Na-K pump in normal red cells.

This paper is concerned with the action of Ca on the K permeability of human red cells, as described by Gardos [4, 5]. This action of Ca in increasing the K permeability is not seen under normal circumstances but only after metabolic alteration of the cells and is, in addition, specific in that Ca affects only K and not Na permeability. Thus, Gardos [4] showed that cells suspended in their own plasma become highly permeable to K after the addition of adenosine and iodoacetic acid (IAA). The addition of EDTA to the plasma protects the cells from the large change in K permeability [5] and EDTA can immediately reverse the change if added after the stimulation in K outflux has begun [11]. These changes in K permeability are known to be due to the specific action of Ca present in plasma and can be duplicated completely using washed cells incubated in Ringer's-type solutions containing Ca, IAA and adenosine [11, 18]. Furthermore, in the pres-

ence of IAA and adenosine, there is no effect of Ca on the outflux of Na beyond the reduction that results from the inhibition of the pump in cells incubated with only IAA and adenosine [16]. This specific change in K permeability can also be brought about by Ca alone in cells depleted of their endogenous energy stores. Thus, in red cells incubated without substrate for 24 hr, Ca acts by itself to increase the K outflux [11, 16] although the addition of IAA will produce a further increase in the K permeability. The role of the IAA and adenosine in acting on fresh cells is thought to be to remove a permeability regulating substance which normally protects the cell from the action of Ca [11, 18]. Since this substance also appears to be removed by starvation, energy-depleted cells were studied exclusively in the present work in order to by-pass the metabolic protective mechanism [*see* 11].

The experiments described below have been undertaken to characterize the membrane site at which Ca acts to induce the changes in K permeability. The results indicate that Ca acts on an altered form of the same mechanism which is normally used in the active transport of K. A brief account of this work has been previously presented [1].

Materials and Methods

Fresh human blood drawn into heparin (0.15 mg/ml) from normal young adults was centrifuged within one hr after collection at approximately $25{,}000 \times g$ for 2 min at 4 °C. After removal of the buffy coat by aspiration, the red cells were washed three times (1 vol cells to 4 vol medium) with a standard medium containing 153 mM NaCl + 20 mM glycylglycine (pH 7.15 at 37 °C). The cells were then suspended in this medium (also containing 0.1 mg% chloramphenicol) at a hematocrit of 40% and incubated without added substrate at 37 °C for 24 hr to deplete them of their endogenous energy stores. In those instances where the unidirectional outflux was to be measured, the desired isotope (^{42}K or ^{24}Na) was present during the entire depletion period. At the end of the depletion period the cells were again washed three times with the standard medium and packed ready for use in the flux experiments. Analysis of the packed cells at the end of the depletion and washing periods showed that the cells contained about 25 mM Na and 70 mM K/liter cells and 73% H_2O. These values of intracellular Na and K represent the cellular content at the beginning of the flux estimations.

The outflux was measured by the methods outlined by Hoffman [10] and adapted for use with intact cells. Thus, approximately 0.2 ml labeled packed cells were added to flasks containing 30 ml of the final incubation medium and allowed to equilibrate for 10 min at 37 °C in a shaker bath before the zero time sample was removed. The zero and subsequent time samples (usually 15-min intervals) were centrifuged at $15{,}000 \times g$ for 2 min and the supernatants pipetted for determination of radioactivity. A sample of the whole suspension was also counted. The outward rate constant, ${}^{0}k_{K}$ or ${}^{0}k_{Na}$, was calculated from the fractional loss of the cellular radioactivity appearing in the medium per unit time. Since the cells in most cases were in an unsteady state due to the loss of KCl with concomitant cell shrinkage, the use of the rate constant provides the most direct way of comparing relative permeabilities.

The K influx was measured using packed cells which had been depleted and washed as described above, but which had not been previously exposed to isotope. The packed cells were treated in the same fashion as described for outflux but were incubated in the final medium at a hematocrit of 16 to 20%. After temperature equilibration, a trace amount of ^{42}K was added and samples taken at different time intervals were centrifuged at $25{,}000 \times g$ for 10 min in lucite tubes. The lucite tubes were so designed that the cells during centrifugation were funneled into a narrow constriction where, after removal of the supernatant, they could be easily pipetted for analysis. The inward rate constant, ${}^{i}k_{K}$, could be calculated from the ^{42}K which entered the cell per unit time. All influx measurements were corrected for the small amount of medium radioactivity trapped with the packed cells and for the decrease in cell volume which resulted from the net loss of KCl. This latter correction was determined by the change in hemoglobin concentration measured by the cyanmethemoglobin method of Drabkin and Austin [2]. The influx of K, ${}^{i}M_{K}$, in mM/(liter original cell vol $\times$ hr), was calculated from the relation, ${}^{i}M_{K} = {}^{i}k_{K} \cdot (\overline{K})_0 \cdot t$, where $(\overline{K})_0$ is the average external concentration of K during the time period, t, in which ${}^{i}k_{K}$ was measured, usually in 15-min intervals.

The binding of tritium-labeled or unlabeled ouabain was performed by exposing the red cells to ouabain during the last 60 min of the 24-hr depletion period. Before exposure, the cells were washed three times in the cold with the standard medium so that cells bound ouabain in a K-free medium. In some experiments, 50 mM of CsCl replaced an equal amount of NaCl in the standard medium. It should be emphasized that the washes of the cells which were routinely performed at the end of the depletion period also removed all unbound ^{3}H-ouabain. The methods used for analyzing tritiated-ouabain bound to red cells have been described previously [12, 13].

Unless otherwise specified, the general procedure used for testing the effects of various conditions on the cation permeability was to place the packed red cells in the standard medium containing 1 mM NaIAA (Sigma Chemical Co.). At the end of temperature equilibration but just before the zero time sample was taken for analysis, sufficient $CaCl_2$ was added to each flask, in quantities of either 0.1 ml or 1.0 ml, to give the desired final concentration of $CaCl_2$, which, in most cases, was 10 mM. Since the change in the K permeability induced by Ca was generally rather large, this procedure insured accurate exposure times and flux periods.

When KCl was added to the standard medium it was substituted for an equal concentration of NaCl. The osmolality of all solutions was $306 \pm 1\%$ mOsm and the pH was kept at 7.15 ± 0.02 at 37 °C. Tritiated-ouabain was obtained from New England Nuclear Corp. Neutralized solutions of ^{42}KCl and $^{24}NaCl$ were obtained from Cambridge Nuclear Corp. Ca was measured in a Perkins Elmer atomic absorption spectrometer (Model 303, Standard Ca hollow cathode lamp) according to the manufacturer's directions. Na and K were determined by flame photometry using Li as an internal standard.

Results

We will consider two experimental approaches to identifying the locus of the membrane site at which Ca acts to alter K permeability: The first type is concerned with certain kinetic characteristics of the process; the second type is concerned with the effect of ouabain.

Fig. 1 shows the effect of external K on the Ca-induced change in K permeability of depleted cells exposed to IAA. This experiment was carried out by incubating washed cells, labeled with ^{42}K, in different media contain-

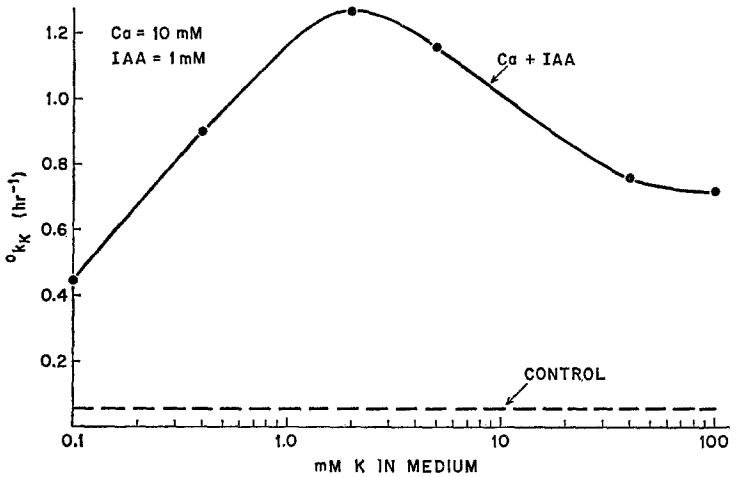

Fig. 1. The change in the Ca-induced K permeability as influenced by the external concentration of K. The K permeability is expressed in terms of the outward rate constant, ^0k_K, in units of reciprocal hr. The K concentration in the medium was varied by replacing Na with K. The external medium also contained 20 mM glycylglycine at pH 7.15. The flux was measured, at 37 °C using labeled depleted cells exposed to 1 mM IAA, over a 30-min period after 10-min equilibration (control cells) and upon the addition of 10 mM $CaCl_2$. *See* text for details of measurement. Note the logarithmic scale of the abscissa

ing IAA and various concentrations of K. After 10-min equilibration at 37 °C (control cells) and upon the addition of 10 mM $CaCl_2$, the rate of appearance of ^{42}K in the medium was measured during the next 30 min. It is apparent that after Ca addition there is a marked stimulation in the K outflux at all concentrations of external K. The increase in K permeability induced by Ca is about eightfold in K-free media rising to approximately 25-fold at 2 mM K. At K concentrations above 2 mM the K outflux decreases somewhat but still remains considerably above controls levels. As known from previous studies [16, 18], the change in K permeability produced by Ca is specific for K and is without effect on Na permeability. Thus, Na outflux, measured under the same conditions as described above for K, was unaffected by Ca or by changes in the concentration of K in the medium. It seems then, from the results presented in Fig. 1, that the transport of K in the Ca-induced situation cannot be characterized by a mechanism based on simple diffusion. If transport occurred by simple diffusion, ^{42}K leaving the cell would do so at a rate independent of the K concentration on the outside of the membrane. In addition, at K concentrations below 2 mM the Ca-stimulated cells increase their K permeability with increasing concentrations of K, similar to the behavior expected if the transport occurred by a mediated process such as a K-K exchange mechanism [7, 8].

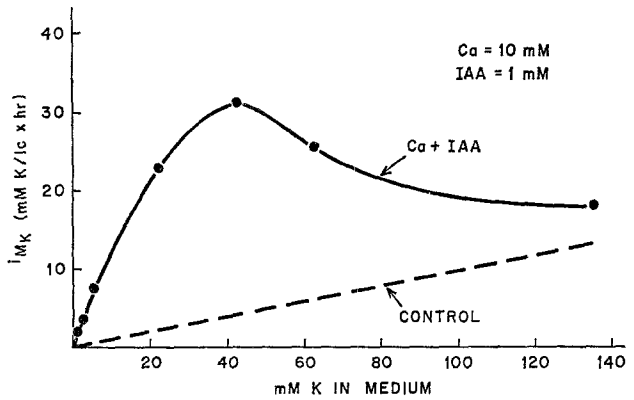

Fig. 2. The change in the influx of K, as induced by Ca, as a function of the external concentration of K. The influx, $^{i}M_K$ [in mM K/(liter × hr)] was measured, at 37 °C using depleted cells exposed to 1 mM IAA, over a 30-min period after 10-min equilibration (control cells) and upon the addition of 10 mM $CaCl_2$. The medium was buffered to pH 7.15 with 20 mM glycylglycine and the K in the medium was varied by substitution for Na. *See* text for details of measurement

Measurements of K influx at increasing concentrations of medium K provide additional support that the Ca-induced change in K permeability is a membrane mediated process. The results of this type of experiment are shown in Fig. 2 where the unidirectional influx of K is plotted against the external concentration of K. It is apparent that K entry increases in a non-linear fashion as medium K is increased to a point where further addition of K results in an inhibition. Thus, the influx appears to behave as if it has a saturable component but this interpretation is complicated by the effects of high K. Even so, the similarity in the change in the Ca-induced K permeability as presented in Figs. 1 and 2 suggests that there is an interaction between the influx and outflux of K.

Fig. 3 shows that coupling between flows can occur since the system exhibits the kinetics of counter-transport; that is, where the uphill movement of one ion species is driven by the downhill movement of another [9, 20]. The experiment presented in Fig. 3 was carried out by incubating depleted cells in low (0.7 mM) and high (135 mM) concentrations of medium K. Since the cells were incubated at a hematocrit of about 20%, changes in the K concentration in the low K medium could not be avoided and the 0.7 mM K value represents the initial K concentration which increased to 3 mM by 50 min and to approximately 12 mM K by the end of the experiment. At zero time, Ca and ^{42}K are added and the cellular uptake of ^{42}K is measured as a function of time. It should be understood that the cells incubating in low K medium shrink with time as a result of the net loss of KCl induced by

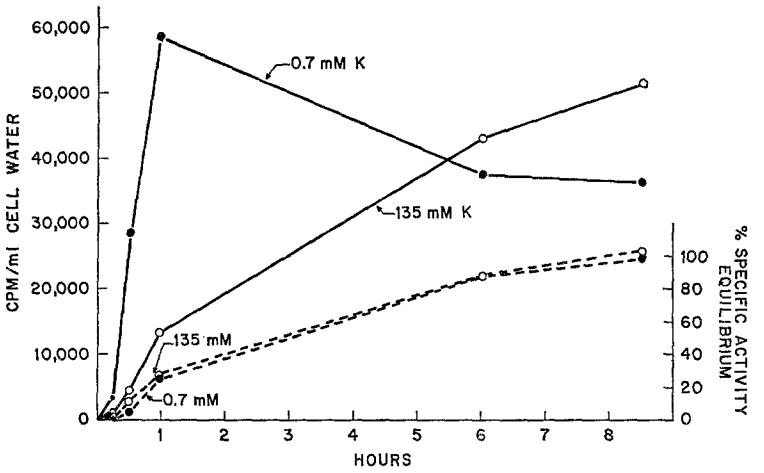

Fig. 3. The Ca-induced rate of uptake of ^{42}K by depleted cells incubated in 1 mM IAA in low (0.7 mM) and high (135 mM) K media. The medium was buffered to pH 7.15 with 20 mM glycylglycine and the K in the medium replaced an equal concentration of Na. The measurements were begun after 10-min equilibration by the addition of 10 mM $CaCl_2$ and a trace amount of ^{42}KCl. Analysis of the cells and media were carried out at the indicated times. The left ordinate represents the cellular uptake of ^{42}K in counts per minute (cpm) in one ml of the original cells, i.e., after correction for volume changes resulting from the loss of K. The right ordinate is the ratio of the cellular to the medium specific activity (cpm/mM) $\times$ 100

Ca. Since no such gradient for K exists for the cells in high K medium only minimum changes in volume occur during incubation. It is apparent that the rate at which the cells approach specific activity equilibrium is the same and independent of the medium K. In contrast, the time course of ^{42}K uptake and content differs in the two instances dependent on the medium K. Thus, for cells placed in a low K medium, the ^{42}K content goes through a maximum, a type of behavior that is expected in a counterflow type of transport. In this instance the net loss of ^{39}K from the cell drives ^{42}K into the cell against its concentration gradient. Since the driving force necessary for the uphill movement is derived from the ^{39}K loss there would be no counterflow in the absence of a K gradient as is the case for the cells incubating in high K medium. It should be mentioned that in other experiments the peak of the cellular ^{42}K increases with increasing concentrations of medium K reaching a maximum at approximately 5 mM K. The time interval taken to reach the peak activity increases slightly with increasing concentrations of K.

Having demonstrated that the Ca-induced increase in K permeability appeared to be a mediated process it became of interest to determine whether this type of transport of K could be driven by the same membrane

Table 1. *The effect of ouabain on the Ca-stimulated K permeability in depleted red cells*[a]

Exp.	Control ${}^0k_K$ (hr^{-1})	Ouabain ${}^0k_K$ (hr^{-1})	% inhibition
12–24	0.21	0.17	16
1–14	0.46	0.41	11
1–16	0.32	0.23	29
2–3	0.30	0.20	33
6–26A	0.52	0.40	24
6–26B	0.41	0.22	46
Mean	0.37	0.27	26

[a] Control cells and cells exposed to 1×10^{-4} M ouabain during the last hr of their energy depletion, were washed in the standard medium which contained 153 mM NaCl and 20 mM glycylglycine (pH 7.15). The outward rate constant of K, ${}^0k_K$, in units of reciprocal hr, was measured in both control and ouabain-exposed cells during incubation, at 37 °C, in the standard medium (in the absence of added ouabain) in the presence of 1 mM IAA and 10 mM $CaCl_2$. (*See* text for details.)

system that controls K transport in normal cells. One approach to the localization of the membrane site would be to see if the Ca-stimulated transport system was sensitive to cardiac glycosides such as ouabain. From the results presented in Table 1, it is clear that the Ca-stimulated K transport system is inhibited by ouabain. While the inhibition is not complete, the absolute increase in the average ouabain-sensitive K outflux is more than 15 times the ouabain-sensitive K outflux observed in normal cells. The average value for the latter is about 0.4 mM K/(liter cells × hr) [7]. Assuming a mean intracellular concentration of 70 mM K/liter cells, for the experiments listed in Table 1, the mean ouabain-sensitive flux is 6.7 mM K/(liter cells × hr). The question can be asked whether, in these circumstances, Ca acts at or creates new ouabain-sensitive sites or whether Ca acts at the locus of the naturally occurring ouabain-binding sites on the membrane.

The question just raised is answered indirectly by the experimental protocol used to test the effects of ouabain. Thus, for the experiments given in Table 1, the cells were exposed to ouabain (1×10^{-4} M) only during the last hr of the depletion period with subsequent washing to remove any traces of free glycoside. Since the ^{42}K outflux was measured in glycoside-free solutions, the observed inhibition by ouabain must have been due to ouabain that was bound to the cells during depletion (that is, before exposure of the cells to Ca) and remained bound throughout the washes and the flux period. The available evidence [12] suggests that ouabain bound to the cells under these conditions is bound primarily at sites associated with the Na-K pump.

The experiment presented in Table 2 was done to compare the extent of ouabain inhibition of the Ca-induced K outflux with the inhibition of the

Table 2. *Comparison of the percent inhibition of the Ca-stimulated K outflux with Na outflux as a function of the amount of bound ouabain*[a]

Labeling conditions		Ca-induced K outflux			Na outflux		
Medium	^{3}H-Ouabain (M/liter)	% I	no. ouab per cell	no. ouab per cell at 100% I	% I	no. ouab per cell	no. ouab per cell at 100% I
Na	2×10^{-8}	52	178	340	—	—	—
Na	7×10^{-8}	91	342	375	—	—	—
Na+Cs	2×10^{-8}	34	87*	255	24	87*	209
Na+Cs	7×10^{-8}	65	127*	195	51	127*	249

[a] Cells were exposed to the indicated concentrations of ^{3}H-ouabain during the last 60 min of the 24 hr depletion period. Exposure of the cells to ^{3}H-ouabain was carried out in two different media: the standard Na medium contained 153 mM NaCl + 20 mM glycylglycine (pH 7.15); the Na + Cs medium was the same except that 51 mM CsCl replaced an equal amount of NaCl. Portions of control and ^{3}H-ouabain exposed cells were also labeled during depletion with either ^{42}K or ^{24}Na for subsequent outflux determinations. At the termination of depletion the cells were washed in the standard Na medium to remove all traces of labeling radioactivity. Thus, the number of molecules of ouabain bound per cell (no. ouab/cell) was determined on cells exposed only to ^{3}H-ouabain and not exposed to ^{42}K or ^{24}Na. Therefore, the number of ouabain molecules bound per cell for the cells labeled in the Na + Cs medium was the same (values indicated with an *) whether their counterparts were to be used in K or Na outflux determinations. The outflux measurements were carried out at 37 °C, with control and ^{3}H-labeled cells incubated in the presence and absence of 1×10^{-4} M ouabain in order to evaluate the percent inhibition (% I). Thus, % I, in this Table, refers only to the ouabain-sensitive flux and not to the percent of the total flux inhibited by ouabain. The Ca-induced K outflux was measured in a medium which contained 153 mM NaCl + 20 mM glycylglycine + 1 mM IAA + 10 mM $CaCl_2$. The medium used in the Na outflux determination contained 17 mM KCl + 136 mM NaCl + 20 mM glycylglycine + 10 mM adenosine (to replete the cells and activate the Na pump). All final incubations were carried out in duplicate with duplicate analyses. The quantity, no. ouab/cell at 100% I, was calculated from the no. ouab/cell for each % I.

pump outflux of Na under conditions where the number of bound ouabain molecules per cell was known. As described in the legend to Table 2, either the outflux of ^{24}Na and/or ^{42}K was measured from cells that had been previously labeled with tritiated ouabain. Other than the type of outflux to be measured, the cells were treated in a fashion as identical as possible for each assay condition. The portion of the Ca-induced K outflux and the Na outflux that was ouabain-sensitive as presented in Table 2, was 27% and 34%, respectively, of the total outflux. With regard to cells labeled in the Na + Cs medium, it is apparent that the percent inhibition of Ca-stimulated K outflux is nearly the same as the percent inhibition of the Na

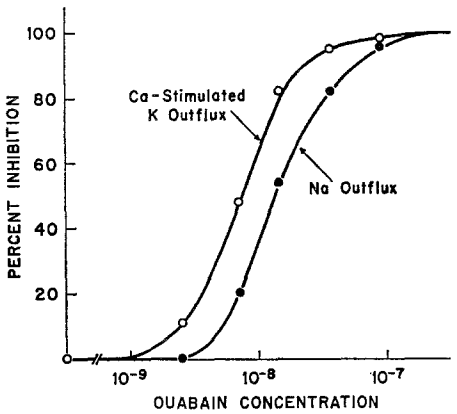

Fig. 4. Comparison of the ouabain-sensitivity of the Ca-stimulated K outflux with the pump outflux of Na. Cells suspended in the standard medium, were exposed to the indicated concentrations of ouabain during the last 30 min of the 24 hr depletion period (37 °C). The standard medium was 153 mM NaCl or 20 mM glycylglycine at pH 7.15. Portions of control and ouabain exposed cells were also labeled with ^{42}K or ^{24}Na during depletion for the subsequent outflux measurements. After depletion the cells were washed in this standard medium to remove any free ouabain or radioactivity. The outflux measurements were carried out, at 37 °C, with control and ouabain exposed cells also incubated in the presence and absence of 1×10^{-4} M ouabain in order to evaluate the percent inhibition. Thus, the percent inhibition given in Fig. 5 refers only to the ouabain-sensitive flux and not to the percent inhibition of the total flux. The Ca-induced K outflux was measured in a medium which contained 153 mM NaCl + 20 mM glycylglycine + 1 mM IAA + 10 mM $CaCl_2$. The medium used in the Na outflux determination contained 17 mM KCl + 136 mM NaCl + 20 mM glycylglycine + 10 mM adenosine (to energy replete the cells and activate the Na pump)

pump flux for either concentration of tritiated ouabain. The fact that the calculated numbers of ouabain molecules bound per cell at 100% inhibition are so close supports the idea that the molecules of ouabain which prevent Ca from acting are bound at pump-associated sites. It should be mentioned that ouabain once bound is not eluted during the outflux measurements described in Table 2. Analysis of the cells for their ^{3}H-ouabain content after incubation for 30 min (using cells which did not contain ^{42}K or ^{24}Na) showed that less than 5% was lost during any of the flux assay conditions. The reason Cs was included in the labeling medium was to reduce the extent of non-specific binding of glycoside; that is, glycoside that is bound to sites that are not involved in transport [12]. This effect of Cs can be seen in Table 2 in the Ca-induced K outflux experiments as the difference in the number of ouabain molecules bound per cell at 100% inhibition obtained by labeling in the Na medium compared to the medium containing Cs. These results eliminate the possibility that Ca might be interacting with the membrane at the non-specific binding sites of glycoside. The results presented

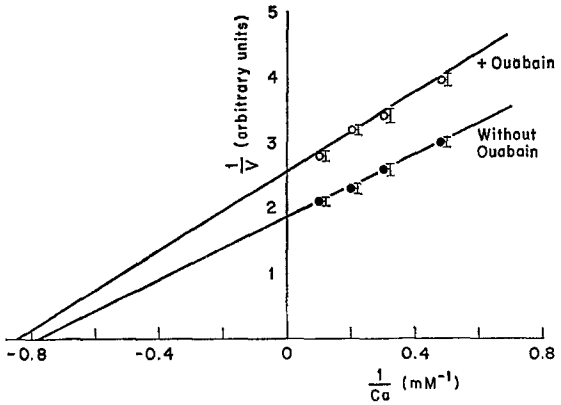

Fig. 5. The effect of different concentrations of Ca on the ouabain inhibition of the Ca-stimulated outflux of K. Cells, suspended in the standard medium, were incubated in the presence and absence of 1×10^{-4} M ouabain during the last hr of the 24-hr depletion period (37 °C). After depletion both batches of cells were washed in the standard medium (153 mM NaCl + 20 mM glycylglycine, pH 7.15) which, in the case of the ouabain-exposed cells, served to remove any remaining free ouabain. The outflux determinations (V) were carried out at 37 °C by incubating control (no ouabain) and ouabain-exposed cells in the standard medium which also contained 1 mM IAA and the indicated concentrations of $CaCl_2$, ranging from 2 to 10 mM. Each point is bracketed by ± 1 SE. The curves are fitted by the method of least squares

in Table 2 also support the conclusion that Ca can act at naturally occurring sites in the membrane.

The results of another experiment, similar in design to the one presented in Table 2, are shown in Fig. 4 where a comparison is made between the ouabain-sensitivities of the Ca-activated system and the Na pump. As before, different portions of the same cells were exposed to ouabain and washed prior to the flux determinations in such a way that all conditions were the same except for the type of flux to be measured. From the similarity in the ouabain-sensitivities of the two types of measurements these results indicate again that the ouabain bound to the cell is inhibitory for both processes. The concentration of ouabain which gave 50% inhibition of the Ca-stimulated K outflux was 0.9×10^{-8} M compared to 1.2×10^{-8} M for the pump component of the Na outflux. A similar comparison was also made between ouabain and the less potent cardiotonic steroid, dihydro-ouabain (kindly provided by Dr. Elwood Titus), evaluating their relative effectiveness in inhibiting the Ca-stimulated K outflux. The concentration of dihydroouabain which gave 40% inhibition was approximately 2.2×10^{-8} M; that is, about three times less effective than ouabain.

Since the magnitude of the Ca-stimulated outflux of K has been found to be dependent on the concentration of Ca [*see* 11, 18] it is possible that

the mechanism of the inhibition attained with ouabain is due to ouabain decreasing the affinity of the membrane for Ca. As shown in the double reciprocal plot of K permeability against the Ca concentration (Fig. 5), the apparent affinity of the transport locus for Ca is not affected by ouabain.

Discussion

The work presented in this paper has been carried out in an attempt to define more clearly the membrane mechanism responsible for the increase in the K permeability induced by Ca. On the basis of the kinetic characteristics of the change in K transport and its inhibition by ouabain it appears that at least one domain for the action of Ca is the Na-K pump complex. Thus, the kinetic behavior already discussed in connection with the first three figures indicated that in the Ca-induced system, K transport is a membrane mediated process. The inhibition of this process by ouabain (Table 1), under circumstances where ouabain is evidently bound only to pump-associated sites (Table 2), served to identify this locus of action of Ca.

It should be mentioned that counterflow of K, as seen for instance in Fig. 3, is not a property that can be demonstrated in normal human red cells presumably because the cells are in the steady state with regard to net cation movements. Unsteady state conditions must be accompanied by rather large changes in unidirectional fluxes (compared to normal) if the phenomenon of counter-transport is to be seen at all. This is the case for the experiment presented in Fig. 3. The ability to detect counter-transport in this circumstance decreases with decreasing Ca since the change in the K permeability depends upon the concentration of Ca. Counterflow of K has been previously demonstrated in human red cells poisoned with Pb [14, 19]. Similar observations have also been made on human red cells treated with propranolol [3]. The interrelationship between these two agents and the Ca-induced system and whether they share a common membrane mechanism has yet to be established.

Let us consider now in more detail the interaction of Ca with the membrane and its inhibition by ouabain. Dr. V.L. Lew has suggested *(personal communication)* that the primary action of ouabain would, in this situation, be to spare intracellular ATP. This means that cells exposed to ouabain would contain more ATP than unexposed or control cells. ATP would afford protection against Ca by activating the outwardly oriented Ca pump [17, 21] ridding the cell (or membrane compartment) at least momentarily of any Ca that has entered. It follows then that since the Ca pump is not inhibited by ouabain [21] the higher the cellular ATP the greater would be the protection against Ca.

There are several reasons for suspecting that the Ca pump *per se* is not involved in the Ca-induced system and that ouabain does not act by protecting ATP breakdown. One reason concerns the time element in the exposure of the cells to ouabain. While it is true that exposure of the cells to ouabain during depletion (as in Table 1) would favor the ATP hypothesis, the same degree of inhibition is obtained when ouabain (1×10^{-4} M) is added only in the final incubation medium; that is, at the time of the flux determination. Thus, any differences in ATP content between the control and ouabain exposed cells would be minimized in this situation. In addition, since the half time for Ca transport is about 5 min [17, 21] it is also of interest that the effectiveness of ouabain in inhibiting the Ca-induced system, in K-free media as in Table 1, is only slightly less during the second 15-min flux period than it is in the first. The effectiveness of ouabain has also been studied as a function of the length of the depletion period. The time-course for the fall in ATP content during depletion (determined by Dr. F. Proverbio) averages 62, 24, 13 and 9 μmoles ATP/liter cells at 18, 24, 30 and 36 hr, respectively. In two different experiments (carried out with 1×10^{-4} M ouabain added only in the medium at the time of the flux determination but otherwise in the same way as described for those presented in Table 1) the percent inhibition with ouabain was, in one, 34, 42 and 42% after 19, 24 and 27 hr depletion, respectively; and, in the other, 16, 26 and 39% after 18, 33 and 39 hr depletion, respectively. These results strongly imply that the effect of ouabain in this system is independent of the cellular ATP and the Ca pump. Another reason for thinking that the inhibitory action of ouabain is direct rather than indirect is connected with the effects of inhibitors such as furosemide and oligomycin, which help to pinpoint the membrane locus of Ca action (*see* below).

The foregoing discussion supports the view that the molecular mechanism of the Na-K pump complex underlies, at least in part, the action of Ca. This is to say that the mechanism responsible in the normal cell for carrying out K-K exchange could be the system stimulated by Ca. Since this mechanism is inhibited by ouabain and since the number of ouabain binding sites is fixed, Ca would then act by increasing the turnover rate of the K-K exchange mechanism. But it should be remembered that this stimulation by Ca occurs only in energy-depleted cells (conditions incidentally, in which there is no K-K exchange in the absence of Ca) and the characteristics of the K transport in this situation, as influenced by Ca, may not reflect the characteristics of this mode of the pump in normal cells.

Since only partial inhibition of the system is obtained with ouabain, it is possible that Ca interacts with loci on the membrane separate from the

Na-K pump. While other loci cannot be excluded, it seems likely from the action of furosemide and oligomycin that the Na-K pump complex is the primary target for Ca. Thus, the Ca-induced K transport system is also inhibited by furosemide [1] and by oligomycin (details to be published in a separate paper). Furosemide (1×10^{-3} M), in the absence of ouabain, inhibits in K-free media to about the same extent as seen with ouabain in Table 1. The extent of the inhibition obtained with the combination furosemide + ouabain is almost double the effect of either agent acting alone indicating that while there is some overlap in their effects, they differ mainly either in their efficiency in inhibiting the same sites or that they inhibit different sites. In marked contrast, oligomycin (6.7 μg/ml) has been found to inhibit the total Ca-induced change in permeability by more than 90%. (All of the effects of furosemide and oligomycin were obtained by adding these agents directly to the medium at the time of the flux determination.) Furosemide [20] and oligomycin [6, 22] are both known to be inhibitors of the Na-K pump; in addition, it is also known *(unpublished studies)* that neither compound competes with ouabain for binding to the membrane; so, presumably, they inhibit the same process in different ways. If this is so, it would imply that when the observed inhibition is only partial (as is the case with ouabain or furosemide) each site becomes only partially inactivated. It is not worthwhile to speculate further about this aspect of the inhibition without additional experimental information.

It should be remembered that all of the effects of Ca, as described in this paper, have been studied under conditions where IAA is present. The presence of IAA is not obligatory for the effects to be seen but the responsiveness or sensitivity of the system to Ca is considerably enhanced by IAA. Thus, it is possible that IAA, acting say by virtue of its sulfhydryl activity, alters the tertiary structure of the protein in the region of pump complex such that the K-K exchange mechanism can turn over more easily.

This work was supported by U.S. Public Health Service Grants HE-09906 and AM-05644 and by the National Science Foundation Grant GB-18924.

Note Added in Proof, December 21, 1971: It has recently been proposed [Glynn, I. M., Warner, A. E., *Brit. J. Pharmacol. Chemother. (In press)*] that fluxes of the sort that are presented in Fig. 3 which are interpreted as showing countertransport can also be interpreted in terms of a model in which the ion flows are independent of each other but are influenced by the changes in the membrane potential that result from the increased K permeability. Conclusions concerning the membrane locus for Ca action, however, are independent of the model chosen for explaining "counterflow" phenomena.

References

1. Blum, R.M., Hoffman, J.F. 1970. Carrier mediation of Ca-induced K and its inhibition in red blood cells. *Fed. Proc.* **29**:663*a*.
2. Drabkin, D.L., Austin, J.H. 1936. Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *J. Biol. Chem.* **112**:51.
3. Ekman, A., Manninen, V., Salminen, S. 1969. Ion movements in red cells treated with propranolol. *Acta Physiol. Scand.* **75**:333.
4. Gardos, G. 1956. The permeability of human erythrocytes to potassium. *Acta Physiol. Sci. Acad. Hung.* **10**:185.
5. — 1959. The role of calcium in the potassium permeability of human erythrocytes. *Acta Physiol. Sci. Acad. Hung.* **15**:121.
6. Garrahan, P.J., Glynn, I.M. 1967. The stoichiometry of the sodium pump. *J. Physiol.* **192**:217.
7. Glynn, I.M., Lew, V.L., Lüthi, U. 1970. Reversal of the potassium entry in red cells, with and without reversal of the entire pump cycle. *J. Physiol.* **207**:371.
8. — Lüthi, U. 1968. The relation between ouabain-sensitive potassium efflux and the hypothetical dephosphorylation step in the "transport ATPase" system. *J. Gen. Physiol.* **51**:385*s*.
9. Heckmann, K. 1965. *See* Discussion. *In:* Mechanism of Hormone Action; A NATO advanced study Institute. P. Karlson, editor. p. 57. Academic Press Inc., New York.
10. Hoffman, J.F. 1962. The active transport of sodium by ghosts of human red blood cells. *J. Gen. Physiol.* **45**:837.
11. — 1966. The red cell membrane and the transport of sodium and potassium. *Amer. J. Med.* **41**:666.
12. — Ingram, C.J. 1968. Cation transport and binding of *T*-ouabain to intact human red cells. *In:* Stoffwechsel und Membranpermeabilität von Erythrocyten und Thrombocyten. E. Deutsch, E. Gerlach and K. Moser, editors. p. 420. Georg Thieme Verlag, Stuttgart.
13. Ingram, C.J. 1969. The binding of ouabain to human red cells. Ph.D. Thesis. Yale University, New Haven, Conn.
14. Joyce, C.R.B., Moore, H., Weatherall, M. 1954. The effects of lead, mercury and gold on the potassium turnover of rabbit blood cells. *Brit. J. Pharmacol. Chemother.* **9**:463.
15. Kregenow, F.M. 1962. Metabolic control of passive transfer and exchange diffusion of sodium and potassium in human red blood cells. M.D. Thesis. Yale University, New Haven, Conn.
16. — Hoffman, J.F. 1962. Metabolic control of passive transport and exchange diffusion of Na and K in human red cells. *Biophysical Soc. (Abstract), Wash.* TD. II
17. Lee, K.S., Shin, B.C. 1969. Studies on the active transport of calcium in human red cells. *J. Gen. Physiol.* **54**:713.
18. Passow, H. 1963. Metabolic control of passive cation permeability in human red cells. *In:* Cell Interface Reactions. H.D. Brown, editor. Scholar's Library, New York.
19. — 1970. The red blood cell: Penetration, distribution and toxic action of heavy metals. *In:* Effects of Metals on Cells, Subcellular Elements and Macromolecules. J. Maniloff, J.R. Coleman and M. Miller, editors. p. 291. Charles C. Thomas, Springfield, Ill.
20. Sachs, J.R. 1971. Ouabain-insensitive sodium movements in the human red blood cell. *J. Gen. Physiol.* **57**:259.
21. Schatzmann, H.J., Vincenzi, F.F. 1969. Calcium movements across the membrane of human red cells. *J. Physiol.* **201**:369.
22. Whittam, R., Wheeler, K.P., Blake, A. 1964. Oligomycin and active transport reactions in cell membranes. *Nature* **203**:720.
23. Wilbrandt, W., Rosenberg, T. 1961. The concept of carrier transport and its corollaries in pharmacology. *Pharmacol. Rev.* **13**:109.