J. Physiol. (1977), **264**, *pp.* 411–428
With 7 text-figures
Printed in Great Britain

THE ALTERATION BY OUABAIN OF CALCIUM MOVEMENTS IN HUMAN RED CELL GHOSTS

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(*Received 13 May 1976*)

SUMMARY

1. The influence of ouabain on net Ca movements was studied in human erythrocyte ghosts by atomic absorption spectrophotometry.

2. Ouabain (10^{-5} M) showed a dual effect, altering both entry and exit of Ca from K-rich ghosts incubated in a high-Na medium in the presence of 10 mM-Ca.

3. Stimulation of Ca entry was observed in the first 15 min at 37° C, whereas during the subsequent 15 min incubation ouabain elicited Ca extrusion. This latter effect was eliminated when the ouabain concentration was raised to 1 mM.

4. Ouabain-dependent Ca movements were abolished by replacing both internal K and external Na with choline. They were also absent from ghosts prepared at a high lytic ratio (1:100) or obtained from ATP-depleted cells.

5. A moderate increase in cell ATP enhanced the effect of ouabain on Ca efflux whilst it was eliminated at higher ATP levels.

6. The actions of ouabain markedly depended on the initial ADP/ATP ratio in ghosts, being optimal at about 2·5.

7. The results suggest that the effects of ouabain on Ca movements are mediated through the Na pump. Reversal of this pump in Na-rich K-free medium may provide the energy for active Ca transport.

INTRODUCTION

The Ca pump of human red cells is responsible for the maintenance of a low permeability to Na and K. This pump is normally energized by ATP. Thus, when ATP-depleted cells are incubated with Ca, there is a net entry and a concomitant increase of K permeability. A supply of ATP either prevents the increase in permeability or restores it to normal by reducing cell Ca to low levels (Romero & Whittam, 1971).

The change of permeability is completely prevented by oligomycin or partially suppressed by ouabain (Blum & Hoffman, 1971; Riordan & Passow, 1971), both well known to be inhibitors of the Na pump of human red cells (Schatzmann, 1953; Whittam, Wheeler & Blake, 1964). However, ouabain seems to have a dual effect, depending on external K. The Ca-stimulated K efflux from guinea-pig red cells is reduced in the presence of external K whereas in K-free medium ouabain increases K efflux (Lew, 1971). It has been suggested that the actions of ouabain are mediated by the intracellular level of ATP, which is in turn regulated by the running direction of the sodium pump (Lew, 1971).

Conflicting with the above view, Blum & Hoffman (1971) reported that as ouabain acted almost immediately upon addition and its action was observed to the same extent in ATP-depleted cells, ATP was not involved.

Since the permeability of red cells to K is regulated by internal Ca, we have investigated the influence of ouabain on net Ca movements in human erythrocyte ghosts. It was found that ouabain elicits a net efflux under conditions compatible with a reversal of the Na pump.

METHODS

Analytical quality reagents were used whenever possible and were obtained from British Drug Houses Ltd., England. ATP, ADP and the luciferin-luciferase enzyme complex were purchased from Sigma Chemical Co., U.S.A. In all solutions pH was adjusted at room temperature within a range of $\pm 0{\cdot}04$ units.

Preparation of ghosts

Red cells from human blood which had been stored in the cold in acid citrate-dextrose solution for 4–6 weeks, were washed until free of glucose and packed as described elsewhere (Romero, 1974).

One volume of packed cells was lysed at 4° C in 30 vol. of a medium containing (mM): $MgCl_2$, 2; Tris-acetate buffer, 10; pH 7·0. The lytic ratio was increased in some experiments to 1:100. After 1 min vigorous stirring, enough 3 M-KCl (or choline chloride) solution was added to regain isotonicity and the suspension was then incubated for 30 min at 37° C (Hoffman, Tosteson & Whittam, 1960).

Ghosts containing different ATP concentrations were prepared from cells incubated in a Na-medium at 37° C either for 30 min with 15 mM inosine plus 5 mM adenine, in the presence or absence of 10 mM-Na phosphate (Whittam & Wiley, 1967) or 3–14 hr in the presence of 10 mM-KCl without added metabolites. In the latter case, 1 μg/ml. chloramphenicol was also present (Blum & Hoffman, 1971).

Following resealing, the ghosts were washed twice by centrifuging at 11,000 *g* for 10 min at 4° C and resuspending in ice-cold 160 mM-NaCl containing 20 mM-Tris-HCl (pH 7·6).

One volume of these ghosts was incubated with 40 vol. of fresh medium. When required Ca was added to a final concentration of 10 mM, which is sufficient to obtain a net gain (Romero & Whittam, 1971). Ouabain (10^{-5} M) was used for inhibiting the Na pump (Blum & Hoffman, 1971). In some experiments, the concentration of ouabain was increased to 1 mM to ensure complete inhibition. Duplicate samples were incubated in a water-bath for 15, 30 and 45 min at 37° C. These times were

chosen in order to obtain a net gain of 0·5–1 μmoles Ca/ml. ghost, which produces half maximum activation of the Ca pump in intact cells (Romero & Whittam, 1971).

After incubation, the ghosts were washed twice as described above and finally lysed in water for chemical analysis (original lysate). A similar treatment was employed with non-incubated ghosts.

Analytical procedures

Determination of haemoglobin. Oxyhaemoglobin was measured at 540 nm in suitable dilutions of the original lysate in 0·01 N-NH_4OH (Wooton, 1964).

Measurements of Ca. Ca was determined in trichloroacetic acid extracts of the original lysate, using a Varian Techtron 1000 atomic absorption spectrophotometer set at 422·5 nm. K (10 mg/ml.) was present in both samples and standards to overcome ionization and in most experiments, La (2 mg/ml.) was added as a releasing agent (Ramakrishna, West & Robinson, 1968).

The differences between the Ca content of duplicates were usually less than 15 %. An alternative procedure was also used, which involved deproteination by perchloric acid, its removal as $KClO_4$ and addition of 20 mM-EDTA (K salt). The results corresponded closely with the first method, which was routinely adopted.

Determinations of K were done by flame emission at 766·3 nm with the above-mentioned instrument.

Assays of ATP. Aliquots (0·4 ml.) of original lysates were deproteinized by heating for 4 min in a water-bath at 97° C (Strehler, 1965). Samples were immediately immersed in an ice-bath for 10 min and stored at −4° C. ATP was measured within 24 hr.

ATP was determined by light emission using the luciferin–luciferase system and a Hewlett-Packard 10602 A scintillation detector, operating at 1100 V. The electrical pulses from the photomultiplier were fed into a 5202L H-P digital counter. The reaction was started by adding 20 μl. of the sample to 75 μl. of the enzyme complex already contained in a mixing chamber placed immediately above the photocathode. Counts were accumulated for 10 sec and then related to the ATP concentration of convenient standards. This method allowed detection of 10^{-12} moles ATP.

The ATP concentration in the samples was expressed as μmole/l. ghost water, assuming a 90 % water content. The Na concentration of ghosts, after adequate corrections, was used to estimate the amount of trapped fluid. Duplicate samples usually agreed to within less than 20 %.

Determination of ADP/ATP ratio. A known volume (2 ml.) of original lysate was deproteinized with 5 % perchloric acid. Thereafter, the acid was precipitated as $KClO_4$, the supernatant solution was lyophilized and the residue was dissolved in 20 μl. water.

The nucleotides were analysed by high-pressure liquid chromatography, using a Varian chromatograph (LCS 1000) equipped with a double-beam detector operated at 254 nm. The column was packed with a pellicular anion-exchange resin and a linear gradient of eluents was employed (Scholar, Brown, Parks & Calabresi, 1973). The column flow was 42·3 ml./hr and the temperature 70° C. The peaks were identified by internal standards and the concentration was determined by comparing the peak area with that of convenient standards.

The ATP concentration determined by this method closely agreed with that described previously.

RESULTS

Partial inhibition by ouabain of the raised K permeability. Earlier work on red cells and ghosts showed that the Ca-dependent increase in K permeability is inhibited by ouabain (Blum & Hoffman, 1971; Riordan & Passow, 1971; Lew, 1971). With the intention of confirming these observations, net K efflux from ghosts incubated in a Na-rich K-free medium was studied.

After 45 min in the absence of Ca, ghost K was decreased by nearly 20 μequiv/ml. ghosts (Fig. 1, upper graph). The major part of K loss occurred during the first 15 min incubation. Such a large efflux is due mainly to leakage from ghosts which had not recovered a low permeability (Bodeman & Passow, 1972).

In the presence of Ca, ghost K was further decreased by about 70 μequiv K/ml. ghosts after 45 min and ouabain did not prevent the increase in permeability. During the first 15 min, however, ouabain (10^{-5} M) reduced K loss slightly but statistically significantly. This effect was abolished by a hundredfold increase in ouabain concentration.

Alteration by ouabain of Ca movements. In view of the above results for K loss, Ca movements were studied under identical conditions. After 45-min incubation with Ca, the internal content progressively rises from 0·1 to 1 μmole Ca/ml. ghosts (Fig. 1, lower graph). By contrast, when Ca and ouabain (10^{-5} M) are added together, ghost Ca (in μmole/ml. ghosts) is further increased by nearly 0·3 during the first 15 min incubation and then diminished by about 0·4 during the subsequent 15 min incubation. Thereafter, Ca is regained by the ghosts.

The stimulation of Ca efflux is abolished when ouabain is raised to 1 mM. At this concentration, the enhancement of Ca influx is persistent.

The results may indicate that ouabain, at low concentrations, has the dual action of increasing Ca influx and stimulating Ca efflux from ATP-poor ghosts. They also show that ouabain, at high concentration, stimulates only Ca entry.

Substitution of Na and K by choline. In order to investigate if the actions of ouabain have specific ionic requirements, ghosts were prepared containing either K or choline as the main internal cation.

The replacement of Na and K by choline completely prevented the ouabain-associated Ca movements (Table 1). Similar results were obtained when K-rich ghosts were incubated in an all-choline medium.

These findings demonstrate that the effects of the glycoside require external Na.

Influence of an increased lytic ratio. The existence of specific ionic requirements for the dual action of ouabain and the presence of a ouabain-

sensitive K efflux into K-free, Na-media suggest a relationship between the effects of ouabain and the reversed Na–K exchange through the Na pump. Since this exchange does not occur in ATP-depleted cells (Glynn, Lew & Lüthi, 1970), K-rich ghosts were prepared at a higher lytic ratio (1:100) to lower their ATP content.

As was expected, the glycoside had no effect on Ca movements, thus suggesting requirements similar to that for reversal of the Na pump.

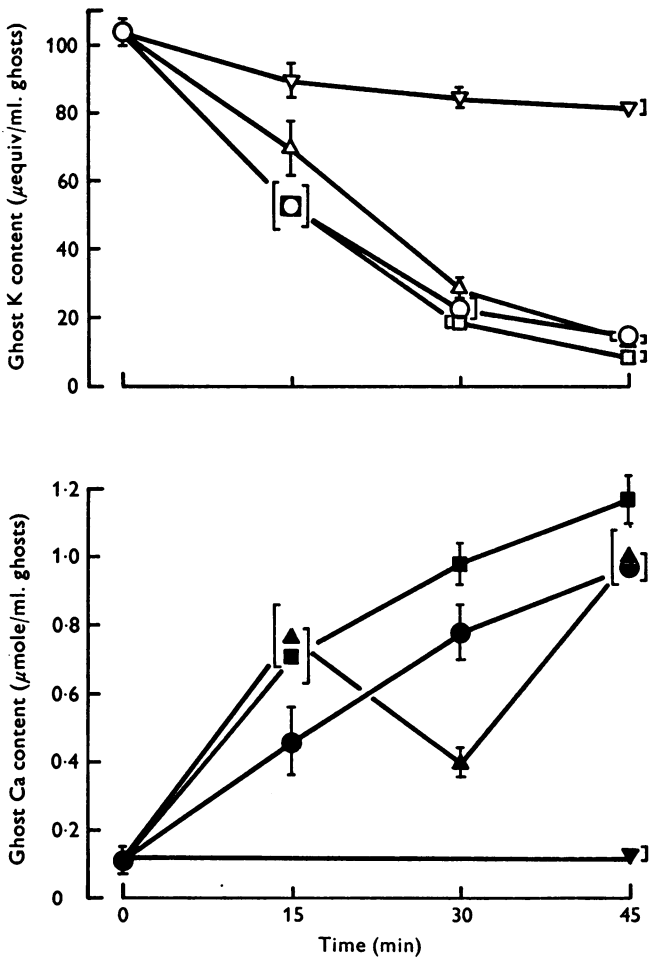

Fig. 1. The effects of ouabain on K loss and net Ca movements. High-K ghosts were incubated at 37° C in a medium containing (mM): NaCl, 160; Tris-HCl, 20; pH 7·6. Ghost K (open symbols) and Ca content (filled symbols) were determined after incubation with no additions (▽, ▼), 10 mM-$CaCl_2$ (○, ●) or 10 mM-$CaCl_2$ with either 10^{-5} M (△, ▲) or 10^{-3} M ouabain (□, ■). Results from five experiments are shown. Vertical bars indicate ± 1 S.D. of mean.

Dependence of ouabain actions on cell ATP. The above findings imply that ATP may be involved. To investigate this possibility, the ATP content of the original cells was altered by incubating either without metabolites or with adenine plus inosine in the presence or absence of inorganic phosphate (P_i). ATP synthesis is markedly stimulated by P_i since this ion is required at the first step of inosine metabolism (Whittam & Wiley, 1967). In this way, cells containing low, moderate and high ATP concentrations were obtained, the AT Pcontent being about 9, 40 and 1,600 μM, respectively. Ghosts from the various types were incubated under conditions used in previous experiments.

TABLE 1. Abolition of ouabain effects by replacing Na and K by choline

Cations replaced by choline	Additions to incubation medium	Ghost Ca content (μmole/ml. ghosts) Incubation time (min) 0	15	30
External Na	None	0·1 ± 0·01	0·6 ± 0·08	0·8 ± 0·08
	Ouabain added		0·7 ± 0·10	0·9 ± 0·01
External Na and internal K	None	0·1 ± 0·02	0·4 ± 0·03	0·7 ± 0·07
	Ouabain added		0·5 ± 0·07	0·7 ± 0·06

Ghosts with either K or choline as the main internal cation were incubated at 37° C in a medium containing (mM): choline chloride, 170; $CaCl_2$, 10; Tris-HCl, 20; pH 7·6, in the presence or absence of ouabain (10^{-5} M). The results shown are mean values ± 1 S.D. of at least four experiments.

Ouabain (10^{-5} M) had no effect on ghosts from low-ATP cells, but when the ATP concentration was increased to 40 μM, the dual action became evident (Fig. 2). Thus, the glycoside enhanced Ca influx by about 0·3 μmole Ca/ml. ghosts during the first 15 min incubation and stimulated an efflux of the same magnitude at the end of incubation.

The above effects were not observed in ghosts made from cells fully replenished with ATP (Fig. 3, lower graph). When ouabain was raised to 1 mM, however, stimulation of Ca influx was attained.

In view of the latter results, it was of interest to study the net K movements from these ghosts.

As expected, K loss (in μequiv/ml. ghosts) was not affected by 10^{-5} M ouabain, being roughly 30 after 45 min (Fig. 3, upper graph). This loss was further increased by 20 when ouabain was raised a hundredfold. Such a stimulation of K loss is compatible with an increased Ca influx under similar conditions.

The results suggest that ATP may be indispensable for the dual action of ouabain. There is a cellular ATP level below or above which ouabain

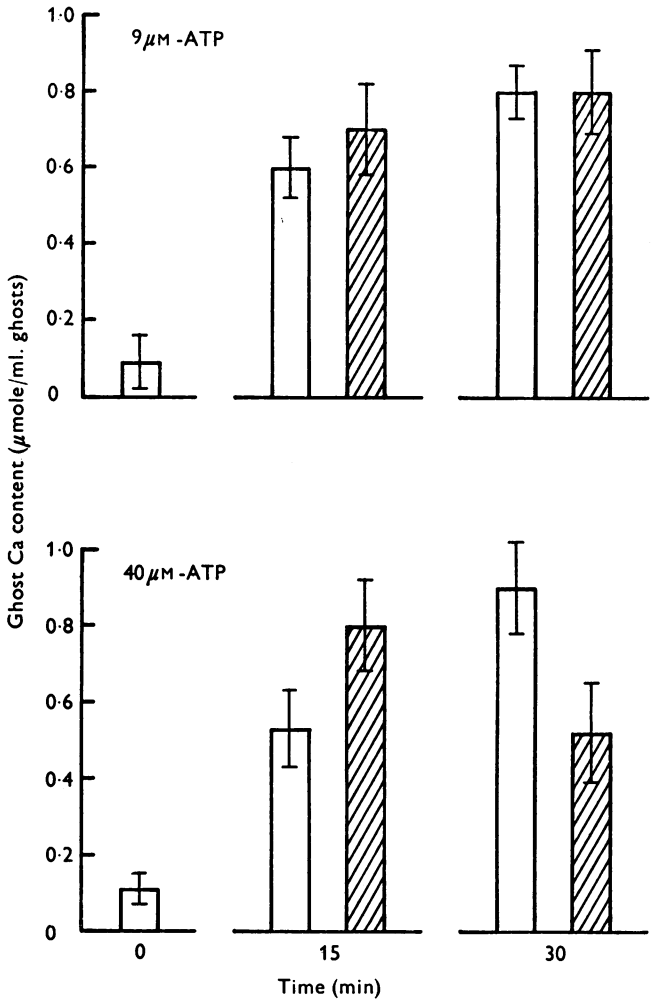

Fig. 2. Loss of the effects of ouabain by metabolic depletion. Red cells from blood stored in the cold for about 6 weeks were washed in 160 mM-NaCl containing 20 mM Tris-HCl (pH 7·6) and divided into two lots. One portion was incubated for 30 min at 37° C in the presence of 15 mM inosine plus 5 mM adenine. The other portion was incubated in Na-medium containing 10 mM-KCl for 3–14 hr at 37° C in the absence of metabolites. Cells treated in this way had an ATP content of about 40 and 9 μmole/l. cell water, respectively. Ghosts were prepared from these cells and the effects of ouabain (10^{-5} M) on internal Ca were assessed after incubation in Na-medium containing 10 mM-$CaCl_2$ for the length of time indicated in the graphs. The Ca content of ghosts from 9 (upper graph) and 40 μM-ATP cells (lower graph) is shown by the vertical columns. Shaded columns represent incubation with ouabain. Results from four experiments are given as mean values ± 1 S.D.

cannot induce net Ca movements. On the other hand, stimulation of Ca influx appears to be induced at increased ATP levels only by high ouabain concentrations.

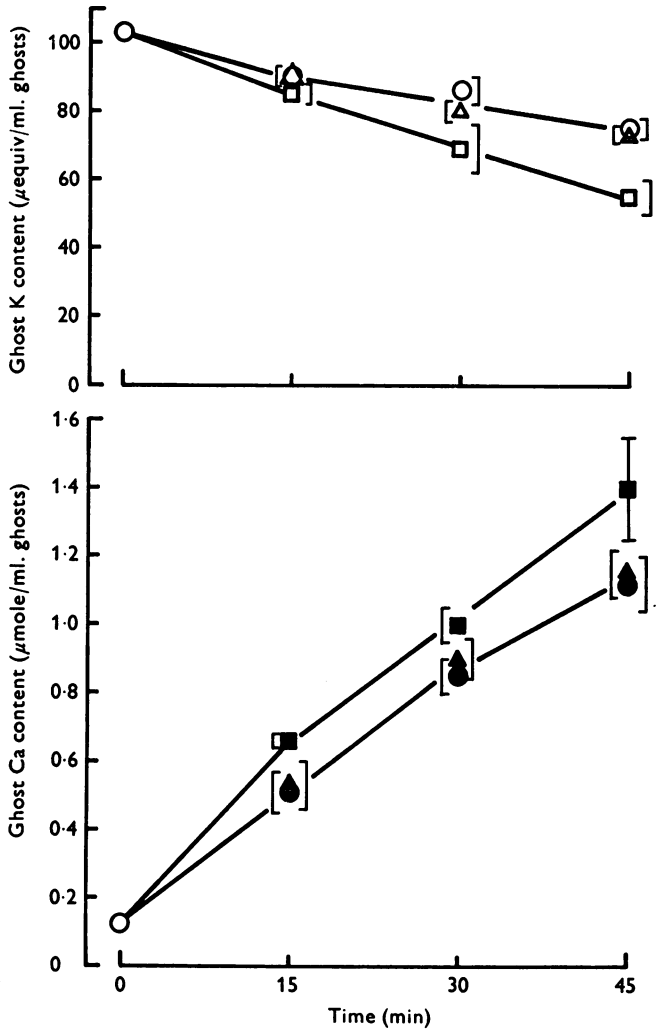

Fig. 3. The alteration of ouabain effects by preliminary incubation with metabolites and P_i. K-rich ghosts were obtained from cells which had been incubated with metabolites as described in Fig. 2, but in the presence of 10 mM-Na phosphate. The ghosts were then incubated in a medium containing (mM): NaCl, 160; Tris-HCl, 20; pH 7·6, for the length of time shown above. Ghost K (open symbols) and Ca content (filled symbols) were determined after incubation with 10 mM-$CaCl_2$ (○, ●) or 10 mM-$CaCl_2$ with either 10^{-5} M (△, ▲) or 10^{-3} M ouabain (□, ■). Results from four experiments are shown. Vertical bars indicate ± 1 S.D. of mean.

The above findings stress a similarity with the sodium pump which requires a certain level of intracellular ATP, below or above which no reversal occurs (Glynn, Hoffman & Lew, 1971).

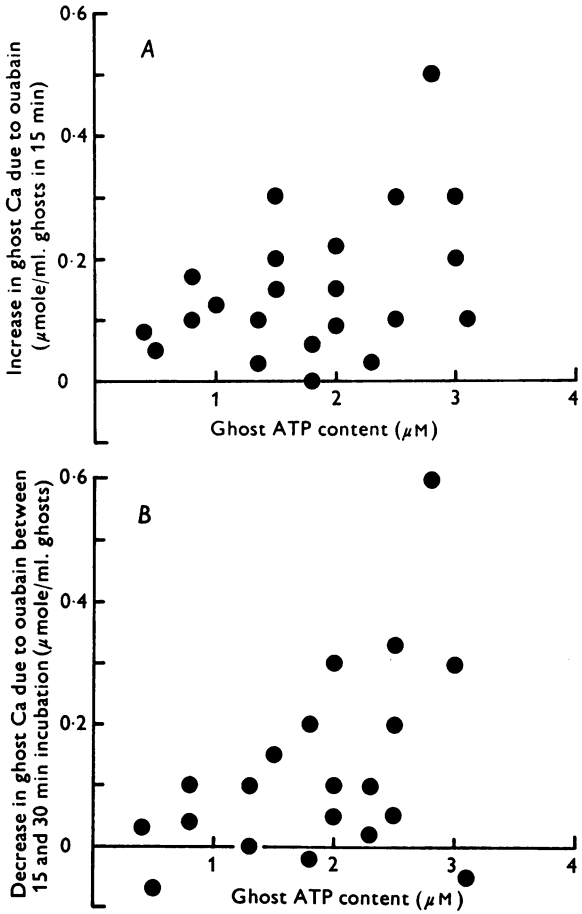

Fig. 4. The lack of dependence on ghost ATP of ouabain-stimulated Ca movements. Ghosts were prepared from cells having different ATP concentrations and incubated at 37° C for 30 min in Ringer solution containing (mM): NaCl, 160; $CaCl_2$, 10; Tris-HCl, 20 (pH 7·6), with and without ouabain (10^{-5} M). The ouabain-dependent Ca movements after the first 15 min (part *A*) and between 15 and 30 min incubation (part *B*) were determined and plotted against the initial ghost ATP concentration. Each point corresponds to the average from a different experiment run in duplicate.

The effects of ouabain and ghost ATP. In order to determine the extent of the dependence on ATP, ghosts were obtained from cells having different ATP concentrations. The actions of ouabain (10^{-5} M) on Ca movements

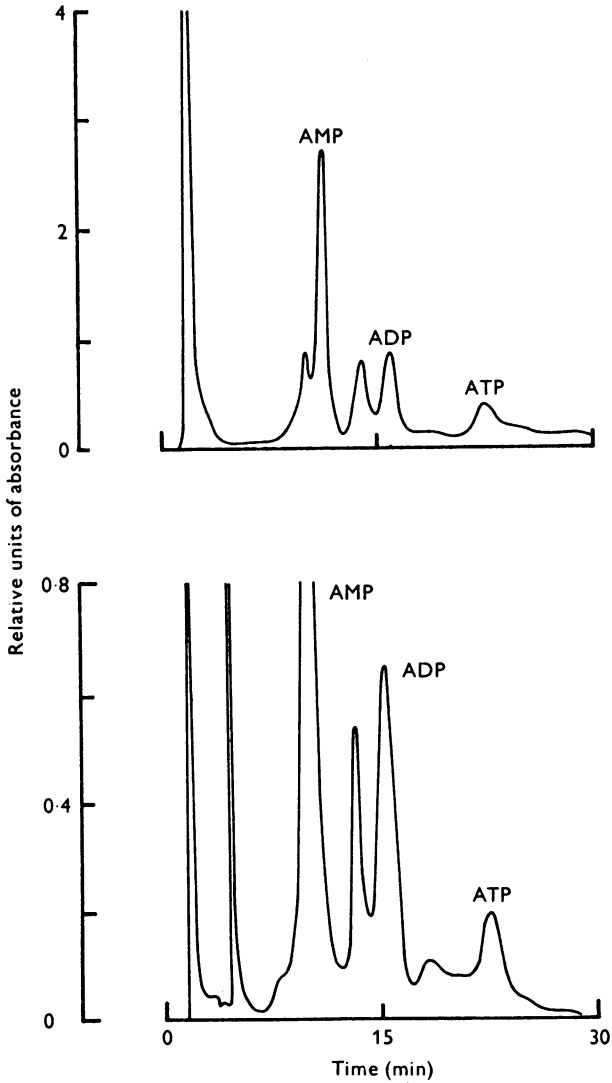

Fig. 5. The alteration of ADP/ATP ratio during resealing. Red cells from human blood which had been stored in the cold for 6 weeks, were washed free of glucose and incubated for 30 min at 37° C in the presence of 15 mM inosine plus 5 mM adenine. They were then lysed in 30 vol. of a medium containing (mM): $MgCl_2$, 2; Tris-acetate buffer, 10; pH 7·0. After 1 min lysis, the ghosts were resealed for 30 min incubation at 37° C in the presence of 160 mM-KCl. Thereafter, they were washed twice in ice-cold 160 mM-NaCl containing 20 mM Tris-HCl (pH 7·6) and finally lysed in 1 vol. water. For nucleotides determinations, samples taken before (upper graph) and after resealing (lower graph), were treated as described in the text and analysed by high-pressure liquid chromatography.

were assessed after incubation and then related to the initial ATP content of ghosts.

As was expected, no correspondence was found (Fig. 4). On the other hand, ghost ATP seemed independent of the ATP concentration in the original cells. Thus, the ATP level of ghosts prepared from cells containing 0·05, 0·1, 0·5, 1·0 and 1·5 mM-ATP was between 1 and 3 μM. These observations indicate that ATP is metabolized to a greater extent during the resealing process and, therefore, different ADP/ATP ratios are to be expected in the ghosts. To investigate such changes adenine nucleotides were analysed before and after resealing.

A chromatographic profile of nucleotides in whole haemolysate and in the corresponding resealed ghosts is shown in Fig. 5. It can be observed that the amount of ADP is increased in the ghosts whilst ATP is reduced. The findings clearly show that the ADP/ATP ratio alters during resealing.

Correspondence between ouabain actions and ADP/ATP ratio. The backward running of the Na pump can be shown in energy-poor cells (Glynn & Lew, 1970) where ADP and P_i contents are moderately high. Furthermore, reversal of the pump seems to require a certain ADP/ATP ratio (Glynn *et al.* 1971). To determine whether ouabain-dependent Ca movements are also affected by ADP/ATP changes, the effects of ouabain were related to the initial nucleotide ratio in the ghosts used above.

The stimulation by ouabain of net Ca movements showed a marked dependence on the ADP/ATP ratio. Thus, when it was 1 or lower than this value, the actions of the glycoside were abolished (Fig. 6). An optimal effect was obtained for both efflux and influx by raising this ratio to about 2·5. At higher values, the effect on Ca efflux was abolished whilst the action on influx seemed to persist, although at a much lower extent.

The results suggest that the dual action of ouabain involves a common mechanism. In addition, they further stress a parallelism between ouabain effects and reversal of the Na pump.

DISCUSSION

The permeability of erythrocytes to alkali ions is determined by the intracellular Ca level, which is in turn regulated by Ca pump activity (Romero & Whittam, 1971). In this context, the partial inhibition by ouabain of the raised K permeability should be associated with a decrease in cell Ca.

The experiments reported here demonstrate that ouabain affects the Ca content of ghosts, altering both influx and efflux. Perhaps, the most important of these findings is the stimulation by a low ouabain concentration of an outwardly directed Ca movement. Before going any further,

we must consider first the possibility of whether this movement is an artifact.

The human erythrocyte becomes permeable to Ca as it enters the cell (Romero & Whittam, 1971). It is possible that the same phenomenon occurs in ghosts. Ghosts containing more Ca would also lose more during

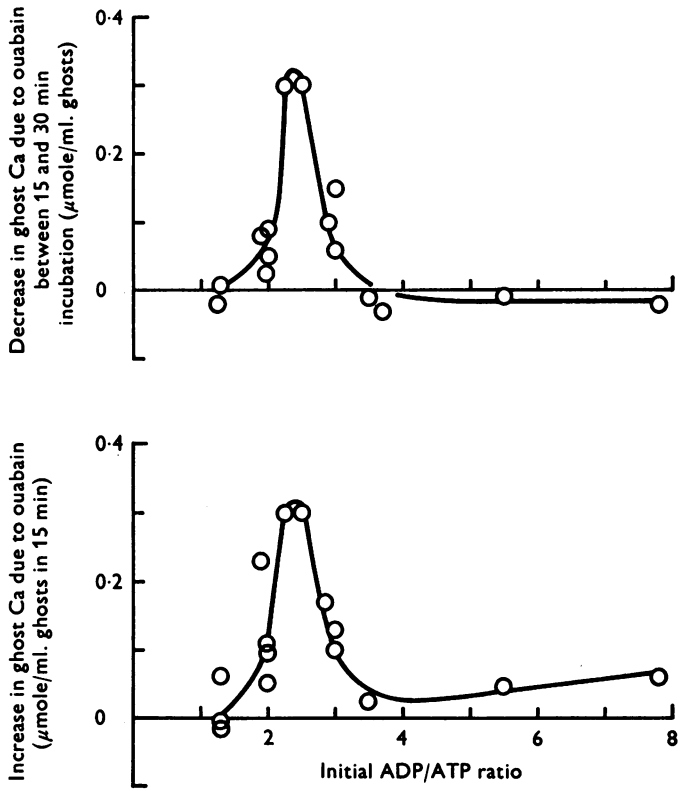

Fig. 6. The dependence of ouabain-stimulated Ca movements on the initial ADP/ATP ratio. Ouabain-dependent Ca movements were determined in ghosts made from cells containing different ATP concentrations, as described in the text of Fig. 4. These movements were then related to the initial ADP/ATP ratio in the ghosts. The effects of ouabain (10^{-5} M) on entry and exit of Ca are shown in the lower and upper graph, respectively. Each point corresponds to the average from a different experiment run in duplicate.

the washing procedure before analysis. The experiment described in Fig. 1 clearly demonstrates that Ca is regained after having been expelled during the previous 15 min incubation. It also shows that the Ca content of

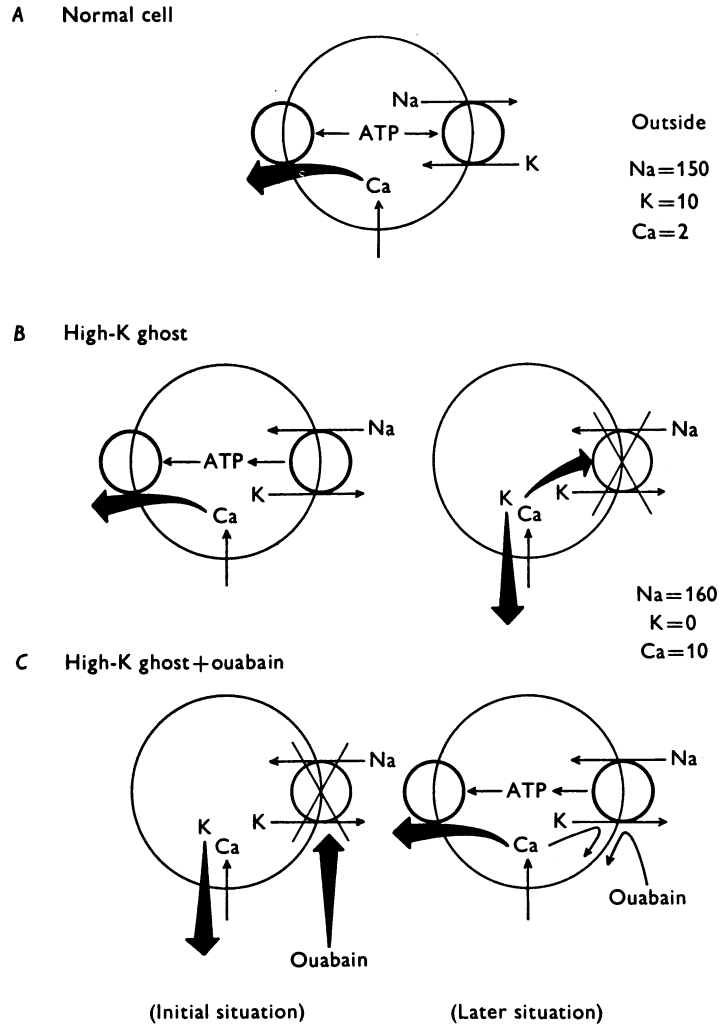

Fig. 7. Model to illustrate the activation of Ca efflux by ouabain. *A*, in the normal cell, ATP resulting from metabolic activity will activate both Na and Ca pumps. *B*, reversal of the sodium pump would occur during incubation with Ca until inhibitory levels of this ion are reached in the ghosts. *C*, in the presence of ouabain, the sodium pump is immediately inhibited but the increasing level of internal Ca would be relieving this inhibition progressively. ATP synthesized by pump reversal would activate the calcium pump, getting rid of internal Ca and restoring membrane permeability to normal.

ghosts incubated with 1 mM ouabain continues increasing after 15 min incubation. Such findings cannot be explained assuming a raised Ca permeability and, therefore, exclude the possibility of an artifact.

The ouabain-dependent extrusion had the following characteristics.

(*a*) It was abolished when choline replaced both external Na and internal K, thus showing specific ionic requirements.

(*b*) It was not observed when the original cell ATP content was reduced by lysing at a higher ratio (1:100) or when ghosts were prepared from metabolically depleted cells.

(*c*) Increasing the intracellular ATP level by previous incubation with adenine plus inosine caused a marked stimulation of the efflux, but it was abolished at higher ATP concentrations.

(*d*) The extrusion did not depend on ghost ATP but on the initial ADP/ATP ratio.

The above requirements are coincident with those for reversal of the Na pump. Associated with this mode of operation, a net synthesis has been postulated both in erythrocytes (Lew & Glynn, 1970) and ghosts (Lant & Whittam, 1968), which may provide the energy for active Ca transport.

The immediate question that arises is why a net Ca efflux is elicited by ouabain, when both forward and backward running of the Na pump should be blocked and, therefore, no ATP synthesis would be expected.

In this context, it is important to stress that the effect described above was observed only at a low ouabain concentration (10^{-5} M) and after 15 min incubation. It seems likely that under such conditions the Na pump is partially inhibited.

In the course of incubation with Ca, external K is increased as a consequence of both a raised K permeability and leakage from poorly resealed ghosts. It is a known feature of the Na pump that inhibition by cardiac glycosides can be overcome by raising external K (Glynn, 1957). This possibility, however, seems unlikely under the present conditions by the following reasons. First, the final K concentration in the incubation medium would be too low (1–2 mM for an haematocrit of 2·5 %) to become effective at a high external Na (Schatzmann, 1965). Secondly, the effect of K is only observed at very low concentrations of cardiac glycosides, i.e. below 10^{-5} M (Glynn, 1957).

The presence of a relatively high Ca concentration might create a situation different to that where the Na pump activity is completely blocked by ouabain even at concentrations lower than 10^{-5} M (see Blum & Hoffman, 1971). Perhaps Ca, by competing with ouabain, relieves the inhibition of the Na pump. In such a case the pump would run backwards,

exchanging K for external Na. From these movements enough ATP may be synthesized to activate the Ca pump, getting rid of cell Ca and restoring membrane permeability to normal (see Fig. 7, part *C*).

In favour of this hypothesis is the fact that the stimulation of Ca efflux is abolished by a hundredfold increase in ouabain concentration. This finding appears consistent with a Ca–ouabain competition for Na pump sites.

The Na pump of human erythrocytes is inhibited by a moderately high internal Ca concentration (Dunham & Glynn, 1961). It is therefore expected that in the presence of Ca, the Na pump will be progressively inhibited by the increased ionic Ca inside the ghosts (see Fig. 7, part *B*). The minimal amount of free Ca required for maximal inhibition has not been determined. However, it is possible to make a rough estimate from the results of Dunham & Glynn (1961). These authors showed that the ouabain-sensitive ATP hydrolysis is completely blocked by 0·2 mM-Ca. The ghosts used were repeatedly washed with water. This procedure would eliminate endogenous Ca chelating compounds present in the original cells. If chelation by ATP is neglected, then the Ca concentration just mentioned would practically represent the top limit of free Ca required for Na pump inhibition.

In the present work, it has been suggested that the backward running of the Na pump provides the energy required for Ca pump activity. Ca extrusion occurs from ghosts containing about 0·8 mM-Ca (see Fig. 1) and the doubt arises of whether the pump is already inhibited at this concentration. We do not know what the ionic Ca content would be in these ghosts. However, it will be certainly lower than 0·8 mM due to both presence of residual chelating compounds and adsorption to the ghost membrane.

The findings reported in this paper are not in agreement with the hypothesis of Blum & Hoffman (1971) that ouabain acts independently of ATP or the Ca pump. In addition, the same authors obtained an inhibition of K efflux from poisoned cells lower than that found in this work with ghosts after 15 min incubation. Such discrepancies may be attributed to differences in experimental conditions, involving long periods of preliminary incubation with the glycoside and the use of cells, where a similar rise in internal Ca would result in a lower free concentration due to a greater binding by the intact cell.

Ouabain reduces the Ca-dependent K efflux by nearly 20 μequiv/ml. ghosts after 15 min incubation. This inhibition suggests that a component of K loss occurs via the Na pump working in reverse. The magnitude of the reduction is equivalent to 80 μequiv-K/ml. ghosts $\times$ hr, far greater than that of 0·36 obtained in the absence of Ca with ghosts from

energy-poor human erythrocytes (Lant, Priestland & Whittam, 1970). Comparison of these values would suggest that Ca increases the rate of pump reversal. This suggestion, however, is difficult to reconcile with the fact that 1 mM ouabain does not reduce K loss under similar conditions, as would be expected from complete inhibition of pump reversal.

There is another interpretation for the reduction of K loss which does not involve an increased rate of pump reversal. Thus, ATP synthesized by the Na pump would lead to a diminution of K loss by both chelating Ca and stimulating an active Ca transport.

Another finding which needs discussion is the enhancement of Ca entry by ouabain. This stimulation cannot be attributed to an increase in the Ca driving force associated with the raised K permeability. In the presence of ouabain (10^{-5} M) K loss is reduced and so is the change in membrane potential.

The increased Ca entry is not due to an exchange for internal Na or K. Pilot experiments have shown that Ca influx is not affected by raising internal Na or K, providing some precautions (i.e. incorporation of EGTA inside ghosts) are taken to prevent the change in K permeability.

The stimulation was found only in K-rich ghosts incubated with external Na and was markedly dependent on the initial ADP/ATP ratio in ghosts. Moreover, conditions that favoured an increased entry were practically similar to those where the stimulation of Ca efflux occurred. These observations suggest that reversal of the Na pump is also involved in this phenomenon.

It seems likely that ouabain, by inhibiting pump reversal, would stop the energy supply for the Ca pump and thus favour a raised Ca influx.

To make the results consistent, it is necessary to postulate that a rise in internal Ca relieves the inhibition by ouabain of the Na pump. In this way, the glycoside would be fully inhibitory at the beginning of incubation but as internal Ca rises, this inhibition is progressively overcome.

It is important to point out that the effect described above was also observed at a high ouabain concentration (1 mM). This was obtained in ghosts prepared from either energy-poor cells (see Fig. 1) or P_i-loaded cells (see Fig. 3). In the latter case, however, ouabain was not effective at a lower concentration. Such a finding supports the idea that the Na pump is not completely blocked by 10^{-5} M ouabain during the course of incubation with Ca.

Another interesting finding is that ouabain has different effects on K loss, depending on concentration and whether or not the ghosts were made from P_i-loaded cells, fully replenished with ATP. Thus, ouabain at a low concentration reduces K loss from ghosts prepared from energy-poor cells and seems to enhance it at higher concentrations. Conversely, when

ghosts are made from P_i-loaded cells, a low ouabain concentration is ineffective whilst increasing K efflux at higher concentrations.

This work has shown that ATP is metabolized during the resealing of K-rich ghosts, presumably through adenylate kinase and phosphatase activities. A greater ADP/ATP ratio is thus expected in ghosts containing larger amounts of ATP, i.e. those made from P_i-loaded cells.

Chelation of Ca by ATP synthesized by pump reversal would reduce K loss at low ouabain concentrations. At high concentration, by contrast, the Na pump is fully inhibited and no ATP is synthesized. Such a situation would increase K efflux by raising free internal Ca.

This paradoxical effect of the glycoside somewhat resembles that reported by Lew (1971) in guinea-pig red cells. This author showed that in the presence of external K, when the pump is running forward, ouabain reduces K loss whilst increasing it in the absence of K, when the pump is running backwards.

It is tempting to comment on these findings in the light of the results reported in the present paper. In the former case, ouabain would stimulate indirectly an active Ca transport by sparing ATP and thus a reduction of K efflux is expected. In the latter case, by contrast, ouabain would inhibit indirectly the Ca pump by blocking Na pump reversal and therefore a raised K efflux would be expected.

The financial support received from Consejo Nacional de Investigaciones Científicas y Tecnológicas (Grant DF-S1-0214) and Consejo de Desarrollo Científico y Humanístico (Grant 362) is acknowledged. We are also grateful to Dr R. N. Priestland for helpful discussion, Dr I. de December for valuable help during nucleotides analysis, Miss E. Romero for skilled technical aid and the Blood Bank Unit of Vargas Hospital for providing the blood.

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