Calcium Transport in Intact Human Erythrocytes

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ABSTRACT Intact human erythrocytes can be readily loaded with calcium by incubation in hyperosmotic media at alkaline pH. Erythrocyte calcium content increases from 15–20 to 120–150 nmol/g hemoglobin after incubation for 2 h at 20°C in a 400 mosmol/kg, pH 7.8 solution containing 100 mM sodium chloride, 90 mM tetramethylammonium chloride, 1 mM potassium chloride, and 10 mM calcium chloride. Calcium uptake is a time-dependent process that is associated with an augmented efflux of potassium. The ATP content in these cells remains at more than 60% of normal and is not affected by calcium. Calcium uptake is influenced by the cationic composition of the external media. The response to potassium is diphasic. With increasing potassium concentrations, the net accumulation of calcium initially increases, becoming maximal at 1 mM potassium, then diminishes, falling below basal levels at concentrations above 3 mM potassium. Ouabain inhibits the stimulatory effect of low concentrations of potassium. The inhibitory effects of higher concentrations of potassium are ouabain insensitive and independent of the external calcium concentration. Sodium also inhibits calcium uptake but this inhibition can be modified by altering the external concentration of calcium. The efflux of calcium from loaded erythrocytes is not significantly altered by changes in osmolality, medium ion composition, or ouabain. It is concluded that hypertonicity increases the net uptake of calcium by increasing the influx of calcium and that some part of the sodium potassium transport system is involved in this influx process.

INTRODUCTION

Human erythrocytes contain approximately 15 μmol calcium per liter packed red cells (10). This concentration is thought to be maintained by a low membrane permeability to calcium and an active calcium pump (17). Methods that have previously been used to increase calcium concentration of these cells have involved either reversible hemolysis (9), chemical agents to alter membrane permeability (6), or energy depletion (20). The studies reported here describe a method for increasing the calcium content that involves hyperosmotic incubation at alkaline pH. Since this method is a selective process for calcium accumulation, it does not produce changes in intracellular sodium and potassium. The calcium uptake is, however, influenced by the monovalent cation composition of the incubation media. All the results are consistent with the hypothesis that hypertonicity increases the influx of calcium.

METHODS

Blood was collected with heparinized syringes, and all experiments were begun within 15 min of venipuncture. The plasma and buffy coat were removed before the cells were

washed in incubating solutions. For convenience all incubations were performed at 20° with moderate shaking and a cell-to-medium ratio of 1/10–1/20. Detailed protocols are given in the figure legends. All cations were added as their chloride salts.

In net accumulation studies cells were prepared for sodium, potassium, calcium, hemoglobin, and ATP determinations by washing six times at room temperature with 300 mosmol/kg solutions containing 5 mM Tris, pH 7.4, and tetramethylammonium (TMA) chloride. Sodium and potassium concentrations were determined by standard flame photometric techniques. Calcium measurements were done by an atomic absorption spectrophotometer equipped with a heated graphite atomizer as previously described (12). ATP determinations were accomplished by the semiautomated procedure of Dufresne and Gitelman (5). Hemoglobin concentrations were determined by the method of VanKampen and Zijlstra (19). Cell chloride concentrations were determined by conductimetric titration of chloride (14). Cell water determinations were done by drying to constant weight (13).

RESULTS

Preliminary experiments demonstrated that the incubation of erythrocytes in hypertonic solutions was associated with an increased calcium uptake. This could be accomplished by changing osmolality with either sodium chloride or sucrose. The representative study in Fig. 1 shows the content of calcium after 2 h of incubation when the osmolality is adjusted with sodium chloride. Cellular concentrations are expressed in terms of hemoglobin so that the units of measurement are independent of alterations in cell volume. These measurements were obtained after incubation at 20°C in the presence of 50 mM calcium. The content of calcium is increased with both hypo- and hypertonic incubations. The most striking uptake is obtained with 475 mosmol/kg at pH 7.7. Small reductions from this point in either osmolality or pH are associated with a marked decrease in calcium accumulation.

The specificity of this process for calcium uptake was evaluated by measuring sodium, potassium, and ATP concentrations of red cells during hypertonic incubation with and without 20 mM calcium. Panels A and B of Fig. 2 show only small changes in the concentrations of sodium and potassium over the time course. Panel C of Fig. 2 displays the time-dependent process of calcium accumulation. The reduction in ATP content shown in panel D of Fig. 2 is linear and independent of the calcium entry. The small potassium loss that is present during a 2-h incubation is dependent upon internal content of calcium. Fig. 3 demonstrates that the magnitude of this potassium leak is related to the concentration of internal calcium regardless of the external conditions.

Fig. 4 displays two separate experiments involving different donors which further evaluate the effect of pH on the entry of calcium. Calcium uptake is a curvilinear function of increasing pH over the range of 7.2–7.8. There is no change in the entry of calcium at pH values below 7.2. These observations prompted an examination of chloride ratios of erythrocytes in isotonic and hypertonic solutions to evaluate whether hypertonicity might also contribute to the increased polarization of the membrane associated with increased pH. However, hypertonicity does not appear to alter membrane potential. After 2-h incubations at 20°C at pH 7.6, the ratios of the internal to external chloride

concentrations are 0.635 ± 0.007 at an osmolality of 300 mosmol/kg and 0.625 ± 0.007 at 400 mosmol/kg.

The influence of monovalent ions on calcium uptake in hypertonic solutions was subsequently examined. Fig. 5 displays the net uptake of calcium as a function of the external calcium concentration at different external potassium concentrations. Net calcium uptake is a nonlinear function of the external calcium concentration and does not show saturation over the range tested. The

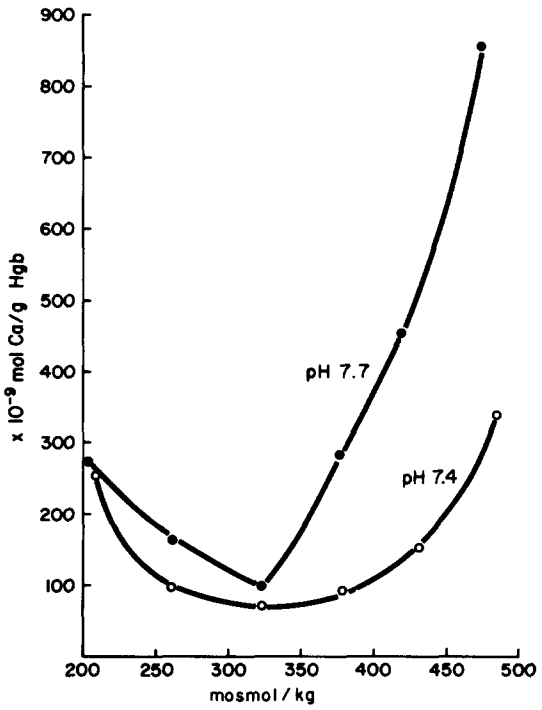

FIGURE 1. Intracellular calcium as a function of osmolality. Erythrocytes were incubated for 2 h at 20°. The incubation media contained 50 mM calcium, 10 mM glucose, 10 mM HEPES(*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid)-Tris buffer at a pH of either 7.7 (closed symbols) or 7.4 (open symbols) and the appropriate amount of sodium to produce the indicated osmolality. All cations were added as chloride salts.

addition of 1 mM potassium to the incubation medium is associated with a uniform increase in calcium uptake in cells incubated with 6 mM or greater calcium. In contrast, less calcium uptake is observed over the same external calcium concentrations when the external potassium has been raised to 5 mM.

The diphasic response to potassium prompted an examination of the influence of ouabain on this process. In these experiments potassium concentration was varied and the external calcium concentration was fixed. Each of the panels in Fig. 6 represents separate experiments with erythrocytes from different individuals. The upper curve in each panel displays calcium accumulation in the

absence of ouabain. In both cases, the calcium content increases as the external potassium increases between 0.1 and 1 mM. Above 1 mM there is a decrease in calcium uptake. The center curve in each panel represents the effect of potas-

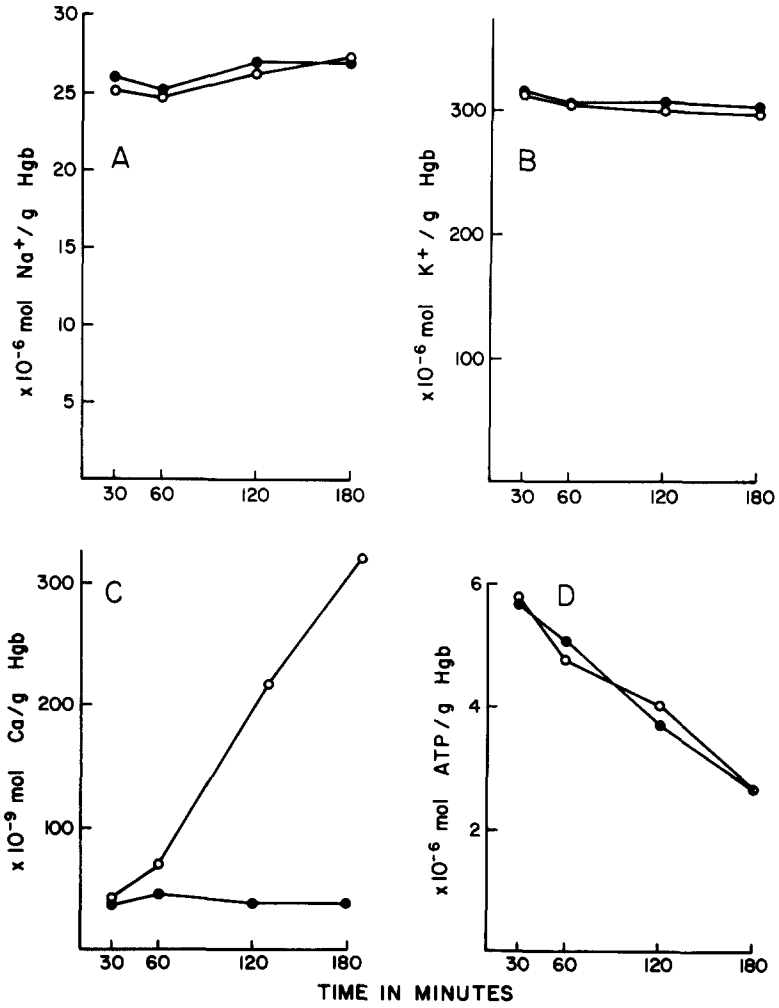

FIGURE 2. Cellular ion and ATP contents as a function of incubation time in the presence or absence of external calcium. Incubation media contained 20 mM HEPES-Tris buffer pH 7.8, 100 mM sodium, 10 mM glucose, either 0 (closed symbols) or 20 mM (open symbols) calcium and the appropriate amount of TMA to give a final osmolality of 400 mosmol/kg. Panel A displays intracellular sodium values; panel B, potassium values; panel C, the calcium values; panel D, the ATP values.

sium in the presence of ouabain. Under these conditions the stimulatory effect of potassium between 0.1 and 1 mM is absent. Calcium values in the 3–10 mM potassium range are similar in the presence and absence of ouabain. Although the shapes of the experimental curves are different in these two studies, the

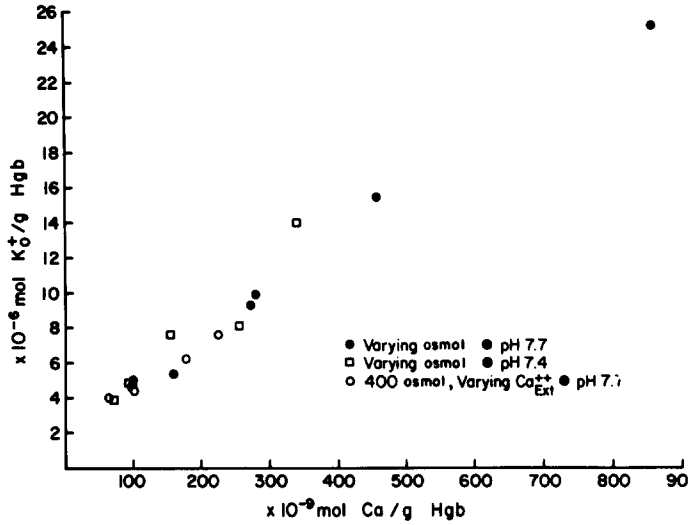

FIGURE 3. Potassium loss as a function of the net calcium accumulation. The supernatant potassium was measured after 2 h of incubation and expressed in terms of the hemoglobin concentration of the incubation mixture. The calcium accumulations were done as described in Fig. 1. The open circles represent incubations at pH 7.4 in the presence of 50 mM calcium at different osmolalities. The closed circles represent incubations under the same conditions except the pH was 7.7. The squares represent incubations at 400 mosmol/kg pH 7.7 with varying concentrations of calcium.

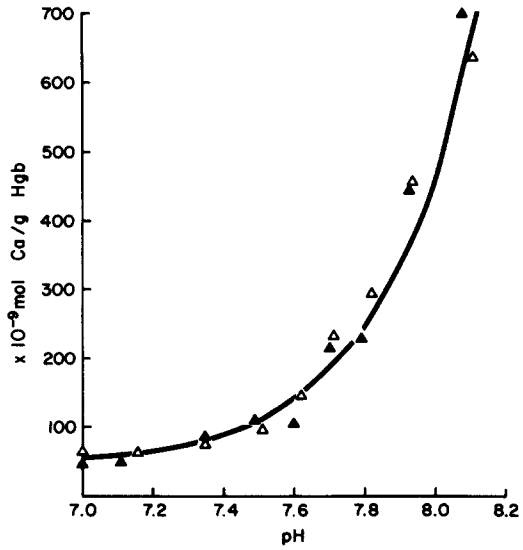

FIGURE 4. Cellular calcium of two different donors as a function of pH. The intracellular calcium concentrations were measured after 2 h of incubation at 20° in media containing 50 mM calcium, 120 mM sodium, and 20 mM HEPES-Tris buffer adjusted to different pH values. The pH values were measured at the end of the 2-h incubation. The open and closed symbols distinguish the two donors.

derived ouabain-sensitive curves, the bottom curves in each panel, are similar with a peak at approximately 1 mM potassium. Fig. 7 summarizes these potassium and ouabain effects in several experiments with different donors. There is a tendency for cells incubated with 1 mM potassium and ouabain to accumulate less calcium than cells incubated in the absence of added potassium ($P < 0.05$) which may reflect the difficulty in maintaining potassium-free conditions throughout the incubation period. More importantly, cells incubated for 2 h with 1 mM potassium contain significantly more calcium than cells incubated

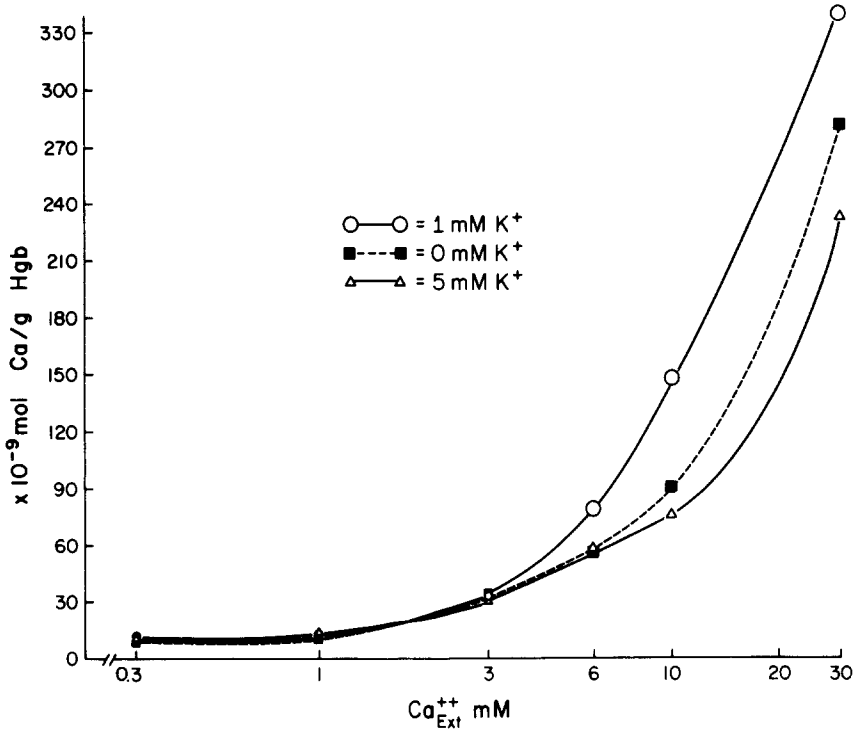

FIGURE 5. Intracellular calcium as a function of the external calcium in the absence and in the presence of 1 and 5 mM potassium. Measurements were done after 2 h of incubation at 20° in media containing 20 mM HEPES-Tris buffer pH 7.8, 100 mM sodium, 10 mM glucose, the indicated calcium and potassium concentration adjusted to a final osmolality of 400 mosmol/kg with TMA.

either in the absence of potassium ($P < 0.025$) or in the presence of 1 mM potassium and ouabain ($P < 0.005$).

The inhibitory effects of higher concentrations of potassium (concentrations in excess of 3 mM) are ouabain insensitive. These inhibitory effects of potassium were further evaluated by examining their dependence upon the external calcium concentration. In Fig. 8, panel A displays the effects of potassium on calcium uptake in the presence of ouabain at two different external calcium concentrations, 40 and 10 mM. With both external calcium concentrations an increase in external potassium is associated with a decrease in calcium uptake. The results of each experiment have been normalized in panel B by expressing

the calcium uptake as a percentage of the delta calcium value with a 3 mM potassium concentration. These calculated curves for the two external calcium concentrations are the same, indicating that the degree of inhibition associated with potassium is independent of the external calcium concentration.

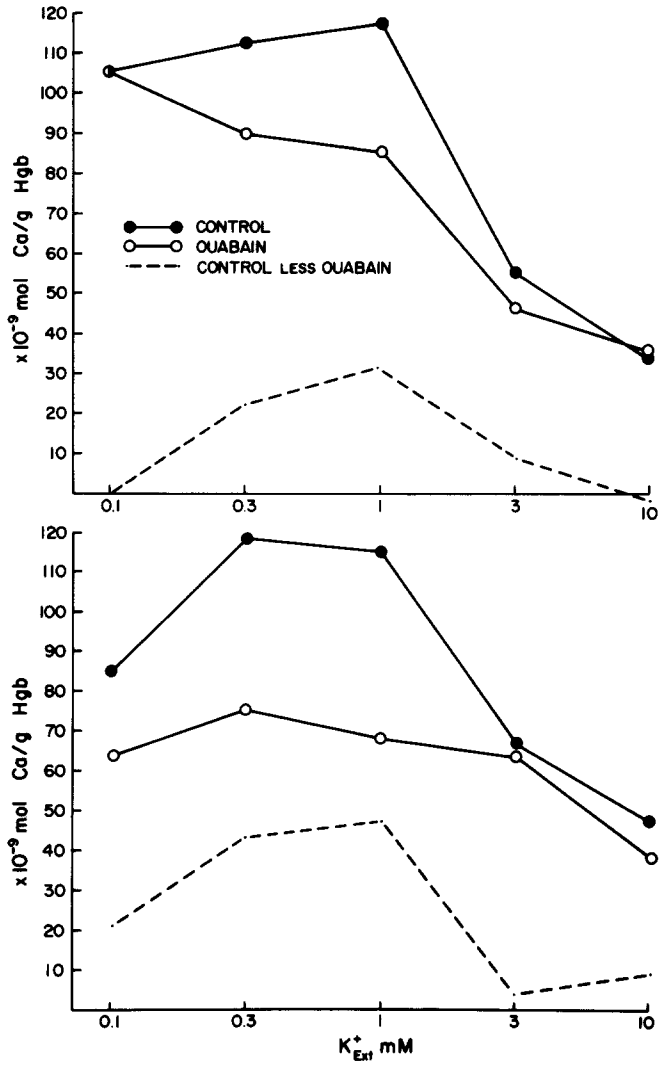

FIGURE 6. Cellular calcium as a function of external potassium. Each panel represents a separate experiment with a different donor. Incubations were for 2 h at 20°. Incubation media contained 10 mM calcium, 120 mM sodium, 20 mM HEPES-Tris buffer adjusted to pH 7.8, the indicated amount of potassium, and sufficient TMA to produce 400 mosmol/kg. 2×10^{-4} M ouabain was used.

The influence of sodium on the process of calcium uptake was evaluated in a similar fashion. In comparison to potassium, inhibition with sodium requires a much higher cation concentration. Furthermore the curves in Fig. 9 show that the inhibitory pattern associated with sodium is dependent upon the external

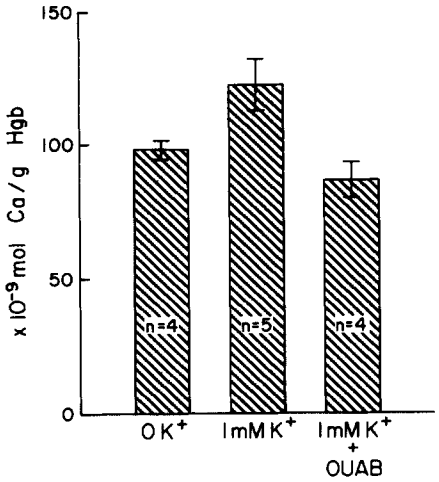

FIGURE 7. The effect of potassium and ouabain on calcium accumulation. Incubation conditions were the same as described in Fig. 5. The results are from separate experiments with different donors. Standard errors and number of individuals are given with each bar. The bar on the left displays the calcium values obtained in the absence of added potassium, the center bar, the calcium values obtained in the presence of 1 mM potassium, the bar on the right, calcium values obtained in the presence of 1 mM potassium and 2×10^{-4} M ouabain.

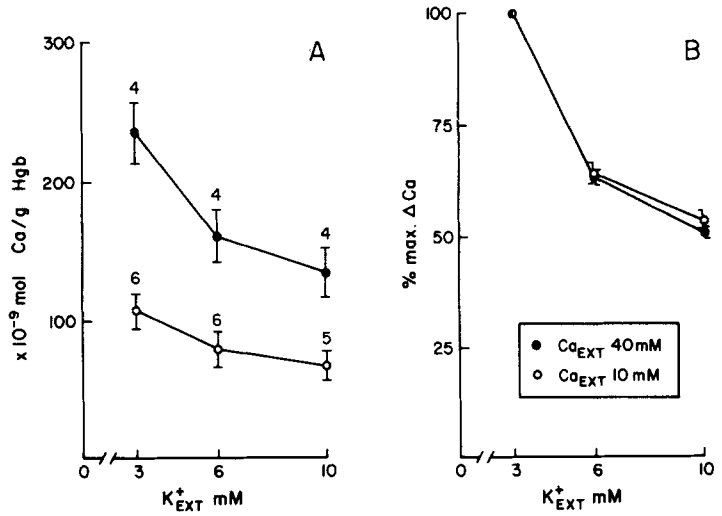

FIGURE 8. Inhibitory effects of potassium on calcium accumulation in the presence of two different external calcium concentrations. Incubations were for 2 h at 20°. Incubation media contained 2×10^{-4} M ouabain, 20 mM HEPES-Tris buffer pH 7.8, 100 mM sodium, either 10 mM (open symbols) or 40 mM (closed symbols) calcium, the indicated potassium concentration, and enough TMA to give a final osmolality of 400 mosmol/kg. Panel A expresses erythrocyte calcium concentration as a function of the external potassium concentration. In panel B, the calcium uptake in each experiment was normalized as the percentage of the delta calcium values obtained at the 3 mM potassium concentration. Standard errors and n values are given for each point.

sodium concentration of calcium. The accumulations with 10 mM calcium are markedly inhibited as the external sodium goes from 10 to 70 mM while the change in the accumulation with 40 mM calcium lags behind over the same range. The normalized results, displayed in panel B, confirm the difference in these inhibitory patterns indicating that sodium produces a decrease in calcium uptake that is dependent on the concentration of external calcium.

Having established these effects of hypertonicity, pH, potassium, ouabain, and sodium on the uptake of calcium, comparison studies were performed to

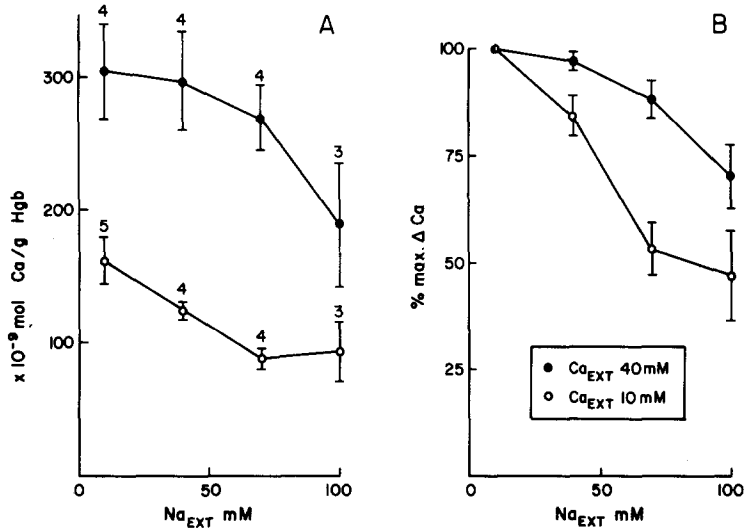

FIGURE 9. Inhibitory effects of sodium on calcium accumulation in the presence of two different external calcium concentrations. Incubations were for 2 h at 20°. Incubation media contained 2×10^{-4} M ouabain, 20 mM (closed symbols) calcium, the indicated amount of sodium, and enough TMA to give a final osmolality of 400 mosmol/kg. Panel A expresses erythrocyte calcium concentration as a function of the external sodium concentration. In panel B, the calcium uptake in each experiment is normalized as a percentage of the delta calcium values obtained at the 10 mM sodium concentration. Standard errors and *n* values are given for each point.

determine if any of these parameters influenced the efflux rate of calcium. These efflux experiments were performed under conditions similar to those employed with the net accumulation studies except that calcium was omitted from the incubation solutions after hypertonic loading. The calcium effluxes are displayed in Fig. 10 for two different donors in isotonic and hypertonic solutions. Although the cells contain different amounts of calcium the rates of calcium efflux proceed linearly from 15 to 75 min. Within each experiment the efflux rates for calcium are independent of the tonicity of the incubating solutions. Table I summarizes the experiments that were performed to determine the effects of pH, potassium, sodium, and ouabain on calcium efflux. In comparison to the results observed during net uptake studies, the effects of these parameters on efflux rates are small and variable. Furthermore only

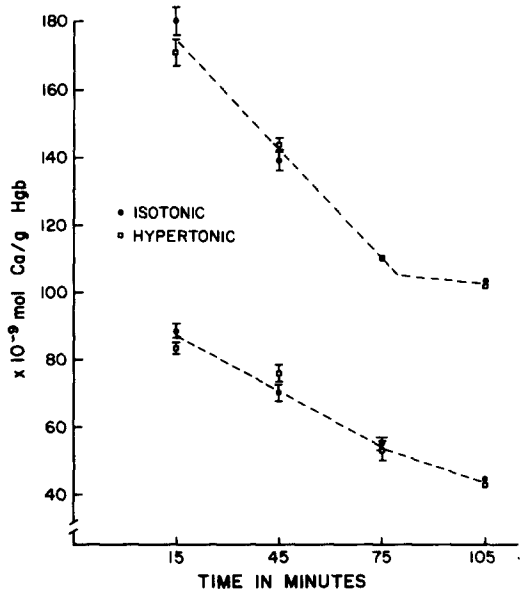

FIGURE 10. Calcium content of calcium-loaded erythrocytes as a function of the incubation time in calcium-free solutions that are either hyperosmotic or isoosmotic. Two separate experiments are shown with erythrocytes from different donors. Erythrocytes were loaded with calcium by incubating for 2 h at 20° in media containing 20 mM HEPES-Tris buffer pH 7.8, 20 mM calcium, 100 mM sodium, 1 mM potassium, 10 mM glucose, and 90 mM TMA. The final osmolality was 400 mosmol/kg. After this incubation the cells were washed twice in 300 mosmol/kg solution of TMA with 20 mM HEPES-Tris buffer pH 7.4, then washed once and incubated in either 300 (closed symbols) or 400 (open symbols) mosmol/kg solutions containing 20 mM HEPES-Tris buffer pH 7.8, 100 mM sodium, 10 mM glucose, and sufficient TMA to give the indicated osmolality. At the end of the incubations the cells were washed three times with 300 mosmol/kg solution of TMA containing 5 mM Tris buffer pH 7.4.

TABLE I

SUMMARY OF EFFLUX EXPERIMENTS

Experimental variable	Exp. no.	Control rate	Experimental rate	Difference in rate	Average rate difference
			$\times 10^{-9}$ mol Ca/g Hgb/h		
pH	1	35.22	32.22	−3.00	−4.00
	2	44.86	39.86	−5.00	
55 mM K	1	43.38	45.14	1.76	
	2	33.70	42.58	8.88	4.09
	3	26.30	27.94	1.65	
200 mM Na	1	34.50	29.55	4.95	
	2	33.44	33.60	−0.16	0.68
	3	18.74	21.50	−2.76	
Ouabain	1	33.60	33.36	−0.24	
	2	39.16	38.36	−0.80	−0.52

Cells were calcium loaded as described in Fig. 10. The control incubation solution contained 100 mM sodium, 1 mM potassium, 20 mM HEPES-Tris buffer pH 7.8 at 20°. Final osmolality was set to 400 mosmol/kg with TMA. In the pH experiments the experimental group's pH was 7.2. Units of measure are nanomoles per gram hemoglobin per hour.

potassium produces a change in efflux rate that would contribute to the alteration in the uptake of calcium observed during loading circumstances.

DISCUSSION

These results demonstrate that hypertonic incubation provides a rapid and convenient technique for incorporating calcium into human erythrocytes. There are several reasons for believing that this technique has promoted calcium entry into the cell interior: *(a)* The process is time dependent, thus excluding a simple absorptive phenomena. *(b)* Calcium uptake in hypertonic incubations is associated with an augmented efflux of potassium (Fig. 3). It will be recalled that the effects of increased intracellular calcium have been linked to increases in potassium permeability in reconstituted erythrocyte ghosts (3). *(c)* Certain calcium entry phenomena observed with hypertonic loading can be correlated with other calcium uptake studies. The increased uptake of calcium associated with pH values above 7.2 in hypertonic media corresponds to Romero and Whittam's (15) findings in energy-depleted cells. Also, the inhibition of calcium uptake produced by external potassium during hypertonic incubation is comparable to the inhibitory effects of potassium on ^{45}Ca entry in metabolically depleted cells observed by Lew (11).

In addition, our studies provide some insight into the mechanisms that contribute to this uptake of calcium under hypertonic conditions. We attribute the predominant effects of our major variables in net uptake studies to influx phenomena, since these parameters have either no effect or small effects on the rate of calcium efflux. This relative constancy of the efflux process simplifies our evaluation of the uptake phenomena we have observed. We would caution, however, that the absence of effects on efflux does not exclude the possibility that hypertonicity is associated with an increase in diffusion of calcium through the membrane. The efflux rates we have observed could be a consequence of an active calcium pump. This pump mechanism, in the absence of a sufficient concentration gradient for calcium, could account for our inability to observe the effects of diffusion on the efflux process.

It is likely that some component of the sodium potassium transport system is involved in calcium uptake under hypertonic conditions. This view is supported by the similar behavior of both transport processes. The stimulation of calcium influx by external potassium concentrations below 3 mM (Figs. 6 and 7) is similar to the stimulation produced by external potassium for both potassium influx and sodium efflux (8, 16). Furthermore, both of these effects of external potassium in the red cell are totally inhibited by 10^{-4} M ouabain. It is also of interest that this mechanism offers an alternative explanation for the calcium-dependent, ouabain-sensitive potassium diffusional process described by Blum and Hoffman (4). Since calcium-dependent increases in the permeability of potassium require increases in internal calcium, factors that affect the calcium influx would be expected to affect the magnitude of this diffusional process. Thus it is possible that the inhibitory effects of 10^{-4} M ouabain and the stimulatory effects of 2 mM potassium observed in their study could be attributed to alterations in the entry of calcium similar to those displayed in Figs. 6 and 7.

We have additionally observed inhibitory effects of sodium, potassium, and

hydrogen ions on the uptake of calcium during hypertonic incubations. The sodium inhibition appears to be of the competitive type since it can be modified by altering the external concentrations of calcium. In this regard, the human erythrocyte resembles other tissues in which a competitive relationship has been reported between calcium and sodium transfer (1, 2, 12). This relationship can be described in nervous tissue and dog erythrocyte by a carrier mechanism which appears to involve a coupled sodium calcium exchange. If a similar mechanism is operative in the human erythrocyte we would have anticipated that increases in external sodium would have augmented calcium efflux. Our failure to influence calcium efflux by raising the external sodium suggests that the interaction between sodium and calcium in the human erythrocyte occurs by a somewhat different and perhaps unrelated mechanism.

The inhibitory effects of potassium and hydrogen ions appear to be independent of the external calcium concentration. We do not know how these effects are mediated. One possibility to be considered is that increases in the concentrations of these ions would tend to diminish a driving force for calcium entry. We do not believe this is a likely explanation, since these alterations in membrane potential would be insignificant when compared to the large concentration gradient of calcium that is required to achieve calcium uptake. Alternatively, we would suggest that alterations in potassium and/or hydrogen ion concentration may be associated with small changes in the permeability of the membrane to calcium. This would explain why the pH and potassium effects appear to be independent of the external calcium concentration and are additive to the effects of sodium and ouabain.

The fundamental question that remains is how has exposure to hypertonicity altered the membrane properties of the erythryocyte to enable us to observe the uptake of calcium. None of our data bear directly on this point. The possibilities to be considered include alterations in ionic strength, membrane hydration, or the concentration of a critical metabolic intermediate which may alter the properties of the erythrocyte membrane (18). If calcium influx is related to a binding process, it is possible that an alteration in surface charge or a conformational change in a binding protein might alter the mobility, affinity, or capacity of some membrane ligand for calcium. Finally, it seems appropriate to consider whether physical distortion of the membrane per se can induce calcium accumulation. Altered morphology is a common feature which could relate the increased content of calcium associated with sickle cells (7) to hypotonically treated dog erythrocytes (12) or normal cells exposed to hypertonicity.

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