Studies on the Active Transport of Calcium in Human Red Cells

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ABSTRACT The Ca^{++} transport mechanism in the red cell membrane was studied in resealed ghost cells. It was found that the red cell membrane can transport Ca^{++} from inside the cell into the medium against great concentration gradient ratios. Tracing the movement of ^{45}Ca infused inside red cells indicated that over 95% of all Ca^{++} in the cells was transported into media in 20 min incubation under the optimum experimental conditions. The influence of temperature on the rate constant of transport indicated an activation energy of 13,500 cal per mole. The optimum pH range of media for the transport was between 7.5 and 8.5. As energy sources, ATP,[1] CTP, and UTP were about equally effective, GTP somewhat less effective, and ITP least effective among the nucleotides tested. The Ca^{++} transport does not appear to involve exchange of Ca^{++} with any monovalent or divalent cations. Also, it is not influenced by oligomycin, sodium azide, or ouabain in high concentrations, which inhibit the Ca^{++} transport in mitochondria or in sarcoplasmic reticulum. In these respects, the Ca^{++} transport mechanism in the red cell membrane is different from those of mitochondria and the sarcoplasmic reticulum.

The energy-linked transport of Ca^{++} in phosphorylating mitochondria and in the sarcoplasmic reticulum has been well-established during the past decade (Brierley, 1963; Brierley et al., 1964; Engstrom and De Luca, 1964; De Luca and Engstrom, 1961; Chance, 1965; Hasselbach, 1964; Hasselbach and Makinose, 1961; Ebashi and Lipmann, 1962; Lee, 1965; Lee et al., 1966). Since the free Ca^{++} concentration gradient between extra- and intracellular fluid is extremely large in tissues such as muscle and nerve, the existence of a similar Ca^{++} pump mechanism in the cell membrane which is responsible for Ca^{++} extrusion from these cells against an electrochemical gradient has been suggested (Reuter and Seitz, 1968; Baker et al., 1969). However, no direct evidence was available for an active, outward Ca^{++} trans-

[1] ATP, adenosinetriphosphate; CTP, cytidine triphosphate; ITP, inosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

port located in a cellular membrane until Schatzmann in 1966 reported the active, ATP-dependent Ca^{++} transport mechanism in red cell ghosts. Vincenzi and Schatzmann (1967) showed that Ca^{++}-activated ATPase was responsible for this Ca^{++} extrusion mechanism. This system was found to transport strontium in addition to Ca^{++} (Olson and Cazort, 1969). In view of the important role played by Ca^{++} in numerous cellular functions, including the excitation-contraction coupling processes in muscle, understanding of the active mechanism for outward transport of Ca^{++} in the cellular membrane would have a physiologically important significance.

Recently, Schatzmann and Vincenzi (1969) further extended their original findings and presented many characteristics of the Ca^{++} extrusion mechanism in red cells. They studied Ca^{++} movement by measurement of the Ca^{++} content of cells and media. We have investigated the transport mechanism by simultaneously measuring both ^{45}Ca flux and Ca^{++} content of cells and media, while Schatzmann and Vincenzi measured only the latter. This and other differences in experimental conditions enable us to present experimental data demonstrating some of the important aspects of the transport mechanism which were only conjectured by Schatzmann and Vincenzi (1969). In addition, the nucleotide specificity, the optimum pH range, and other characteristics of the Ca^{++} transport mechanism in red cells are reported in this paper. Since the present work was completed before the publication of the work of Schatzmann and Vincenzi (1969), some data which were found to overlap theirs have been omitted.

METHODS

Citrated human red cells which had been stored for 20–25 days in the cold were washed four times with saline solutions, hemolyzed, and resealed according to the method of Schatzmann (1966), with some modification. Particular attention was directed to eliminating white cells as much as possible. For this reason, the white buffy layer formed on centrifuging the washed red cells was eliminated completely, with a considerable loss of red cells. Schatzmann's method consists of hemolyzing red cells in 5 volumes of hypotonic solution, then restoring the isotonicity with KCl. The hemolyzing solution contained 10 mM Tris-buffer, pH 7.4, and appropriate amounts of $MgCl_2$, ATP, and $CaCl_2$ to attain the desired concentrations of these agents in resealed cells. All lysing solutions contained ^{45}Ca at 0.05 μc/ml. After 5 min of lysing, the isotonicity was restored by the addition of 3 M KCl solution. After 5 min standing at 25°C, the mixtures were placed in ice baths. The resealed ghosts were separated from the medium by centrifugation at 10,000 *g*, washed twice with an ice-cold incubation medium (six times the volume of red cells), and suspended in 2 volumes of the incubation medium. Incubation was started by warming this final cell suspension to 37°C. After the resealing process, the volume of the ghost cells decreased from the original volume, apparently due to shrinking during the process. The shrinkage was somewhat greater when ATP and Ca^{++} were present in

the lysing solution. Red cells and medium were separated by centrifugation in one portion of the final suspension before warming to 37°C and were used for measurement of volume ratio, ^{45}Ca and Ca^{++} content at 0 time. For incubation, the volume ratio (medium:ghost) of approximately 2 was employed, except in those few experiments in which the ratio was made to be about 1. The volume ratio was measured in each experiment before and after incubation. After incubation, cell suspensions were placed in ice to stop the reaction. Cells were separated by centrifugation (at 10,000 *g*), washed twice with 5 volumes of an ice-cold solution containing the same components as the incubation medium except that it contained no Ca^{++}, resuspended in 5 volumes of water, and hemolyzed. During the washing procedure, very little ^{45}Ca was lost. The radioactivity of appropriate amounts of the final hemolyzing solution, and the original supernatant of the incubation mixture were measured in a Nuclear Chicago gas flow thin-window counter. For measurement of Ca^{++} content, appropriate amounts of the final hemolyzing solution and the original supernatant of the incubation mixture were placed in crucibles, ashed overnight at 700°C, dissolved in water, and the Ca^{++} contents of aliquots (usually 0.1 ml) were measured according to the method of Lee et al. (1969) employing murexide in an Aminco Chance dual beam spectrophotometer (American Instrument Co., Silver Spring, Md.).

In most experiments, red cells were depleted of endogenous ATP before hemolysis and resealing. This involved preincubation of red cells in a glucose-free Krebs solution containing 1 mM iodoacetate for 2 hr at 37°C. This procedure was shown to deplete endogenous ATP (Whittam, 1958), and these cells will be called ATP-depleted cells.

The standard conditions of incubation were as follows: The ATP-depleted red cells were lysed in a solution which contained 10 mM Tris-buffer, pH 7.4, 1 mM $MgCl_2$, 1 mM ATP, 0.8 mM Ca^{++}, and ^{45}Ca, resealed by addition of 3 M KCl, and then centrifuged and washed twice with an incubation mixture before final incubation. The incubation mixture contained 1 mM Ca^{++}, 10 mM Tris-buffer, pH 7.4, 140 mM NaCl, 10 mM KCl, and 2 mM $MgCl_2$. The incubation was carried out at 37°C. The hemolyzing solution under the standard conditions contained 0.8 mM Ca^{++} and ^{45}Ca because it was found that this concentration of Ca^{++} gave concentrations of approximately 1 mM in resealed cells due to cell shrinkage during the resealing process.

Disodium salt of ATP neutralized by Tris to pH 7.0 was used in all experiments except when the Na^+-free lysing medium was used. In the latter case, Tris-ATP was used.

RESULTS

^{45}Ca efflux from ATP-depleted cells and accumulation of ^{45}Ca in the supernatant during an 80 min incubation period at 37°C are shown in Fig. 1. In the presence of ATP and Mg^{++} inside the cells, there was rapid extrusion of ^{45}Ca from the cells into the medium. Only 5% of the total ^{45}Ca content remained inside the cells after 30 min incubation, and accumulation of radioactivity in the medium accounted for all the loss from the cells. In the absence of ATP in the cells, both ^{45}Ca efflux from the cells and accumulation of ^{45}Ca in the medium were very slow. Before incubation, the total ^{45}Ca in

cells without ATP was lower than that with ATP. This was probably due to the fact that ATP in cells formed a complex with ^{45}Ca, so that a smaller amount of ^{45}Ca was lost during washing. Table I presents the summary of

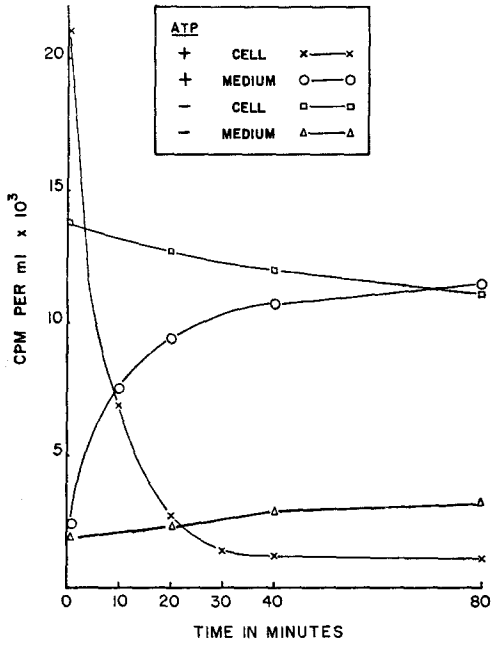

FIGURE 1. Movement of ^{45}Ca during incubation of resealed red cell ghosts. +ATP, lysing solution contained 1 mM ATP. −ATP, lysing solution did not contain ATP.

TABLE I

EXTRUSION OF CALCIUM AND ^{45}Ca FROM RESEALED RED CELL GHOSTS

Incubation time	Total radioactivity		Total radioactivity in medium	Ca^{++} concentrations from flux data*		Ratio (flux) (data)	Ca^{++} concentrations murexide method		Ratio (murexide method)
	Cell	Supernatant		Cell	Supernatant		Cell	Supernatant	
min	*cpm* ± *SE*	*cpm* ± *SE*	%	*mM*	*mM*		*mM* (±*SE*)	*mM* (±*SE*)	
0	27,106 ±1,450	6,024 ±372	18	—	—	—	1.06 ±0.09	1.02 ±0.02	0.96
20	3,510 ±210	27,010 ±2,562	82	0.137	1.42	10.3	0.23 ±0.03	1 43 ±0.12	6.3
80	1,499 ±169	32,020 ±1,920	97	0.058	1.47	25.4	0.07 ±0.02	1.49 ±0.20	21.3

* Calculated from radioactivity data in red cells on assumption that no passive Ca^{++}-^{45}Ca exchange took place.

results obtained in eight experiments in which both ^{45}Ca and total Ca^{++} contents of cells and medium were measured in the same samples. As expected from the experimental procedures, almost all radioactivity was found in red cells, and very little in the supernatant (medium) at 0 time. After 20

min of incubation, approximately 82% of all radioactivity was found in the medium, and after 80 min, about 97% of all radioactivity was found there with 3% remaining in the cells. Schatzmann and Vincenzi (1969) showed in their study on ^{45}Ca flux that the red cell membrane was very poorly permeable to Ca^{++}. We made a study similar to theirs and our results were in complete agreement. Also data in Fig. 1 show that in the absence of ATP, only a very small amount of ^{45}Ca is lost into the medium from cells. On the assumption that there was no passive diffusion on exchange of Ca^{++} during incubation and that the movement of ^{45}Ca from cells to medium was entirely due to extrusion of Ca^{++} by the active pump mechanism, Ca^{++} concentrations of cells and medium were calculated from the radioactivity data in cells (the second column in Table I) and presented in Table I as Ca^{++} concentrations from flux data. These calculated values were very close to those obtained by actual measurement of total Ca^{++} content of cells and medium employing murexide in a dual beam spectrophotometer (compare actual and calculated values in Table I). This indicated that under the prevailing experimental conditions the ^{45}Ca efflux reflected the amount of Ca^{++} transported rather accurately, and Ca^{++}-^{45}Ca exchange by passive diffusion through the cellular membrane could be safely ignored.

The ratios of Ca^{++} concentrations in medium and cells based on the ^{45}Ca flux data were somewhat higher than the ratios based on the measurement of Ca^{++} content. This difference was probably due to one or both of the following factors: First, there was some small degree of passive exchange of $^{45}Ca \leftrightarrow Ca^{++}$ between medium and cells. Second, a certain amount of Ca^{++} bound to red cells was nonexchangeable. Since it is reasonable to assume that the proportion of bound Ca^{++} to free Ca^{++} was greater in cells than in the medium, the Ca^{++} concentration ratios (medium:cells) calculated on the basis of flux data were probably not far from the true ratios for free Ca^{++}. It should be mentioned that Ca^{++} bound to the red cell membrane under conditions similar to the present experimental conditions was found to be 0.059 mM by Gent et al. (1964), and 0.016 mM by Harrison and Long (1968). Thus, it appears that most of the Ca^{++} found in this study at the end of incubation may be bound to the cell membrane and this contributes to the difference in calculated and actual ratios presented in Table I.

Effect of Lack of Depletion of Endogenous ATP

In the standard type of experiment described above, red cells were first depleted of endogenous ATP by pretreatment with iodoacetate before hemolysis. That the presence of a certain amount of endogenous ATP is likely if there is no iodoacetate pretreatment is shown in experiments presented in Fig. 2. Without ATP in the lysing solution, no significant Ca^{++} transport out of iodoacetate-pretreated cells was observed. However, there was about 63%

transport out of the nonpretreated cells when no ATP was added to the lysing solution. Since approximately the same degree (58%) of Ca^{++} was transported in iodoacetate-treated cells to which 0.3 mM of ATP was added in the lysing solution, it appears that noniodoacetate-pretreated cells contain approximately 0.3 mM of endogenous ATP.

Effect of Mg^{++} and ATP Concentrations in Cells

The ^{45}Ca transport was investigated in ATP-depleted cells lysed in solutions containing various amounts of Mg^{++} and ATP. Experiments were performed under the standard conditions, except for varying concentrations of Mg^{++}

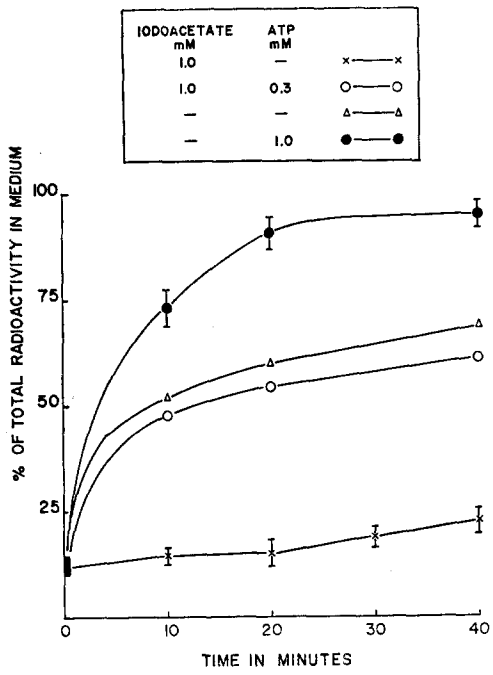

FIGURE 2. Effect of preincubation with iodoacetate. None + ATP (1 mM), the initial preincubation with iodoacetate was omitted. Other conditions, standard. None − ATP, no preincubation with iodoacetate and no ATP in lysing solution. Other conditions, standard. Iodoacetate − ATP, standard conditions except omission of ATP from solution. Iodoacetate + ATP (0.3 mM), standard conditions, except 0.3 mM ATP in lysing solution. Each point represents average of four experiments. Bars indicate standard error (SE).

and ATP in the lysing solution. Results are shown in Fig. 3. The radioactivity of media and cells lysed in a solution containing Mg^{++} 2 mM and ATP 2 mM indicated that over 80% of cellular Ca^{++} was transported into the media during the first 10 min of incubation. On the other hand, during the same period, approximately 50% of cellular Ca^{++} was transported in cells lysed in solutions containing 0.3 mM Mg^{++} and 2 mM ATP, 2 mM Mg^{++} and 0.3 mM ATP, or 0.3 mM Mg^{++} and 0.3 mM ATP. This indicated that the rate of transport was determined by the concentration of both Mg^{++} and ATP or of Mg^{++} alone. After 10 min of incubation, ^{45}Ca continued to be transported in cells with 0.3 mM Mg^{++} and 2 mM ATP, but no significant extra amount of ^{45}Ca was transported in cells with 0.3 mM Mg^{++} and 0.3

mM ATP, presumably due to exhaustion of ATP. A significant amount of Ca^{++} was transported in cells lysed in a solution containing ATP (0.1 or 0.3 mM) but no Mg^{++}. This was probably due to the presence of endogenous Mg^{++}. In the hemolysis and resealing process approximately one-sixth of the original cellular Mg^{++} would probably be retained in the resealed ghosts. This probability is supported by the finding that the addition of 1 mM EDTA to a lysing solution containing 2 mM ATP and 2 mM Ca^{++} but no Mg^{++} abolished ^{45}Ca transport almost completely during the subsequent incubation, in spite of the presence of ATP and Ca^{++} in excess of EDTA in the cells.

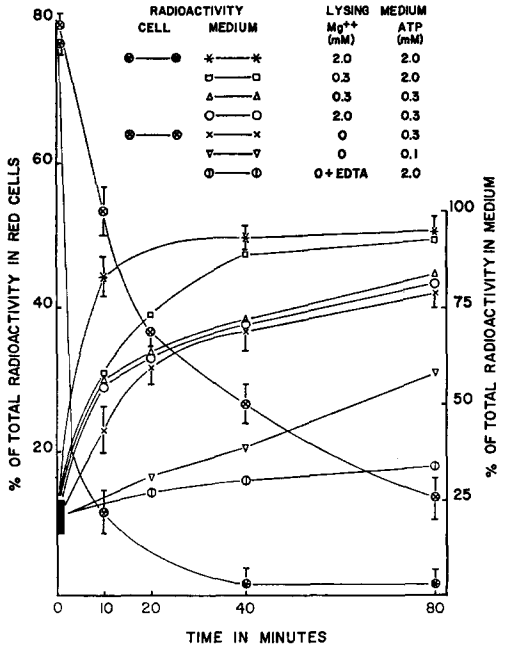

FIGURE 3. Effect of varying concentrations of Mg^{++} and ATP in lysing solutions on Ca^{++} transport. Other conditions, standard. Each point, average of four experiments with SE.

It is interesting to note that extremely little ^{45}Ca was found in media in experiments with EDTA. In these experiments, the lysing solution contained 1 mM EDTA, 2 mM ATP, and 2 mM Ca^{++} (with ^{45}Ca), other conditions being standard. In this condition, the inside Ca^{++} concentration must be fairly high, and yet no significant amount of ^{45}Ca was found in the media. This supports, along with other data presented here, Schatzmann's finding that the red cell is very poorly permeable to Ca^{++}.

Effect of Temperature

Under the standard conditions of incubation, the effect of temperature on Ca^{++} transport was studied, and the results are shown in Fig. 4. The rate of transport increased progressively as the temperature was increased. The half-

time was calculated from a time in minutes required for 50% extrusion of Ca^{++} from the cells. The half-times of radioactivity loss from cells between 0 and 80 min of incubation were 7 min at 40°C, 14 min at 30°C, and 32 min at 20°C. These values corresponded to rate constants, K, of 0.1 min^{-1}, 0.05 min^{-1}, and 0.021 min^{-1} at 40°, 30°, and 20°C, respectively. The activation energy was estimated on the basis of the Arrhenius equation:

$$\ln K = \frac{E}{RT} + \text{constant}$$

where R, T, and E are the gas constant, temperature, and activation energy, respectively. A straight line was obtained when the reciprocal of the absolute

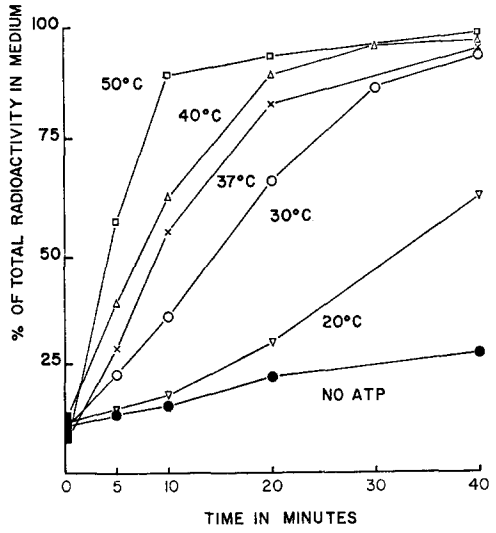

FIGURE 4. Effect of temperature on Ca^{++} transport. Conditions of incubation, standard. Each point, average of three experiments.

temperature, T, was plotted against the logarithm of the rate constant, K. From the slope of this line, the activation energy (E) was calculated and was found to be 13,600 cal/mole. Q_{10} was calculated according to the method of Giese (1957), and was found to be 3.16. Schatzmann and Vincenzi reported an activation energy of 25,030 cal and a Q_{10} of 3.5. The difference may be due to different methods of calculation. They used the initial rate of Ca^{++} extrusion, and we the half-time of ^{45}Ca loss from the cells. It should be mentioned that at 50°C, there was a considerable amount of leakage of hemoglobin after incubation but ^{45}Ca did not go back to the apparently damaged cells.

Influence of Concentration Gradient on Ca^{++} Transport

The ratio $\frac{[Ca^{++}]_o}{[Ca^{++}]_i}$ was varied from about 80 to 1 by employing various con-

centrations of Ca^{++} in the lysing solution and keeping the initial Ca^{++} concentration of media constant at 1 mM. It is impossible to assess the free Ca^{++} concentration inside the cells by any direct method of measurement at present. Therefore, it was assumed in this case that the free Ca^{++} concentration inside the cells was in equilibrium with the lysing solution at the beginning of incubation because during the stage of hemolysis when intra- and extracellular medium were freely exchangeable, 5 min with shaking would

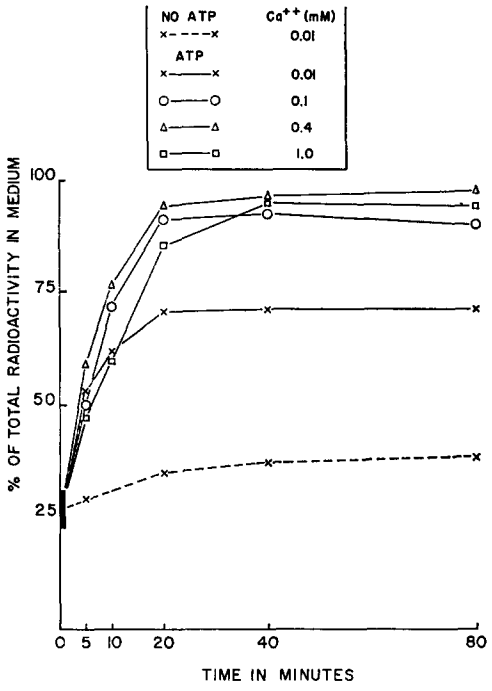

FIGURE 5. Effect of varying Ca^{++} concentrations in lysing solutions on Ca^{++} transport. Other conditions, standard. Each point, average of two experiments.

be enough to establish the equilibrium between Ca^{++} concentrations inside and outside the cells. Except for varying concentrations of Ca^{++} in lysing solutions, the standard conditions of incubation were employed. Results are shown in Fig. 5. In cells lysed in 0.01 mM Ca^{++}, about 60% of intracellular ^{45}Ca was found in the medium after 20 min incubation. On the other hand, very little increase in ^{45}Ca in the medium was found when ATP was omitted from the lysing solution, with other conditions remaining the same. This result suggests that some ^{45}Ca may be actively transported even against an extremely high concentration gradient.

With 0.1 and 0.4 mM Ca^{++} in lysing solutions, the concentration gradient ratios were 10 and 2.5, respectively, and in both cases, the ratios were over 20 after 40 min incubation, indicating that the active transport took place. These data indicate that the Ca^{++} pump mechanism is operative even when the initial Ca^{++} concentration gradient (medium:cells) is extremely high.

Effect of pH of Incubation Media

The pH of the incubation media was varied from 5 to 9.5 under standard conditions, and Ca^{++} transport was measured. As shown in Fig. 6, the range of optimum pH was 7.5–8.5. However, fairly active transport was observed at pH 5 and 9.5, indicating a wide range of hydrogen ion concentrations that permit the Ca^{++} transport mechanism to function.

Nucleotide Specificity

CTP, GTP, UTP, or ITP were used in place of ATP in the reaction mixture under the standard experimental conditions, except that the volume ratio (medium:cells) was 1. Results are shown in Fig. 7. All nucleotides were used

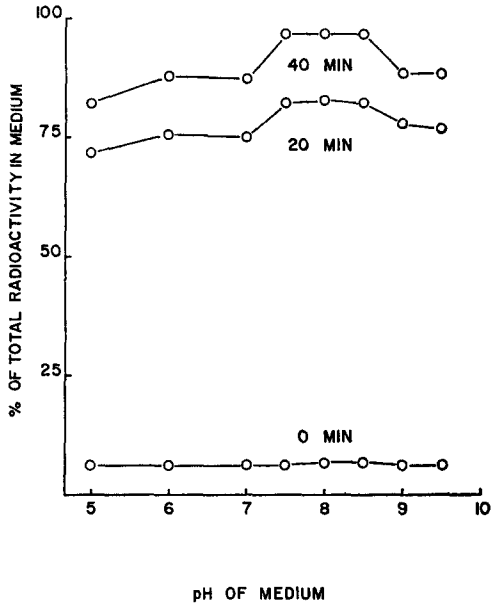

FIGURE 6. Effect of pH of incubation media on Ca^{++} transport. Other incubation conditions, standard. Each point, average of three experiments.

in a concentration of 1 mM. It was found that CTP and UTP were just as effective as ATP. GTP was somewhat less effective and ITP was the poorest among the nucleotides tested. The above results are at variance with those of Olson and Cazort (1969), who observed no difference among ITP, CTP, and ATP as energy sources for Ca^{++} transport in red cells.

Effect of High Ca^{++} Concentrations on the Transport Mechanism

The Ca^{++} concentrations of both lysing solutions and media were increased concomitantly from 1 mM to 2 or 4 mM, other conditions being standard. Results presented in Fig. 8 show that the higher the Ca^{++} concentration em-

ployed, the lower were the maximum ratios of concentration gradient established by the transport mechanism. The maximum ratios, $\frac{[Ca^{++}]_o}{[Ca^{++}]_i}$, which were established at the end of 60 min incubation were 2.1, 5.8, and 30, with Ca^{++} concentrations of 4 mM, 2 mM, and 1 mM, respectively. Also, the time required for the attainment of the maximum ratio is longer in experiments employing high Ca^{++} concentrations. It should be mentioned that with higher concentrations of Ca^{++} the amount of ATP would be the limiting factor for the extrusion of Ca^{++}.

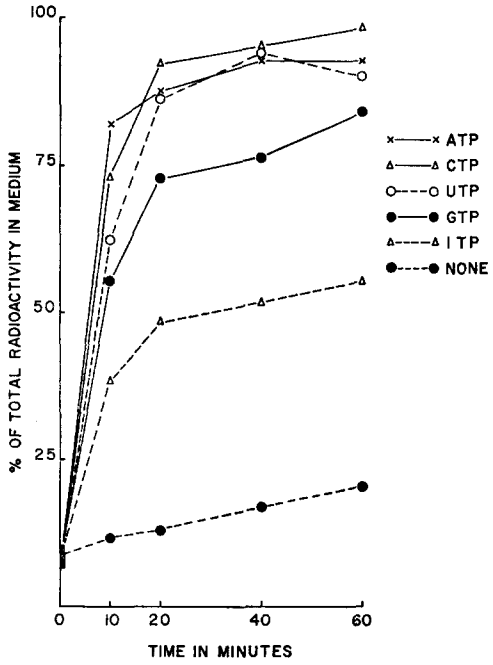

FIGURE 7. Effect of various nucleotides in lysing solution on Ca^{++} transport. Conditions of incubation, standard, except for the replacement of ATP in lysing solutions with other nucleotides and volume ratio (medium:cell) of 1. Each point, average of three experiments.

Effect of the Composition of Lysing Solution and Incubation Media

Red cells were resealed in a lysing solution in which Na^+ replaced K^+. The main intracellular cation was Na^+. When these cells were incubated under the otherwise standard conditions, these "Na^+ cells" extruded Ca^{++} just as effectively as regular "K^+ cells" (Fig. 9). In other experiments, 100 mM choline replaced all the Na^+ in the incubation medium. The Ca^{++} transport from regular K^+ cells to the choline medium occurred as effectively as under the standard conditions (Fig. 9).

Effect of Drugs

Effects of oligomycin, sodium azide, fluoride, ouabain, and digitoxin were studied. None of these drugs had a significant effect on Ca^{++} transport up to a concentration of 10^{-5} M. Drugs were added in both cells and medium.

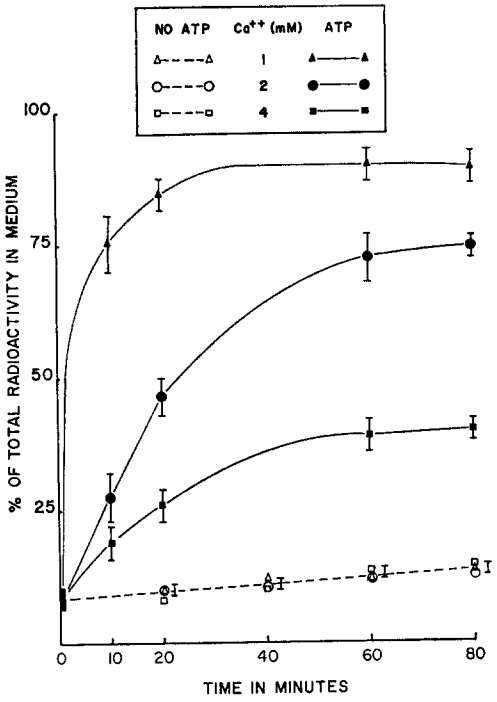

FIGURE 8. Effect of altering Ca^{++} concentrations of lysing solutions and incubation media on Ca^{++} transport. Ca^{++} (mM) 1, 2, and 4 indicate that both lysing solution and incubation medium contained 1 mM, 2 mM, and 4 mM Ca^{++}, respectively. Other conditions, standard, except volume ratio which was 1.2. Each point, average of four experiments ± SE.

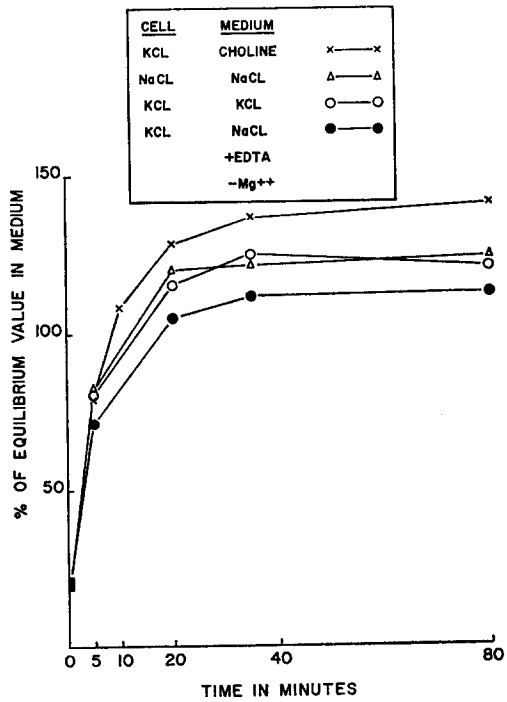

FIGURE 9. Effect of altering compositions of lysing solutions or incubation media on Ca^{++} transport. Cell indicates composition of lysing medium and medium indicates composition of incubation media. Each point, average of three experiments.

DISCUSSION

The analysis of Ca^{++} movement between ghost cells and media indicates clearly that Ca^{++} is transported from ghost cells into media against the concentration gradient. This is also supported by findings (Fig. 1, Table I) that cells containing no ATP lost only a negligible amount of ^{45}Ca and very little ^{45}Ca was found in the media. These data completely support Schatzmann and Vincenzi (1969), who reported that Ca^{++} was extruded from red cells by an active transport mechanism. However, there are important differences between our data and theirs obtained under similar experimental conditions. Their data showed that the Ca^{++} contents of cells and media were approximately 0.6 mM and 1.4 mM, respectively, in one set of experiments; and approximately 0.8 mM and 1.5 mM, respectively, in another set. These values gave concentration gradient ratios (medium:cell) of 2.33 and 1.87. The concentration ratios established after 60 min incubation in our experiments were more than 10-fold greater than these ratios. Different experimental conditions as mentioned below should be considered in this connection: (*a*) Schatzmann and Vincenzi used red cells whose endogenous ATP had been depleted by preincubation in glucose-free medium for 12–17 hr at 37°C. In our study, ATP was depleted by preincubation in a glucose-free medium containing 1 mM iodoacetate for 2 hr at 37°C. We tried many ways to deplete endogenous ATP, and the method employed by Schatzmann and Vincenzi was one of them. However, in our hands, red cells depleted of ATP in their manner transported Ca^{++} very poorly, resulting in low concentration gradient ratios, and we attributed the poor results to damage of the cell membrane during the long preincubation period, because a large amount of hemoglobin leaked out of these ATP-depleted cells during the experimental incubation. On the other hand, iodoacetate–ATP-depleted cells did not show any significant loss of hemoglobin during the experimental incubation. (*b*) We washed red cells after incubation with Ca^{++}-free solution (100 mM NaCl, 20 mM Tris-buffer, pH 7.4), while they did not wash. However, it should be mentioned that comparison of data for ^{45}Ca and Ca^{++} content for red cells washed and for those unwashed indicated a difference of less than 10% in their values. (*c*) Another factor may be the degree of removal of white cells before the use of red cells. It was known that red cells contain very little Ca^{++} (15 βmoles per liter [Harrison and Long, 1968]), and white cells (buffy layer) contain more than 20-fold more Ca^{++} than red cells. In our study with ^{45}Ca it was found that more than 100 times more ^{45}Ca was bound to the white cell layer (the buffy coat) than to the red cells. Schatzmann removed this layer during washing before experiment. However, we took extreme care to remove all white cells as thoroughly as possible after washing and centrifuging, even though sacrificing considerable amounts of red cells in the process.

This may have helped to lower the Ca^{++} values in red cells in our study. (*d*) The volume ratio (medium:cell) of average 2.01 was used in our study, and 4–5 in theirs. (*e*) 1 mM ATP was used in our study, and 2 mM in theirs. This may have resulted in retention of more Ca^{++} in the form of a Ca^{++}-ATP complex inside the cells in their study, if ATP was not used up during their 60 min incubation. These differences in experimental conditions probably account for the difference in the concentration gradient ratios.

In view of the low ratios in their study, Schatzmann and Vincenzi discussed many possibilities which could cause the Ca^{++} movement they observed without necessarily involving the active transport mechanism. After careful consideration, they concluded that red cells extruded Ca^{++} by an active transport mechanism. In the present study, the total Ca^{++} content of cells was less than one-twentieth of that found in the medium under our standard conditions. Since it is highly probable that some Ca^{++} would be bound inside cells, the Ca^{++} concentration gradient ratios (medium:cell) would be more than 20. Cells are electrically negative to medium under the present experimental conditions (Schatzmann, 1966). Thus, Ca^{++} was transported against both electrical and chemical gradients. This is also clear from ^{45}Ca flux data. Since ^{45}Ca was infused inside the cells at the beginning of the experiments, the specific activity of ^{45}Ca in the medium could not be greater than that inside the cells, and the final distribution of ^{45}Ca showed less than 5% inside the cells. Thus, the present data show unequivocally the presence of an active outward transport mechanism for Ca^{++} in the red cell membrane, which was suggested originally by Schatzmann (1966). It should be mentioned that in separate experiments we have found the Ca^{++}-activated ATPase to be about two to three times more active than the Na^{+}-K^{+}–activated ATPase.

The effect of temperature on Ca^{++} transport shows that Q_{10} is 3.19 and the activation energy is approximately 1.3 kcal/mole. As mentioned previously, these values are lower than those corresponding values reported by Schatzmann and Vincenzi (1969). The difference may be due to the different methods of calculation used, and the different ranges of temperature employed. Both these values are much higher than the activation energy of 5600 cal for ^{45}Ca efflux in heart muscle reported by Reuter and Seitz (1968). As reported previously by Schatzmann (1966), the presence of an energy source such as ATP is necessary for the transport. When red cells transported Ca^{++} without the addition of ATP to the lysing solution, it was due to the fact that the endogenous ATP was present in the cell and the amount was estimated to be of the order of 0.3 mM in those resealed cells used in this study (Fig. 3). Thus, when cells were depleted of endogenous ATP by iodoacetate treatment, practically no Ca^{++} transport took place. Mg ATP appears to be the substrate of transport ATPase (Fig. 2), but CTP and UTP are

just as effective as ATP as the energy source (Fig. 7). GTP and ITP may also be used as an energy source. However, ITP is the poorest substrate for the transport. This is at variance with the findings of Olson and Cazort (1969), who reported that GTP and ITP can support calcium transport as well as ATP can. Their data indicate that the maximum concentration gradient ratios, ($[Ca^{++}]_o$:$[Ca^{++}]_i$), attained by the transport mechanism are all below 2.5. Also, they started experiments after 10 min preincubation at 37°C. Under the standard conditions of incubation in our study, over 80% of Ca^{++} transport took place within 10 min of incubation. These factors may have obscured the nucleotide specificity under their experimental conditions.

In most of the experiments when the initial $[Ca^{++}]_o$ and $[Ca^{++}]_i$ were both around 1 mM, the concentration gradient ratios of over 30 were established in a 20 min incubation period in the presence of Mg ATP inside the cells. This assumes that all cells were resealed after 3 M KCl solution was introduced into the lysing mixture. However, if some red cells spontaneously resealed before the addition of 3 M KCl solution, then the equilibrium between free Ca^{++} concentrations inside and outside the cells may not have been attained. There is no way to assess this possibility in the present experiments. It was also noted that the active Ca^{++} transport took place even when the concentration gradient ratio ($[Ca^{++}]_o$:$[Ca^{++}]_i$) was greater than 80 at the beginning of experiments (Fig. 5). These data indicate that the Ca^{++} pump mechanism of the red cell membrane can perform a steep uphill transport work. It appears that the optimum pH range of the incubation medium is between 7.5 and 8.5.

Evidence indicates that the transport mechanism of Ca^{++} in mitochondria and sarcoplasmic reticulum involves the exchange of Ca^{++} with other cations (Engstrom and De Luca, 1964; Brierley, 1963; Chance, 1965; Carvalho and Leo, 1967). Also, it has been suggested that the Ca^{++} transport mechanism in nerve and cardiac muscle tissue may involve an exchange of Ca^{++} with Na^+ (Reuter and Seitz, 1968; Baker et al., 1969). In view of this, the possibility of exchange phenomenon between Ca^{++} and monovalent ions (Na^+, K^+, and H^+) or between Ca^{++} and Mg^{++} was considered. It was found that replacement of all monovalent ions in incubation media by choline did not influence the Ca^{++} outward transport, indicating that the presence of a concentration gradient of Na^+ or K^+ is not necessary for the transport of Ca^{++}. Also, the pH of the nonbuffered incubation medium did not change during the incubation period. This indicates that Ca^{++}-H^+ exchange does not take place during the transport processes. The addition of EDTA with no Mg^{++} added in the incubation medium did slow down the transport somewhat, but not markedly. It appears then that no exchange of Ca^{++} with monovalent ions or with Mg ions takes place during the transport process.

It also appeared that the Na^+-K^+ concentration gradient across the membrane was not needed for the Ca^{++} transport to occur, since neither the replacement of intracellular K^+ by Na^+ nor of extracellular Na^+ by K^+ under the standard experimental conditions had any influence on the Ca^{++} transport. This also indicated that no significant competition between Ca^{++} and Na^+ for the carrier system occurred at the intracellular surface of the membrane. The Ca^{++} transport system is not inhibited by oligomycin, sodium azide, and ouabain in high concentrations which influence the Ca^{++} transport mechanism in mitochondria or sarcoplasmic reticulum (Katz and Repke, 1967; Wadkins and Lehninger, 1963; Carsten and Mommaerts, 1964). In these respects, the red cell Ca^{++} pump mechanism is different from the Ca^{++} transport mechanisms in mitochondria or sarcoplasmic reticulum.

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