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STIMULATION OF CALCIUM TRANSPORT IN INSIDE-OUT VESICLES OF HUMAN ERYTHROCYTE MEMBRANES BY A SOLUBLE CYTOPLASMIC ACTIVATOR

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Summary

Transport of Ca^{2+} by inside-out vesicles requires both Mg^{2+} and ATP and can be linear over 16 min at 37°C. This basal rate of transport may be doubled however by an activator found in membrane-free erythrocyte hemolysate. This activator is probably the same protein(s) which has been shown to activate (Ca^{2+} + Mg^{2+})-ATPase in erythrocyte membrane fragments (Bond, G.H. and Clough, D.E. (1973) Biochim. Biophys. Acta 323, 592—599).

The human erythrocyte has a Mg^{2+} + ATP-dependent Ca^{2+} transport system which lowers intracellular Ca^{2+} by moving Ca^{2+} across the membrane from the protoplasmic (P) to the exoplasmic (E) surface where Ca^{2+} is released [1—4]. The combined effects of this active pumping mechanism and the presence of a small leak flux of Ca^{2+} from outside to inside the cell enables the cell to maintain steady-state intracellular concentrations of Ca^{2+} which are below 10^{-6} M [5, 6].

In the present report, inside-out vesicles were used as a model to study Ca^{2+} uptake [7, 8] and the activation of this uptake by a substance in membrane-free erythrocyte hemolysate. Vesicles were prepared from washed cells 1—28 days old using the method of Steck and Kant [9]. Sealed vesicles were removed from the Dextran barrier and washed once in approximately 20-fold 0.5 mM EDTA/10 mM Tris, pH 7.6, and centrifuged at 28 000 × *g* for 30 min. The membranes were then washed a second time in 10-fold or more 10 mM Tris, pH 7.6, and centrifuged again as described above. The pellets from this second wash were diluted to 3—4 mg protein/ml in 10 mM Tris, pH 7.6, and combined for storage at 4°C. An aliquot of this suspension was assayed for sidedness and for protein. In the experiments reported, inside-out

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

vesicle preparations ranged from 70—80% pure based on the inaccessibility of acetylcholinesterase [9, 10]. Protein was measured by the method of Lowry et al. [11] using bovine serum albumin as a standard. Ca^{2+} flux experiments were performed the third day after starting a preparation. Measurements were obtained in triplicate using plastic incubation tubes. We began an assay by adding a sample of prewarmed vesicles (1 mg protein/ml in 10 mM Tris, pH 7.6) to prewarmed medium to give a final composition of 132 μg vesicle protein/ml, 60 mM NaCl, 1.5 mM $MgCl_2$, 1.5 mM ATP, 0.1 mM $CaCl_2$, 0.05 μCi ^{45}Ca/ml, 40 mM Tris, pH 7.6, unless otherwise noted. Preincubation and experimental temperatures were 37°C. Preincubation lasted 5—10 min.

Vesicles were separated from the medium by filtration under vacuum using 0.3 μm Millipore filters. Negative pressure was maintained at approximately 280 mmHg by an electric vacuum pump. Filters were prewashed with 4 ml of ice-cold 200 mM sucrose/5 mM Tris, pH 7.5. Following filtration of 0.5 ml of incubation medium onto the prewashed filters, vesicles were rapidly washed with 4 ml of the same ice-cold sucrose/Tris solution. When the filtration procedure was completed (approx.35 s for a triplicate determination), filters were placed into liquid scintillation vials for counting. In order to determine time-dependent Ca^{2+} uptake, counts in time zero samples taken just after the addition of vesicles to the incubation medium were subtracted from all samples taken after time zero. In the case of Mg^{2+} + ATP-dependent uptake, counts in time zero samples represent 30—40% of the total counts seen after 4 min incubation.

We prepared hemolysate from whole cells washed three times with 10 vols. of 175 mM Tris, pH 7.5. 2 ml of packed cells were then hemolysed in 38 ml of ice-cold deionized H_2O and centrifuged at 28 000 × g for 30 min [16].

Inorganic chemicals were reagent grade or better. Organic chemicals were from commercial sources. ATP was from Sigma or Boehringer. The water used to make solutions was deionized to purities in excess of 1 M Ω/cm and was filtered to remove bacteria.

Fig. 1 shows the uptake of Ca^{2+} into the vesicles over a 16-min period. The upper three lines show Mg^{2+} + ATP-dependent Ca^{2+} uptake observed in three different membrane preparations. It is not clear why this degree of variation in Ca^{2+} uptake activity is found. This may reflect a variation between different samples of blood or between different preparations. The two points shown closest to the abscissa after 16 min incubation indicate typical Ca^{2+} uptake seen in the absence of Mg^{2+} or ATP. Other experiments not shown indicate that Mg^{2+} + ATP-dependent Ca^{2+} accumulation is almost completely prevented by the Ca^{2+} ionophores X-537A or A 23187 and that the ionophores are active whether they are added at the start or the end of an incubation period. Experiments also show that Mg^{2+} + ATP-dependent Ca^{2+} uptake is essentially unaffected if the usual washing solution is made 1 mM in $CaCl_2$ or 0.5 mM in EGTA.

Fig. 2 shows the effect of the addition of increasing amounts of membrane-free hemolysate to the assay system on Ca^{2+} uptake. This additional uptake responds similarly to that observed in the absence of hemolysate. It is not abolished if the washing solution is made 1 mM in $CaCl_2$ or 0.5 mM in

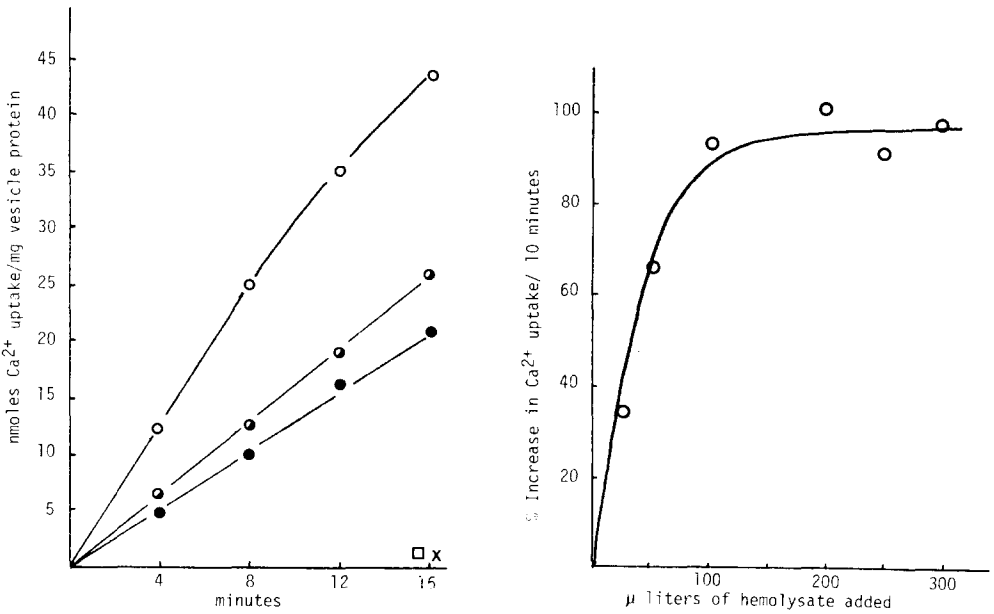

Fig. 1. Ca^{2+} uptake with time. ○, preparation 1, complete system; ◑, preparation 2, complete system; ●, preparation 3, complete system; X, preparation 1, minus ATP; □, preparation 1, minus Mg^{2+}.

Fig. 2. Activation of Ca^{2+} uptake by hemolysate. The curve was drawn by eye and the final incubation volume was 1.5 ml (one experiment).

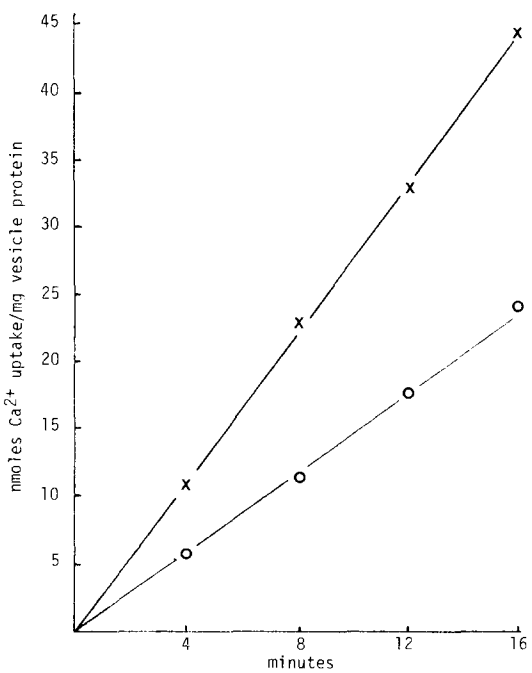

Fig. 3. Stimulated Ca^{2+} uptake with time. X, complete system plus 500 μl of hemolysate; ○, complete system. The final incubation volume was 3 ml with 93 μg vesicle protein/ml. Other conditions were normal. (One of two similar experiments).

EGTA and no uptake is observed in the absence of Mg^{2+}. While vesicles will take up substantial amounts of Ca^{2+} in the presence of hemolysate in the absence of exogenous ATP, this effect apparently represents ATP contamination in the hemolysate because such uptake is not observed in an assay system using hemolysate made from ATP-depleted cells. We have ruled out significant membrane contamination in hemolysate since samples from experimental tubes with the incubation mixture plus hemolysate, but without added inside-out vesicles do not increase ^{45}Ca counts on Millipore filters with time. In addition, no membranes are observed in hemolysate fractions at 1000× with the phase contrast microscope, although this is a magnification at which inside-out vesicles can normally be observed.

Fig. 3 shows the rate of Ca^{2+} uptake by the vesicles over an incubation period in the presence and the absence of hemolysate. This result suggests that hemolysate does not simply make vesicles less leaky for transported Ca^{2+} since one would not then expect hemolysate to accelerate the initial rate of Ca^{2+} uptake.

It appears that membrane-free hemolysate activates Ca^{2+} uptake in a manner similar to the effect of hemolysate activator on (Ca^{2+} + Mg^{2+})-ATPase [12—16]. Moreover, the activator of Ca^{2+} uptake does not appear to pass PSAC Millipore filters (nominal molecular weight limit, 1000), can be partially purified by a modification of the method of Luthra et al. [16] and is sensitive to prolonged boiling.

We have also observed that the source of ATP is not critical. In two experiments not shown, the results of a transport assay were compared using Boehringer ATP (lot 423502) as an energy source with the results obtained when Sigma ATP (lot 125C-7470) was used instead [17—19]. Under our standard conditions, both sources of ATP equally support Ca^{2+} transport whether in the presence or the absence of the activator.

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