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CHARACTERISTICS AND REGULATION OF ACTIVE CALCIUM TRANSPORT IN INSIDE-OUT RED CELL MEMBRANE VESICLES *

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Summary

Sealed, inside-out human red cell membrane vesicles, prepared by a modified method of Steck (Steck T.L. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), Vol 2, pp. 245—281, Plenum Press, New York), accomplish an ATP and Mg^{2+}-dependent uphill calcium uptake with a reproducible maximum rate of 12—15 nmol/mg vesicle protein per min under physiological conditions. This maximum rate is increased by about 60—70% in the presence of a heat-stable cytoplasmic activator protein (calmodulin) obtained from red cells. Calcium efflux from inside-out vesicles is smaller than 0.01 nmol/mg vesicle protein per min at intravesicular calcium concentrations between 0.1 and 20.0 mM.

In the presence of Mg^{2+}, active calcium uptake is supported by ATP, ITP, or UTP, but not by ADP, AMP, or *p*-nitrophenyl phosphate. The optimum pH for the process is 7.4—7.6, and the activation energy is 19—20 kcal/mol, irrespective of the presence or absence of calmodulin. Calcium uptake in inside-out vesicles is unaffected by ouabain or oligomycin, but blocked by low concentrations of lanthanum, ruthenium red, quercetin and phloretin. K^+ and Na^+, when compared to choline$^+$ or Li^+, significantly increase active calcium uptake. This stimulation by K^+ and Na^+ is independent of that by calmodulin.

Concentrated red cell cytoplasm activates calcium uptake at low soluble protein : membrane protein ratios, while a 'deactivation' of the transport occurs at high cytoplasm : membrane protein ratios. A heat-labile cytoplasmic protein fraction antagonizing calmodulin activation, can be separated by

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Abbreviation: SDS, sodium dodecyl sulfate.

DEAE-Sephadex chromatography. Based on these findings the regulation of active calcium transport in human red cells is discussed.

Introduction

ATP and Mg^{2+}-dependent active calcium extrusion from human red cells is a basic function of the red cell membrane, as a small increase in intracellular calcium concentration is harmful to most cellular functions (see Refs. 1 and 2). Moreover, red cell calcium transport may serve as a model for studying calcium movement in various plasma membranes.

Preparation of sealed, inside-out red cell membrane vesicles [3] provides a new approach for investigating active calcium transport and its regulation by calcium, magnesium, ATP, monovalent ions, and cytoplasmic proteins, reacting with the original internal (and in the inverted vesicles external) membrane surface [4—11]. However, since the protein composition and the transport properties of the vesicles depend considerably on the actual way of the membrane preparation, the characteristics of active calcium transport in inside-out vesicles, as found in different laboratories, are at considerable variance. For example, while in most preparations cytoplasmic proteins significantly stimulate active calcium uptake, Quist and Roufogalis [8] reported the absence of such an effect in their inside-out vesicle preparation. The present paper describes the basic features of inside-out vesicles which are prepared by a simple technique providing high yield without a dextran gradient separation. These vesicles have a high calcium transport capacity with a good experimental reproducibility. The vesicles, due to the presence of low concentration of EDTA (20 μM) during inside-out vesicle formation, are strongly depleted of 'loosely bound' internal membrane proteins, and are especially apt for studying the regulation of active calcium movement.

Materials and Methods

All the chemicals used were of analytical grade. Deionized water and solutions without added calcium contained less than 3 μM calcium.

Inside-out vesicles were prepared on the basis of the method of Steck [3] as follows: Freshly drawn human red cells were washed three times with 150 mM NaCl + 10 mM Tris-HCl (pH 7.4) and then hemolyzed in 8 vols. of the following solution: 20 mM KCl, 20 mM sucrose, 10 mM Tris-HCl (pH 7.4). After centrifugation at 20 000 $\times g$ for 20 min, the sediment was washed four times with a ten times dilution of the hemolyzing solution (centrifugations at 20 000 $\times g$, 20 min). The obtained hemoglobin-free membrane was immediately diluted in 40 vols. of 0.5 mM Tris-HCl + 50 μM β-mercaptoethanol + 20 μM Tris-EDTA (pH 8.5); incubated for 30 min at 4°C and for 15 min at 37°C, sedimented at 20 000 $\times g$ for 30 min and washed once with the same volume of the above 0.5 mM Tris-HCl buffer. The sediment was homogenized by a 27 gauge needle, and washed twice with 10 vols. of 10 mM Tris-HCl (pH 7.4, 10 min centrifugations at 20 000 $\times g$). The sediment was resuspended to obtain a 4—5 mg protein/ml solution in 0.16 M KCl (or other 0.16 M salt solution)

buffered with 5 mM Tris-HCl to pH 7.4. The inside-out vesicles obtained were either used for the transport experiments within 24 h after the preparation, or 0.2—0.5 ml batches of the concentrated vesicle suspension were stored at —30°C. After one single refrigeration and thawing of the vesicles, there was no significant change in the percent of the sealed vesicles in the preparation and the calcium-transporting capacity of the vesicles was unchanged for 3—4 weeks in the refrigerated preparations. (A considerable loss of the calcium-transporting capacity and in the response to the protein activator was observed in inside-out vesicles stored at 4°C longer than 24 h.)

Membrane sidedness and the ratio of sealed vesicles were determined by measuring latent acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase activities as described by Steck [3]. The mean diameter of the vesicles was estimated by phase-contrast microscopy, and their mean volume was determined by measuring [^{14}C]inulin or [^{14}C]AMP distribution (supposing no penetration of these substances into sealed vesicles).

Calcium influx into inside-out vesicles was measured by the Sartorius membrane filtration method as described in Refs. 6 and 9.

Cytoplasmic protein extracts were prepared from NaCl-washed red cells hemolyzed in 10 vols. of distilled water. The membrane was sedimented at 20 000 × *g* for 20 min, and the hemolyzate supernatant was adjusted to pH 6.5 with 1 M HCl. Based on the method of Luthra et al. [12,13] hemoglobin from the supernatant was eliminated by a batch adsorption with CM-Sephadex C-50 at pH 6.5, and the resulting cake was washed with 16 mM imidazole-HCl (pH 6.5) in a coarse-sintered glass funnel under vacuum. The non-adsorbed proteins could be concentrated about 100 times, by repeating the CM-Sephadex treatment and the obtained yellow solution was centrifuged for 30 min at 20 000 × *g* to remove the contaminating membrane fragments. The hemoglobin-free cytoplasmic protein extract was dialyzed for 24 h against 40 vols. of 150 mM KCl + 10 mM Tris-HCl (pH 7.4) and stored frozen. Any further concentration of the membrane-free hemolyzate or the protein extract was carried out by an Amicon ultrafiltration system (Model 8 MC).

DEAE-Sephadex A-50 treatment for a partial purification of the activator protein was carried out based on the method of Jarrett and Penniston [14,15]. 5 ml of cytoplasmic protein extract in 16 mM imidazole-HCl (pH 6.5) was applied to a DEAE-Sephadex A-50 column (1.5 × 10 cm) equilibrated with the same buffer. The concentration of KCl was changed to 0.16 M and the eluted proteins were collected. After washing the column with about three column volumes of this KCl solution the KCl concentration was changed to 0.32 M. The previous procedure was repeated with this KCl solution and with a following 0.64 M KCl solution (KCl was always buffered with 16 mM imidazole-HCl, pH 6.5). The protein peaks eluted at 0.16, 0.32 and 0.64 M KCl concentrations were dialyzed against KCl/Tris buffer (pH 7.4). The 0.64 M KCl-eluted proteins contained the partially purified activator, while the 0.32 M KCl eluted fraction inhibited the protein activation of the calcium transport in inside-out vesicles (see Results). The 0.64 M KCl-eluted protein fraction was incubated in a boiling waterbath for 3 min, the precipitated proteins were sedimented at 5000 × *g* for 10 min, and the supernatant, containing the heat-stable activator protein, was used for stimulating active calcium transport.

SDS-polyacrylamide gel electrophoresis was carried out by M. Hasitz, according to the method of Shapiro et al. [16]. Protein concentration was determined by the method of Lowry et al. [17].

Results

When we examined the protein composition of the ghosts and inside-out vesicles by SDS-polyacrylamide gel electrophoresis, as it was also shown by Steck [3], the most characteristic changes caused by the hypotonic, calcium-free treatment of the ghosts were the almost complete loss of spectrin and hemoglobin (band 7), and a depletion in membrane actin (band 5). It is to be mentioned, that a depletion in glyceraldehyde-3-phosphate dehydrogenase (band 6) was observed only in case of washing the inside-out vesicles in media with elevated ionic strength.

The basic characteristics of our inside-out red cell membrane vesicle preparation can be summarized as follows: The preparation contains 72—78% of inside-out vesicles, 6—10% of right-side-out vesicles, and 12—22% membrane fragments. Acetylcholinesterase activity in the disrupted vesicles (total activity) is 2.5—2.7 μmol/mg protein per min, as measured at 37°C, pH 7.4. The mean diameter of the vesicles by phase-contrast microscopy was estimated to be 1.2—1.5 μm, resulting in a mean volume for spherical bodies of 7—14 μm^3. The mean vesicle volume obtain by [^{14}C]inulin distribution measurements is 9—11 μl/mg vesicle protein. The maximum rate of ATP and Mg^{2+}-dependent calcium uptake in the vesicle preparation is highly reproducible, in the range of 12—14 nmol/mg vesicle protein per min, at 250 μM calcium concentration in the medium. The heat-stable activator protein increases this maximum transport rate of 20—25 nmol/mg vesicle protein per min. The calcium concentration producing half-maximum stimulation of active calcium uptake (K_{Ca}) is 40—45 μM in the control vesicles, and 10—15 μM in the presence of the activator protein, respectively.

In Fig. 1 we demonstrate the time course of the (ATP + Mg^{2+})-dependent calcium accumulation in the control vesicles and in the presence of the partially purified red cell cytoplasmic activator protein. In both cases there is no measurable calcium uptake if either ATP or Mg^{2+} is absent in the media, and active calcium uptake is stopped by the addition of divalent cation-chelating agents, such as EDTA or EGTA, to the medium. In the presence of these non-penetrating chelators there is no significant loss of calcium from the vesicles, while upon the addition of the calcium ionophore A23187 a complete release of the vesicular calcium is produced. These experiments show an uphill accumulation of calcium by inside-out vesicles and, as it can be calculated by using the figures for vesicle volume : protein ratios, intravesicular calcium may be increased up to 10—15 mM without significant decrease in the rate of active calcium uptake. At higher intravesicular calcium levels the rate of calcium uptake decreases and an increase in calcium leakage occurs.

In the following experiments we measured the metabolic substrate dependence of active calcium transport in inside-out vesicles. As it is shown in Fig. 2 ATP and ITP both can be used as energy donors for active calcium uptake, while *p*-nitrophenyl phosphate and AMP are ineffective in this respect. The

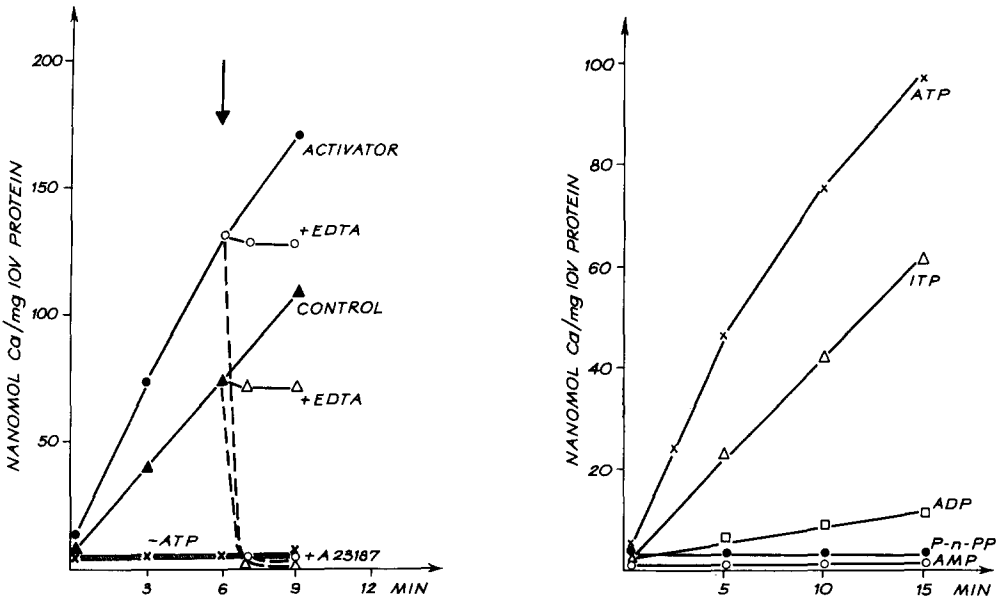

Fig. 1. Active calcium uptake by inside-out red cell membrane vesicles. The incubation media contained 120 mM KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 100 μM $CaCl_2$ ($+^{45}Ca^{2+}$ tracer), and 0 to 1 mM ATP. At the time indicated by the arrow, the media were supplemented with 1 mM EDTA or 5 μM A23187 calcium ionophore. The 'activator' was a DEAE-Sephadex partially purified activator protein from human red cells. Temperature 37° C. Concentration of inside-out vesicle (IOV) protein 20—30 μg/ml medium.

Fig. 2. Effects of nucleotides and *p*-nitrophenyl phosphate (p-n-PP) on the calcium uptake by inside-out red cell membrane vesicles (IOV). The substrates were added in 1 mM concentrations. For the incubation conditions see the legend of Fig. 1.

small calcium uptake produced by ADP is most probably the result of some adenylate kinase activity retained in the vesicle membrane and producing ATP + AMP from ADP. The possibility that the effect of ITP is produced by its conversion to ATP by the enzyme nucleotide diphosphokinase (catalysing the reaction ITP + ADP $\rightleftharpoons$ IDP + ATP) can be most probably excluded, since ADP is absent in the media and addition of ADP to the system does not increase the rate of calcium uptake when the substrate is ITP (data not shown). Table I demonstrates the changes in the rate of active calcium uptake in the presence of ATP, ADP, AMP and ITP, when the media are completed with a hemoglobin-free red cell cytoplasmic protein extract, or by the partially purified activator protein. As it is shown, the percent stimulation of calcium uptake by protein activators is about the same in the presence of ATP and ITP. The large increase in calcium uptake produced by ADP + cytoplasmic protein extract is explained by the adenylate kinase activity in this preparation.

Fig. 3 shows the rate of active calcium accumulation in inside-out vesicles as a function of ATP and ITP concentrations in the media, with or without the cytoplasmic activator protein. The V of the calcium uptake is similar at both energy donor substrates, whereas K_{ATP} is below 50 μM and K_{ITP} is greater than 1 mM, both in the presence or absence of the protein activator.

TABLE I

EFFECTS OF NUCLEOTIDES ON THE ACTIVE CALCIUM UPTAKE BY INSIDE-OUT RED CELL MEMBRANE VESICLES

Calcium uptake was measured in media containing 120 mM KCl, 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 100 μM $CaCl_2$ (+ $^{45}Ca^{2+}$ tracer) and 1 mM of the nucleotide indicated, at 37°C. Inside-out vesicle (IOV) concentration: 20—30 μg IOV protein/ml medium. Figures are presented as mean ± S.D., $n = 6$ in each case, CPE, cytoplasmic protein extract.

Nucleotide (1 mM)	Calcium uptake in IOVs (nmol · mg^{-1} IOV protein per min)		
	Control IOV	+ CPE	+ partially purified activator
AMP	0.5 ± 0.2	0.8 ± 0.3	0.6 ± 0.3
ADP	2.1 ± 0.9	19.2 ± 1.8	3.8 ± 0.6
ATP	12.6 ± 1.1	20.8 ± 1.2	22.4 ± 1.6
ITP	7.1 ± 0.8	11.5 ± 1.0	12.6 ± 1.1
UTP	6.4 ± 0.9	10.5 ± 1.1	11.5 ± 1.0

In the following we analyzed the pH dependence (Fig. 4), the temperature dependence (Fig. 5), and the drug sensitivity (Table II) of the active calcium uptake by inside-out vesicles. We found a fairly broad pH maximum of the process with the peak around pH 7.3—7.5 in the control vesicles, and a slight

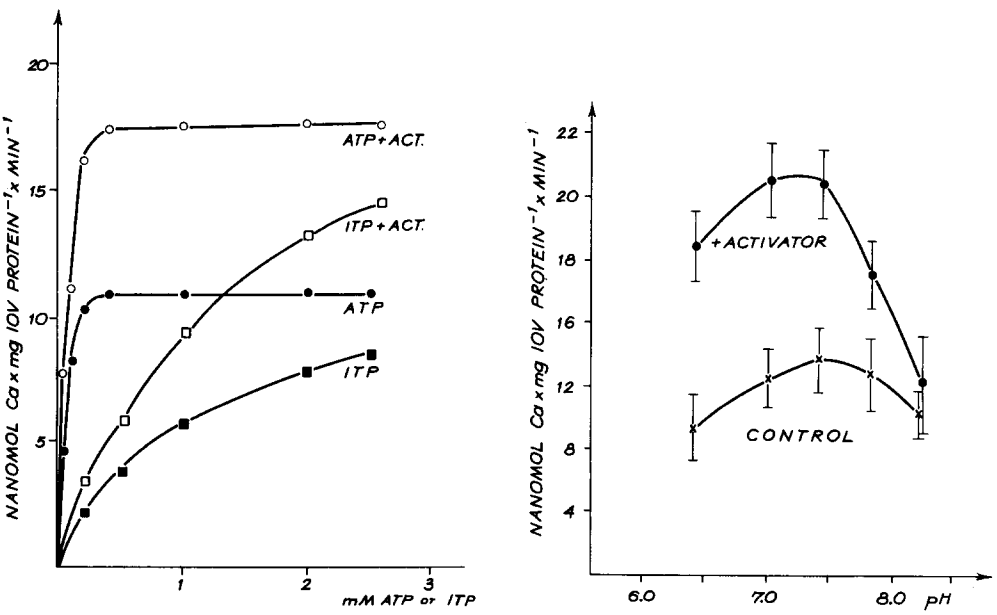

Fig. 3. Dependence of active calcium uptake by inside-out red cell membrane vesicles (IOV) on the ATP and ITP concentration in the medium. The activator (ACT) was partially purified from red cells by DEAE-Sephadex chromatography. For the incubation conditions see the legend of Fig. 1.

Fig. 4. Effect of pH on the rate of active calcium uptake by inside-out red cell membrane vesicles (IOV). The incubation media contained 120 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 100 μM $CaCl_2$ (+$^{45}Ca^{2+}$ tracer) and 20 mM Tris-HCl adjusted to the pH indicated. The activator was partially purified from red cells by DEAE-Sephadex chromatography. Temperature 37° C.

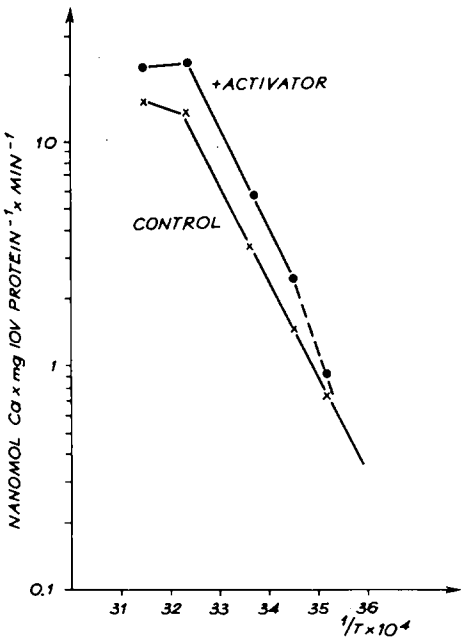

Fig. 5. Effect of temperature on the rate of active calcium uptake by inside-out vesicles (IOV). For the activator and the composition of the incubation medium see the legend of Fig. 1. ATP: 1 mM.

shift of this maximum to acidic regions in the presence of the cytoplasmic activator protein. The most characteristic change in active calcium uptake at pH values greater than 7.8—8.0 is the almost complete loss of its activation by cytoplasmic proteins. The activation energy of the calcium uptake in inside-out vesicles was found to be 19.2 kcal/mol, as a mean of four experiments of this kind, and no significant change in this value was brought about by the activator protein. A loss of protein activation was observed at incubation temperatures lower than 15°C.

TABLE II

EFFECT OF DRUGS ON THE ACTIVE CALCIUM UPTAKE BY INSIDE-OUT RED CELL MEMBRANE VESICLES

For the incubation conditions see Table II. The media contained 0.5 mM ATP and the drugs in the concentrations indicated.

Drugs without effect	Highest concentration applied	Inhibitors		
			Concn. (M)	% inhibition
Ouabain	10^{-3} M *	Lanthanum	10^{-6}	100
Oligomycin	20 μg/ml	$HgCl_2$	10^{-6}	100
Rutin, hesperidin	10^{-3} M	Quercetin	10^{-4}	95
		Phloretin	10^{-4}	95
		Ruthenium red	10^{-5}	95
		p-Chloromercuribenzoate, salyrgan	$2–5 \cdot 10^{-4}$	95

* Also tested by incorporating into inside-out vesicles during preparation.

As it is presented in Table II, ouabain (even if incorporated into the vesicles) and oligomycin are without effect on active calcium uptake. La^{3+} and Hg^{2+}, when reacting with the cytoplasmic surface of the red cell membrane, block calcium transport in concentrations as low as 10^{-6}—10^{-7} M. Ruthenium red, quercetin and phloretin are also powerful inhibitors of active calcium uptake, while several water-soluble flavonoids proved ineffective in this respect. SH reagents inhibit calcium uptake and also cause an increased calcium leakage from the vesicles.

As we previously reported [9], active calcium transport in inside-out vesicles is significantly stimulated by monovalent cations, among which the most effective are Na^+ and K^+. In Table III we show the effect of the cytoplasmic protein activator on active calcium uptake in media containing various monovalent cations. In these experiments we found the percent activation of calcium uptake by the cytoplasmic proteins to be independent of the given salt composition of the medium.

When investigating the effects of the red cell cytoplasmic proteins on active calcium uptake by inside-out vesicles, we used three different soluble protein preparations: (a) concentrated supernatant of the membrane-free hemolyzate; (b) hemoglobin-free cytoplasmic protein extract, and (c) a partially purified heat-stable activator protein. As we demonstrate in Fig. 6, calcium uptake is activated by the concentrated supernatant or by cytoplasmic protein extract at low soluble protein : membrane protein ratios, while there is a 'deactivation' of the process at high protein ratios. (In various experiments the correlation was more pronounced if the soluble protein : membrane protein ratios, rather than only the soluble protein concentrations were plotted.) There is no such deactivation, or loss of activation, if the partially purified activator protein is applied. As can be seen, at physiological membrane : cytoplasmic protein ratios the activating effect of the red cell cytoplasm is almost negligible. These experiments indicate either a strong calcium binding or a specific inhibition of active calcium transport by cytoplasmic proteins of the red cells. In order to further investigate this problem we tested the effects of various proteins on the active

TABLE III

EFFECTS OF ALKALI CATIONS AND THE ACTIVATOR PROTEIN ON THE RATE OF ACTIVE CALCIUM UPTAKE BY INSIDE-OUT RED CELL MEMBRANE VESICLES

The rate of calcium uptake is in nmol · mg^{-1} inside-out vesicles protein per min. The given salt solution is 120 mmol, supplemented with 20 mM Tris-HCl, (pH 7.4), 5 mM $MgCl_2$, 1 mM ATP and 100 μM $CaCl_2$ ($+^{45}Ca^{2+}$ tracer). Temperature 37°C. Figures are presented as mean ± S.D. $n = 5$. Protein activator is partially purified by DEAE-Sephadex chromatography.

Salt	Control		+ Protein activation		% protein activation
	Rate of calcium uptake	% activation by cation	Rate of calcium uptake	% activation by cation	
Choline chloride	7.1 ± 0.9	—	11.6 ± 1.0	—	63
LiCl	6.6 ± 0.7	—7	11.0 ± 0.9	—5	67
KCl	11.9 ± 0.8	67	19.3 ± 1.3	66	62
NaCl	10.9 ± 0.9	53	18.4 ± 1.1	59	69

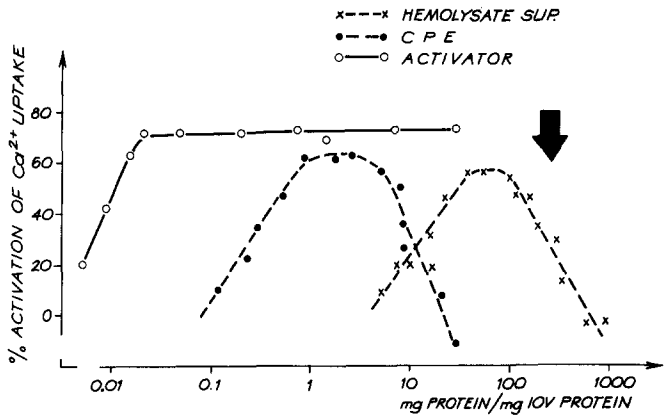

Fig. 6. Effects of red cell soluble proteins on the rate of active calcium uptake by inside-out red cell membrane vesicles (IOV). Hemolyzate supernatant, hemoglobin-free cytoplasmic protein extract (CPE), and activator (partially purified by DEAE-Sephadex chromatography), were prepared as described in Materials and Methods. For the incubation conditions see the legend of Fig. 1. ATP concentration: 1 mM.

calcium uptake in control vesicles and in vesicles treated with the partially purified protein activator (Table VI). In agreement with the findings shown in Fig. 6, there was no significant effect of hemoglobin on calcium uptake, while cytoplasmic protein extract in high concentrations abolished the protein activation. High concentrations of albumin caused some decrease in the rate of calcium uptake but did not alter the percent activation by the partially purified activator protein. By using a protein preparation, fractionated by DEAE-Sephadex chromatography, we could produce a specific elimination of the protein activation of calcium uptake. When investigating the kinetics of active calcium transport in inside-out vesicles (Fig. 7), the increase in V and decrease in K_{Ca} caused by the activator protein could be reversed by high concentrations of cytoplasmic protein extract or by the DEAE-separated 'inhibitor' fraction.

TABLE IV

EFFECTS OF SOLUBLE PROTEINS ON THE ACTIVE CALCIUM UPTAKE BY INSIDE-OUT RED CELL MEMBRANE VESICLES

For the incubation media and conditions see the legend of Fig. 1. Inside-out vesicle (IOV) concentration 40 μg/ml medium. CPE, cytoplasmic protein extract. Figures are presented as mean ± S.D., n = 5.

Additions	Rate of calcium uptake (nmol · mg^{-1} inside-out vesicle protein per min)	
	Control	+ Partially purified activator protein
None	12.6 ± 0.8	20.1 ± 1.3
Hemoglobin 40 mg/ml (100 mg/mg IOV)	11.8 ± 1.0	19.6 ± 1.4
Human albumin 5 mg/ml (125 mg/mg IOV)	8.8 ± 0.7	14.7 ± 0.9
CPE 2.8 mg/ml (70 mg/mg IOV)	9.6 ± 1.1	10.8 ± 1.0
Inhibitor protein 0.1 mg/ml (2.5 mg/mg IOV)	9.0 ± 0.7	9.4 ± 0.9

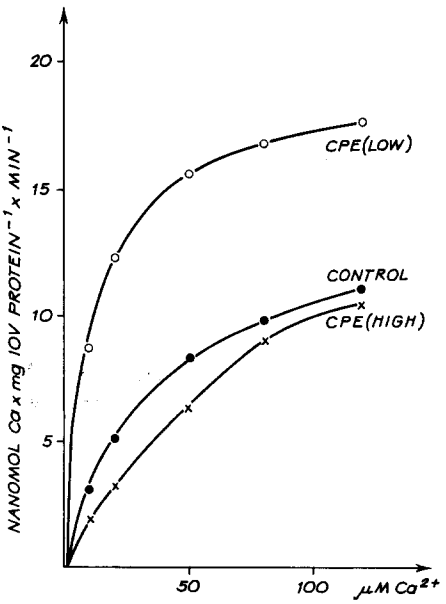

Fig. 7. Effect of hemoglobin-free cytoplasmic protein extract (CPE) on the kinetics of active calcium uptake by inside-out red cell membrane vesicles (IOV). For the incubation conditions see the legend of Fig. 1. ATP concentration: 1 mM. CPE (low): 2 mg/ml inside-out vesicle protein; CPE (high): 30 mg/ml inside-out vesicle protein.

Discussion

In this paper we describe a modified method of Steck [3] for inside-out vesicle preparation and characterize the obtained vesicles in several aspects. The present method has the advantage of simplicity, high yield, and production of vesicles with high calcium-transporting capacity and good reproducibility. The use of low concentration of EDTA in the vesicle preparation media eliminates the possible damage to the calcium pump caused by extremely low concentrations of certain heavy metals in the reagents. The procedure does not require the dextran gradient separation of the inside-out vesicles (dextran gradient centrifugation in our hands caused a significant loss of calcium transport capacity of the vesicles).

The inside-out vesicles show an uphill active calcium uptake which corresponds in its magnitude to the active calcium extrusion in intact red cells, and the low passsive permeability of the vesicle membrane for calcium as well as for EDTA or EGTA (Fig. 1) shows the preservation of the physiological characteristics of the red cell membrane in this preparation. Based on the values for acetylcholinesterase activity (as a tightly bound marker for the membrane surface area), the 12—15 nmol calcium/mg vesicle protein per min maximum calcium transport rate equals about 45—60 μmol/l of red cells per min calcium extrusion rate in the intact red cells. This rate is about 60—75% of that measured in red cells loaded with calcium by the A23187 ionophore method [18,19]. If we regard the rate of calcium uptake in inside-out vesicles measured in the presence of the cytoplasmic activator protein (20—25 nmol/mg vesicle

protein per min), the calculated rate for red cells (80—100 μmol/l of cells per min) is actually somewhat higher than experimentally found.

The stimulation of active calcium uptake in inside-out vesicles by red cell cytoplasmic proteins was first observed by Macintyre and Green [5] and demonstrated later by several research groups [6—8,10]. In studying the features of calcium uptake in inside-out vesicle we used a partially purified protein activator to evaluate the activator-dependent changes in the characteristics of this transport process. It could be demonstrated that both ATP and ITP can be used as energy donor substrates of active calcium transport, although the affinity of the system to ATP is about 20—30 times greater than to ITP. The percent activation observed in the presence of the cytoplasmic proteins was found to be independent of the actual nucleotide substrate used to support calcium uptake. The pH and temperature dependence of active calcium uptake in inside-out vesicles is similar to that observed in ghosts and in intact red cells [19—21], and these characteristics are not significantly influenced by the cytoplasmic activator protein.

When examining the effects of various drugs on active calcium uptake, we could show that the process is insensitive to ouabain, oligomycin and several water-soluble flavonoids, while it is inhibited by the flavonoid quercetin and its 'chemical relative', phloretin. Wüthrich and Schatzmann first showed [22] that quercetin is a potent inhibitor of active calcium uptake in inside-out red cell membrane vesicles and of $(Ca^{2+} + Mg^{2+})$-ATPase in the red cell membrane, and its potency to block calcium transport is somewhat higher than to inhibit active $Na^+ + K^+$ transport. Phloretin is known to inhibit several transport processes in the red cell membrane (see Ref. 2). A high effectiveness in blocking active calcium movement by lanthanum, mercury and ruthenium red is observed in inside-out vesicles, when these agents can react with the cytoplasmic membrane surface, although they possibly block any calcium-dependent membrane function under these conditions.

In the course of studying the regulation of active calcium transport in inside-out red cell membrane vesicles, we examined the combined effects of monovalent cations and the cytoplasmic protein activator on this process. As we previously reported [9], K^+ and Na^+ when compared to choline$^+$, Tris$^+$, or Li^+, significantly stimulate calcium uptake. This stimulation is a saturable function of the K^+ or Na^+ concentration, with a K_{act} of 30—40 mM for both ions. As it is demonstrated in the present report, this K^+ and Na^+ stimulation is independent of the presence of the activator protein, and the percent activation by the cytoplasmic protein is about the same in any of the salt solutions examined. In order to further characterize the protein regulation of active calcium transport, we examined the effects of the red cell cytoplasmic proteins at various soluble protein : membrane protein ratios. As it is shown in Fig. 6 and Table IV, the same cytoplasm preparation can both activate and 'deactivate' calcium uptake. In a previous paper we showed [9] that the cytoplasmic protein activator decreases K_{Ca} and does not alter K_{ATP} of the active calcium uptake. By now it is well documented that the red cell calcium transport activator is a similar (or the very same) protein as the calcium-dependent regulator (calmodulin) shown to regulate various functions in various tissues (see Refs. 7, 10, 23 and 24). This is a low molecular weight (16 000—18 000),

acidic, heat-stable protein, transferring calcium sensitivity to various enzymes. As it is to be reported elsewhere [25] we also found activation of the calcium transport in inside-out vesicles by heat-stable proteins isolated from carp oocytes and sperm, or from crude extracts of rabbit muscle cytoplasm (data not shown). Recently Larsen et al. [26] reported that a calmodulin-binding protein, characterized by Wang and Desai [27] prevented the activation of the calcium uptake in inside-out vesicles by red cell proteins or by exogeneous calmodulin. Au [28] showed the presence of a heat-labile endogenous inhibitor of the $(Ca^{2+} + Mg^{2+})$-ATPase in the red cell membrane. Our present experiments indicate the existence of a heat-labile protein(s) in the red cell cytoplasm which counteract(s) the protein activation of this system. It is unlikely that the observed effect is simply a consequence of some calcium binding by the proteins added, as small amount of the proteins separated by DEAE-Sephadex chromatography can produce a similar effect. Still, the question is clearly open, what kind of a membrane-activator-inhibitor interaction may cause the observed biphasic behaviour in the stimulation of calcium uptake at various soluble protein : membrane protein ratios. It is evident, that a simple activator/activator-binding protein interaction cannot be responsible for the phenomena observed, since their ratio is unchanged in the cytoplasm preparation added. In any case, it is worth emphasizing, that under physiological soluble protein : membrane protein ratios the process of 'deactivation' is predominant, and this fact may be responsible for the higher calcium transpor rates obtained in ghosts (with diluted cytoplasm) (see Ref. 29), and in activator-treated inside-out vesicles, than in intact red cells. The further isolation and characterization of the inhibitory protein(s) is in progress in our laboratory.

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