KINETICS OF ACTIVE CALCIUM TRANSPORT IN INSIDE-OUT RED CELL MEMBRANE VESICLES

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Received 8 March 1978

1. Introduction

Active calcium extrusion has a fundamental physiological role in human red cells since intracellular accumulation of Ca^{2+} is harmful to most cellular functions [1,2]. ATP + Mg^{2+}-dependent active Ca^{2+} transport has been studied in a great deal in resealed ghosts [3–5] and in intact red cells loaded with calcium by various techniques [6,7]. A common shortcoming of these studies is that the ATP-, Mg^{2+}- and Ca^{2+}-binding 'active centre' of the transport system is in the cell interior and thus adjusting the experimental parameters at this site is inherently difficult. A further problem is the intracellular binding of ATP, Mg^{2+} and Ca^{2+} to various cellular buffer systems [4,8,9].

Preparation of a homogeneous population of sealed inside-out red cell membrane vesicles (IOV) as in [10,11] allows us to study the kinetics of active calcium transport in an inverted system, where the conditions for the ATP-, Mg^{2+}- and Ca^{2+}-binding sites can easily be adjusted. Following the first reports on active Ca^{2+} transport measurements in inside-out vesicles [12–14], we have introduced a preparation method producing IOV with high Ca^{2+}-transport activity and describe now the kinetics of active Ca^{2+} transport in this system.

2. Materials and methods

All the chemicals used were of analytical grade. Deionized water and solutions without added $CaCl_2$ contained less than 3 μM calcium.

Inside-out vesicles (IOV) were prepared on the basis of the method in [10] modified as follows: Haemoglobin-free red cell membrane was prepared with chelator-free Tris-buffers, as in [4,15]. In order to obtain IOVs the white membranes ($\approx$ 5 mg protein/ml) were immediately diluted with 20-fold vol. 0.5 mM Tris–HCl buffer + 0.05 mM β-mercaptoethanol (pH 8.5), incubated for 30 min at 4°C and for 15 min at 37°C, then homogenized with a 27 gauge needle. After overnight incubation at 4°C the sealed inside-out vesicles were separated on a dextran gradient [11] and were washed first with 10 mM Tris–HCl + 0.5 mM EDTA, pH 8.0, and then twice with large volumes of 10 mM Tris–HCl, pH 7.4. The vesicles were resuspended at ~ 2 mg protein/ml conc. in 140 mM KCl + 20 mM Tris–HCl, pH 7.4. As measured by ^{14}C inulin distribution, 1 mg IOV protein corresponded to 10 μl vesicles.

In Ca^{2+} influx measurements at 0 min, 5 min and 10 min the vesicles were separated from the medium by filtration through a 0.6 μm pore-size Sartorius membrane filter (SM 11305) which retaines the vesicles completely, and then the filters were washed within 10 s with 8 ml cold 0.16 M KCl. ^{45}Ca activity on the filters was counted in a liquid-scintillation counter as in [14]. On the figures each data point represents a linear regression value obtained by using the triplicates of the above time-points.

3. Results

The vesicles obtained are sealed, inside-out (as judged by acetylcholinesterase accessibility) in

84–88%, they are sealed, right side-out (as judged by GAPD accessibility) in 10–12%, and are leaky in 2–4%. Ca^{2+} transport when measured by the rapid filtration techniques in [14] has the basic features described in [14]: there is an ATP + Mg^{2+}-dependent linear Ca^{2+} uptake by the vesicles up to about 15 min, this uptake is abolished by 10^{-6} M A23187 Ca^{2+}-ionophore and is significantly stimulated by a membrane-free supernatant of the red cell haemolysate. In our present preparations ATP-dependent Ca^{2+} uptake is higher than described previously (it is not much less than expected from data on ghosts or on intact red cells [5]), and the blood-to-blood and day-to-day variations are usually within 30%. When measuring Ca^{2+} efflux from IOVs preloaded to 2–5 nmol Ca^{2+}/mg protein, there was no detectable Ca^{2+} loss during a 10 min incubation period either in the presence or absence of ATP + Mg or EGTA in the medium.

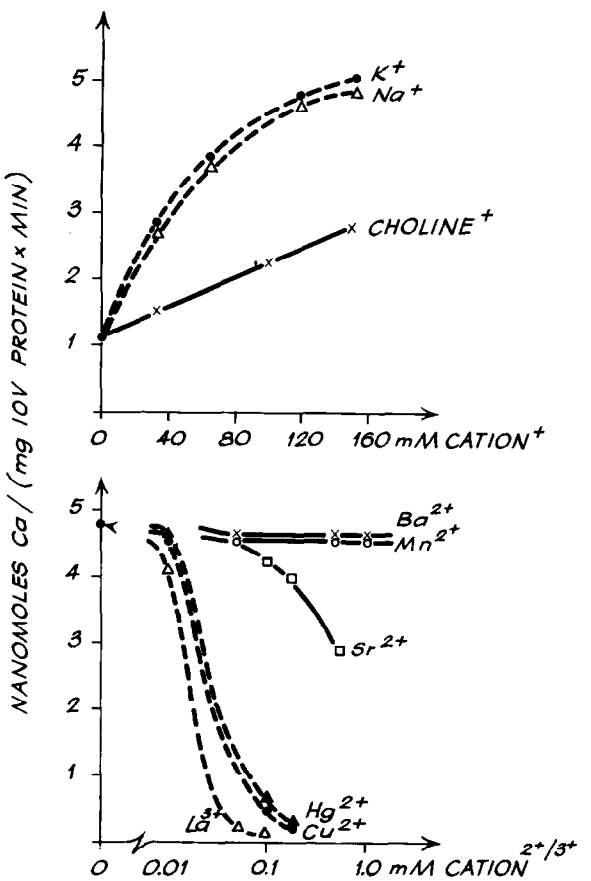

Fig.1. Effects of monovalent, divalent and trivalent cations on active Ca^{2+} uptake by inside-out red cell membrane vesicles (IOV). IOVs in a concentration of 20–30 μg protein/ml medium are incubated at 37°C. Monovalent cations are substituted for isosmotic sucrose, divalent cations and lanthanum are added as chloride salts to the media containing 130 mM KCl. All media are completed with 200 μM $CaCl_2$ (+ 0.1 μCi ^{45}Ca), 500 μM ATP, 2 mM $MgCl_2$ and 20 mM Tris–HCl, pH 7.4. One of three similar experiments.

Figure 1 shows the effect of the changes in the monovalent, divalent or trivalent cation concentrations in the incubation medium on active Ca^{2+} uptake. When substituted for isosmotic sucrose, Na^+ and K^+ increase Ca^{2+} influx in a saturable fashion with half-maximum activation at around 40 mM. The activation is not greater if Na^+ and K^+ are applied in combination, but choline$^+$ produces much less increase in Ca^{2+} uptake than either Na^+ or K^+.

The divalent cations Hg^{2+} and Cu^{2+} produce an almost complete inhibition of Ca^{2+} uptake in concentrations less than 0.2 mM. Ba^{2+} and Mn^{2+} are without effect up to 1.0 mM, whereas Sr^{2+} reduces active Ca^{2+} influx in a competitive manner. An increase in Ca^{2+} concentration shifts Sr^{2+} inhibition to higher Sr^{2+} concentrations and active Sr^{2+} uptake is inhibited by Ca^{2+} (data not shown). The trivalent lanthanum ions block active Ca^{2+} uptake in concentrations less than 0.1 mM.

Figure 2 shows the dependence of active Ca^{2+} uptake by inside-out vesicles on the Ca^{2+} concentration in the medium. Ca^{2+} uptake has a 'high Ca-affinity' component, i.e., a fast increase in Ca^{2+} uptake appears up to about 10 μM of external $[Ca^{2+}]$, then Ca^{2+} influx further increases at increasing $[Ca^{2+}]_o$. Half-maximum activation of Ca^{2+} uptake is produced by 50–70 μM external Ca^{2+}. Upon the addition of a dialysed, membrane-free supernatant of the red cell haemolysate, the affinity of the transport system to Ca^{2+} and the maximum rate of Ca^{2+} uptake significantly increase. A graph of these data on a double-reciprocal plot shows only one, 'high-affinity' component of Ca^{2+} uptake ($K_1/_2 \approx 10$ μM). The same type of stimulation of active Ca^{2+} uptake can be achieved with the clear supernatant of the haemoglobin-free membranes. This supernatant is obtained during IOV preparation and is concentrated on an Amicon-Diaflo apparatus.

Figure 3 shows the effects of ATP and Mg^{2+} on the rate of Ca^{2+} uptake by inside-out vesicles. At an

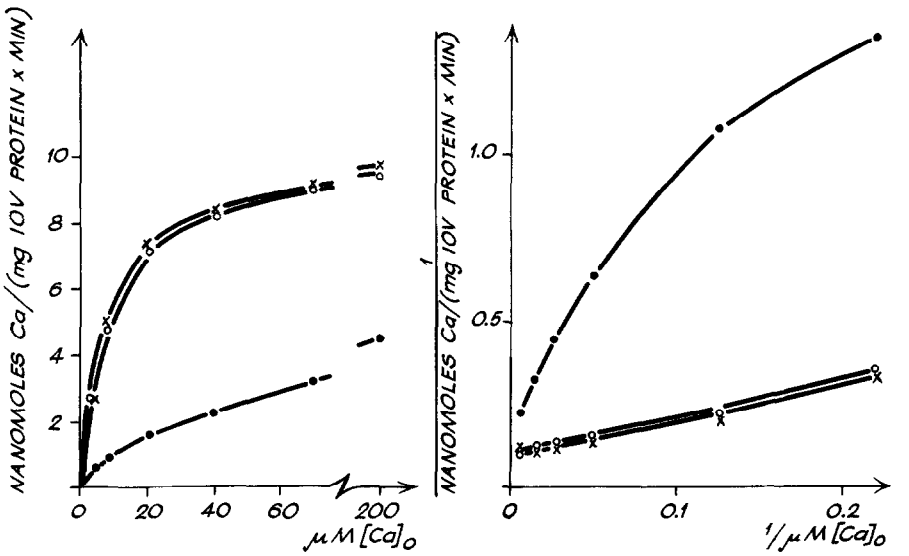

Fig.2. Active Ca^{2+} uptake by inside-out vesicles as a function of the calcium concentration in the medium. IOVs (20–30 μg protein/ml medium) are incubated at 37°C in media containing 130 mM KCl, 20 mM Tris–HCl (pH 7.4), 500 μM ATP, 2 mM $MgCl_2$ and $CaCl_2$ (+ ^{45}Ca) in the concentrations indicated. (•—•) Control. (x—x) Addition of 5% (v/v medium) of a membrane-free supernatant of red cell haemolysate, dialysed against 0.16 M KCl for 46 h at 4°C. Haemoglobin concentration in the dialysate was 1.6 g%. (○—○) Addition of 5% (v/v medium) of a clear supernatant, obtained during IOV preparation from haemoglobin-free membrane, and concentrated to 0.8 mg protein/ml by an Amicon ultrafiltration system (Model 8MC) using an UM 10 Diaflo Ultrafilter.

Note: increase in Ca^{2+} uptake by the supernatant protein is a saturable function of the amount of protein added [14], and here we apply maximum effective doses. Ca^{2+} uptake by IOVs without ATP and/or Mg^{2+} is less than 5% of the active Ca^{2+} uptake either in the presence or absence of the supernatants.

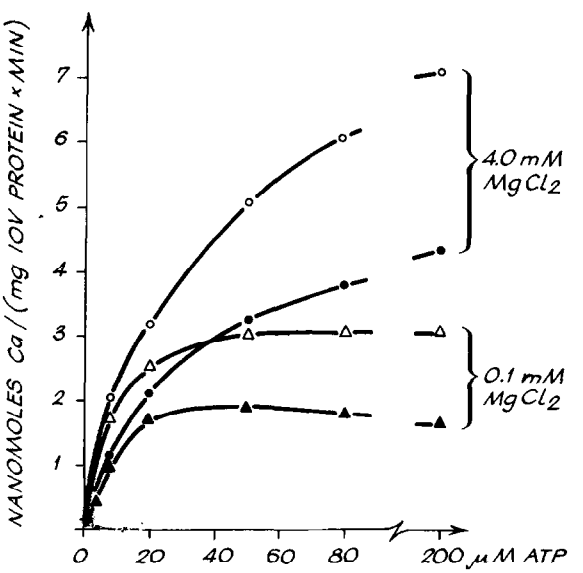

Fig.3. Effects of ATP and Mg^{2+} on Ca^{2+} uptake by inside-out vesicles. IOVs (20–30 μg protein/ml medium) are incubated at 37°C in media containing 130 mM KCl, 20 mM Tris–HCl, pH 7.4, and 200 μM $CaCl_2$ (+ 0.1 μCi ^{45}Ca). (▲—▲) 0.1 mM $MgCl_2$; (△—△) 0.1 mM $MgCl_2$ + 5% (v/v medium) of dialysed supernatant of red cell haemolysate; (•—•) 4.0 mM $MgCl_2$; (○—○) 4.0 mM $MgCl_2$ + 5% dialysed supernatant of red cell haemolysate.

external Ca^{2+} concentration which saturates the transport system, an increase in Mg^{2+} concentration significantly increases the maximum rate of Ca^{2+} uptake and also the $K_1/_2$ for ATP. The calculated $K_1/_2$ for ATP is 6 μM in the presence of 0.1 mM $MgCl_2$ and 25 μM at 4.0 mM $MgCl_2$ concentration in the medium. These half-maximum activation concentrations for ATP do not change in the presence of the dialysed supernatant of the haemolysate,

although Ca^{2+} uptake increases significantly at both Mg^{2+} concentrations examined.

4. Discussion

ATP + Mg-dependent Ca^{2+} uptake by inside-out vesicles provides a useful model system in studying the kinetics and the molecular mechanism of active Ca^{2+} transport of the red cell membrane. Calcium concentration in the vesicles during the uptake experiments is elevated up to mM levels and there is still no outward leakage of Ca^{2+}.

The presence of monovalent cations in the incubation medium significantly stimulates active Ca^{2+} uptake by inside out vesicles. Na^+ and K^+ are more effective in this stimulation than either choline$^+$ or Tris$^+$. These findings are in accordance with the data on Na^+ and K^+ activation of membrane Ca^{2+}-Mg^{2+}-ATPase [16]. Since alterations in monovalent cation concentrations in the incubation media are without effect on Ca^{2+} extrusion from ghosts [4,5] or from intact cells [17] the data suggest a role of monovalent cations at the *cis*-side (normally internal membrane surface) of the Ca^{2+} transport system.

The haemoglobin-free red cell membrane prepared by us has a 'high Ca^{2+}-affinity' Ca^{2+}-Mg^{2+}-ATPase activity [15], i.e., a maximum Ca^{2+}-dependent ATP splitting is observed at Ca^{2+} concentrations below 10 μM. Ca^{2+} uptake by IOVs is half-maximum activated by 50–70 μM of Ca^{2+}, whereas the 'high Ca^{2+}-affinity' character of the transport system is restored by the dialysed supernatant of the haemolysate or by a concentrated supernatant, obtained during IOV preparation from the membrane. In these latter cases the app. $K_1/_2$ for Ca^{2+} is around 10 μM. According to this finding, the activator protein, first described [18] is also present in the cell-membrane fragments showing 'high Ca^{2+}-affinity' Ca^{2+}-Mg^{2+}-ATPase activity, and the activator is detached during IOV preparation. This loss of the activator may be responsible for the conflicting findings on the kinetics of Ca^{2+}-Mg^{2+}-ATPase in various membrane preparations [5,19–21]. The question, whether the activator protein is a physiological regulator of active Ca^{2+} transport or its solubilization is only a preparation artefact, is yet to be resolved.

When examining the effects of ATP and Mg^{2+} concentrations on active Ca^{2+} uptake by IOVs, we find an increased maximum rate of this uptake and also an increase in the $K_1/_2$ for ATP at elevated Mg^{2+} concentrations (see fig.3.). This finding, in accordance with [22] for Ca^{2+}-Mg^{2+}-ATPase in broken membranes, indicates that free ATP may be the substrate for the Ca^{2+}-transport system and that Mg^{2+} activates at another step of the reaction (most probably by accelerating dephosphorylation [23]). As it is shown on fig.3., the supernatant protein does not affect the ATP affinity of the system, although the activator increases Ca^{2+} uptake at both Mg^{2+} concentrations examined. Thus the effect of the activator protein is probably concerned with the alteration of Ca^{2+} binding to the transport system.

Further studies on the kinetics and energetics of active Ca^{2+} transport in inside-out vesicles may lead to a better understanding of the molecular mechanism of this transport system and its possible regulation by intracellular proteins.

Acknowledgements

The investigation was supported by the Scientific Research Council, Ministry of Health, Hungary (6-03-0306-01-1/Gá). We wish to thank Drs R. B. Gunn, Ilma Szász and A. Schubert for valuable comments and Mrs M. Sarkadi for the skilful technical assistance.

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