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

B. SARKADI, J. D. MACINTYRE and G. GÁRDOS

National Institute of Haematology and Blood Transfusion, 1113 Budapest, Hungary and *The University of Chicago, Department of Pharmacological and Physiological Sciences, Chicago, ILL 60637, USA*

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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 (≈ 5 mg protein/ml) were immediately diluted with 20-fold vol. 0.5 mM Tris–HCl buffer + 0.05 mM β-mercapto-ethanol (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

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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.

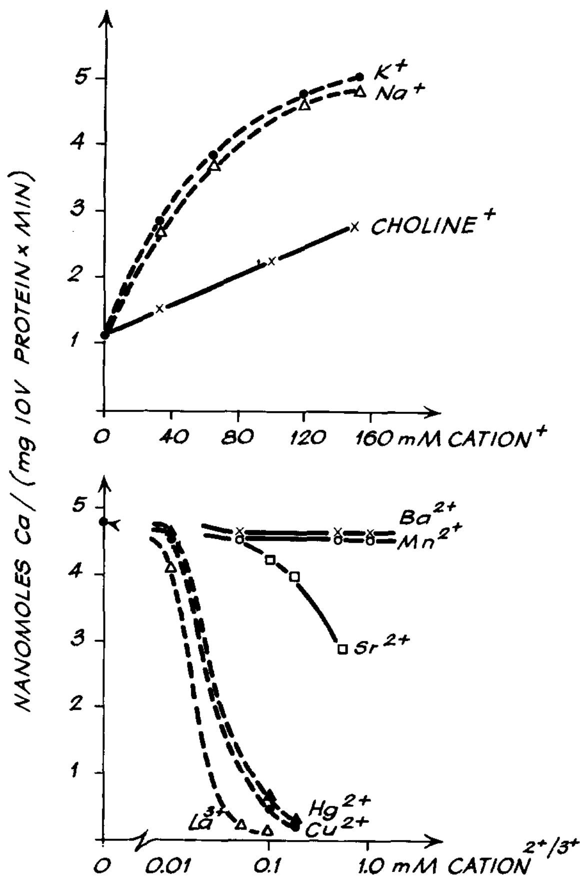

Fig.1. Effects of monovalent, divalent and trivalent cations on active Ca^{2+} uptake by inside-out red cell membrane vesicles (IOV). IOVs in a concentration of 20–30 μg protein/ml medium are incubated at 37°C. Monovalent cations are substituted for isosmotic sucrose, divalent cations and lanthanum are 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 ($K_{1/2}$≈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

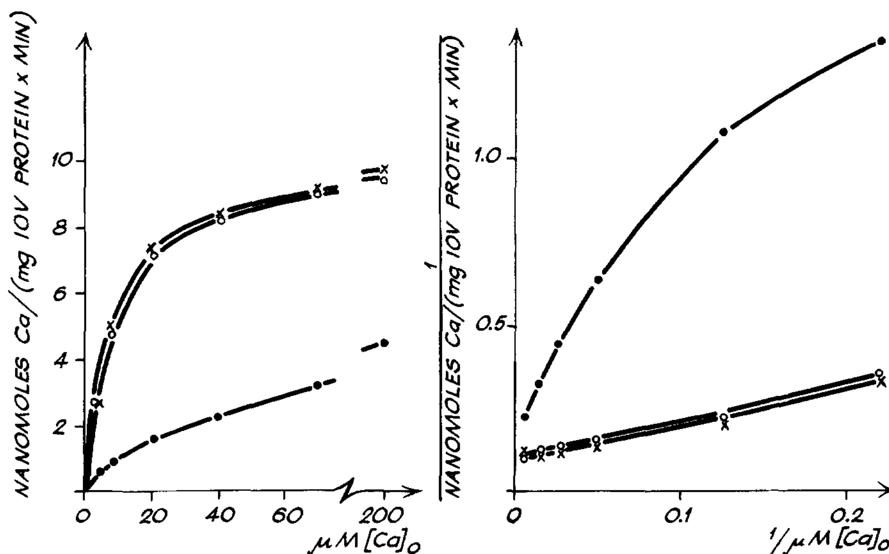

Fig.2. Active Ca^{2+} uptake by inside-out vesicles as a function of the calcium concentration in the medium. IOVs (20–30 μg protein/ml medium) are incubated at 37°C in media containing 130 mM KCl, 20 mM Tris–HCl (pH 7.4), 500 μM ATP, 2 mM $MgCl_2$ and $CaCl_2$ (+ ^{45}Ca) in the concentrations indicated. (•—•) 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%. (o—o) 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.

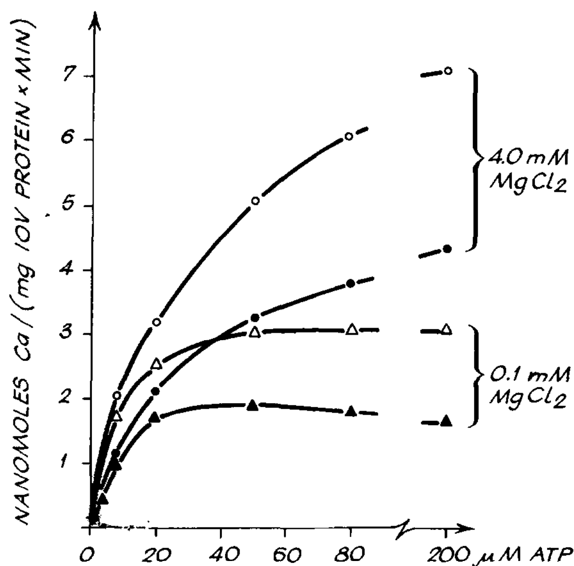

Fig.3. Effects of ATP and Mg^{2+} on Ca^{2+} uptake by inside-out vesicles. IOVs (20–30 μg protein/ml medium) are incubated at 37°C in media containing 130 mM KCl, 20 mM Tris-HCl, pH 7.4, and 200 μM $CaCl_2$ (+ 0.1 μCi ^{45}Ca). (▲—▲) 0.1 mM $MgCl_2$; (△—△) 0.1 mM $MgCl_2$ + 5% (v/v medium) of dialysed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

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