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EFFECTS OF LANTHANUM ON CALCIUM-DEPENDENT PHENOMENA IN HUMAN RED CELLS

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Summary

Lanthanum (0.25 mM) does not penetrate into fresh or Mg^{2+}-depleted cells, whereas it does into ATP-depleted or ATP + 2,3-diphosphoglycerate-depleted cells, into cells containing more than 3 mM calcium, or cells stored for more than 4 weeks in acid/citrate/dextrose solution. In fresh cells loaded with calcium, extracellular lanthanum blocks the active Ca^{2+}-efflux completely and inhibits (Ca^{2+} + Mg^{2+})-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity to about 50%. In Mg^{2+}-depleted cells Ca^{2+}-Ca^{2+} exchange is inhibited by lanthanum. Ca^{2+}-leak is unaffected by lanthanum up to 0.25 mM concentration; higher lanthanum concentrations reduce leak rate. In NaCl medium Ca^{2+}-leak ± S.D. amounts to 0.28 ± 0.08 μmol/l of cells per min, whereas in KCl medium to 0.15 ± 0.04 μmol/l of cells per min at 2.5 mM $[Ca^{2+}]_e$ and 0.25 mM $[La^{3+}]_e$ pH 7.1.

Lanthanum inhibits Ca^{2+}-dependent rapid K^+ transport in ATP-depleted and propranolol-treated red cells, i.e. whenever intracellular calcium is below a critical level. The inhibition of the rapid K^+ transport can be attributed to protein-lanthanum interactions on the cell surface, since lanthanum is effectively detached from the membrane lipids by propranolol.

Lanthanum at 0.2—0.25 mM concentration has no direct effect on the morphology of red cells. The shape regeneration of Ca^{2+}-loaded cells, however, is blocked by lanthanum owing to Ca^{2+}-pump inhibition. Using lanthanum the transition in cell shape can be quantitatively correlated to intracellular Ca^{2+} concentrations.

Abbreviations: EDTA, ethylenediamine tetraacetate; EGTA, ethyleneglycol-bis-(β-aminoethylether)-N,N′-tetraacetate; I_m, morphological index; SITS, 4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulfonic acid.

Introduction

In biological systems a great number of Ca^{2+} effects are abolished by lanthanum. In human red cells lanthanum is known to block active Ca^{2+}-extrusion [1,2], and (Ca^{2+} + Mg^{2+})ATPase activity of the isolated cell membrane [3]. Previously we used these effects as basis for estimating the stoichiometry of the Ca^{2+} pump in intact red cells [2]. Since lanthanum arrests active Ca^{2+} transport without penetrating the red cell membrane [1,2], Ca^{2+}-permeation measured in its presence can be regarded as passive Ca^{2+} transport. Thus the application of lanthanum enables us to study both the passive permeation of Ca^{2+} and the various effects of increased intracellular Ca^{2+} concentration. In human red cells intracellular Ca^{2+} induces characteristic permeability and shape changes: selective, rapid K^+ transport [4—6] and sphaeroechinocytosis [7—9]. In the present paper we report experiments concerning the effects of lanthanum on these cellular functions.

Materials and Methods

Chemicals: lanthanum chloride, sodium hydrogen sulfite, EDTA, inosine (Reanal, Budapest), (±)-propranolol (Sigma Chemical Company, St. Louis, Mo.,), EGTA (SERVA Feinbiochemica, Heidelberg), SITS (BDH, Poole), iodoacetate and iodoacetamide (Fluka AG, Buchs) were of reagent grade. A23187 was kindly provided by Eli Lilly and Co. (Indianapolis, Ind.).

K^+ was determined by flame photometry in an EEL (Evans Electroselenium Ltd., Essex) photometer.

^{42}K (0.159 Ci/g), ^{86}Rb (0.23 Ci/g) and ^{140}La (9.5 Ci/g) were counted in a Beckman Biogamma spectrometer, whereas ^{45}Ca (2.15 Ci/g) in an Intertechnique SL-30 liquid scintillation spectrometer.

Freshly drawn defibrinated human blood was washed as indicated in the text. For ATP depletion cells were incubated with 5 mM iodoacetamide + 10 mM inosine for 3 h at 37°C. For exhaustive phosphate ester depletion the technique described earlier [10] was followed: Red cells were exhausted of 2,3-diphosphoglycerate by a 4-h pretreatment at 37°C with 2.5 mM iodoacetate + 15 mM sodium hydrogen sulfite followed by a 2-h incubation with 2.5 mM idoacetate + 10 mM inosine in KCl medium. Cellular ATP level was determined by the luciferine-luciferase method of Kimmich, Randles and Brand [11] by using the enzyme preparation of SERVA Feinbiochemica. 2,3-diphosphoglycerate was measured with the aid of the Calbiochem kit.

Cells were loaded with Ca^{2+} by treatment with ionophore A23187 and with ^{42}K or ^{86}Rb by making use of the rapid initial uptake of these tracer cations due to membrane hyperpolarization during Ca^{2+}-induced K^+-efflux as described earlier [6]. Cells were depleted of Mg^{2+} following the idea of Flatman and Lew [12], by incubating the cells with 0.1 mM A23187 and 10 mM EDTA for 15 min at 37°C under vigorous shaking. The ionophore was removed by thorough washing in ice-cold 0.5% albumin containing 0.16 M KCl. Mg^{2+} was determined in a Perkin-Elmer 460 atomic absorption spectrophotometer.

Membrane lipids were extracted according to the method of Nayler [13] and

phospholipid phosphate was determined by the method of Allen as modified by Rhodes [14].

Ca^{2+}-influx was studied as reported earlier [15], except that in the presence of lanthanum the haematocrit did not exceed 15% and 0.5% albumin was included in the medium to avoid any aggregation.

Ca^{2+} pump and (Ca^{2+} + Mg^{2+})-ATPase activities were measured in intact cells as already described [2].

Tracer ^{42}K or ^{86}Rb efflux from preloaded cells was followed as in a previous study [6].

For the assay of Ca^{2+} and La^{3+} binding (exchange) by lipids Nayler's [13] technique was adopted with slight modifications reported elsewhere [15]. ^{45}Ca or ^{140}La respectively were included in the water phase (0.25 ml) and mixed with the lipid phase (1 ml) by vigorous shaking. The latter consisted of red cell membrane lipids (lipid-*P*: 10—15 μg/ml) extracted with chloroform/methanol (2 : 1).

The morphology of glutaraldehyde-fixed red cells was examined under a phase contrast microscope. The percentual distribution of the various forms of disk-sphere transformation [16] was determined. The percentual value of the biconcave disks was multiplied by 1.0, that of the intermediate forms by 0.5 and the microspheres by 0.0. The sum of these products was called morphological index, I_m. It gives a value of 100 for a homogeneous cell population consisting of biconcave disks, 0 for a homogeneous microsphaerocyte population and values between 0 and 100 for heterogeneous populations.

Results

1. Uptake of lanthanum by the red cells

If red cells are incubated in an isosmotic medium supplemented with 0.2 mM $LaCl_3$, both fresh and ATP-depleted cells bind an appreciable amount of lanthanum. In both cases a rapid release of lanthanum occurs if, after a delay, 0.2 mM EGTA is added to the medium (Fig. 1A). A careful study shows that while there is a complete release of lanthanum by EGTA from fresh cells, an easily measurable amount of lanthanum (20 μmol/l of cells in 1 h) remains attached to phosphate-ester-depleted cells (Fig. 1B). A similar (20—30 μmol/l of cells per h) lanthanum uptake was observed in Ca^{2+}-loaded red cells containing 3—5 mM Ca^{2+} and in cells from blood stored in acid/citrate/dextrose solution more than 4 weeks. Propranolol, a drug moderately increasing Ca^{2+} influx into fresh human red cells, fails to elicit any La^{3+} uptake. There is no lanthanum penetration into cells depleted of Mg^{2+} by the A23187 ionophore method (with normal cellular ATP) either.

2. Effect of lanthanum on the (Ca^{2+} + Mg^{2+})-ATPase activity of red cells

Fig. 2 shows the (Ca^{2+} + Mg^{2+})-ATPase activities in Ca^{2+}-loaded red cells in the presence of lanthanum. Lanthanum, if it does not penetrate into the cell inhibits (Ca^{2+} + Mg^{2+})-ATPase to about 50%. Parallel with its penetration the inhibition first increases, whereas later cell damage and haemolysis occur and ATPase determination becomes erroneous. With 1—3 mM intracellular calcium

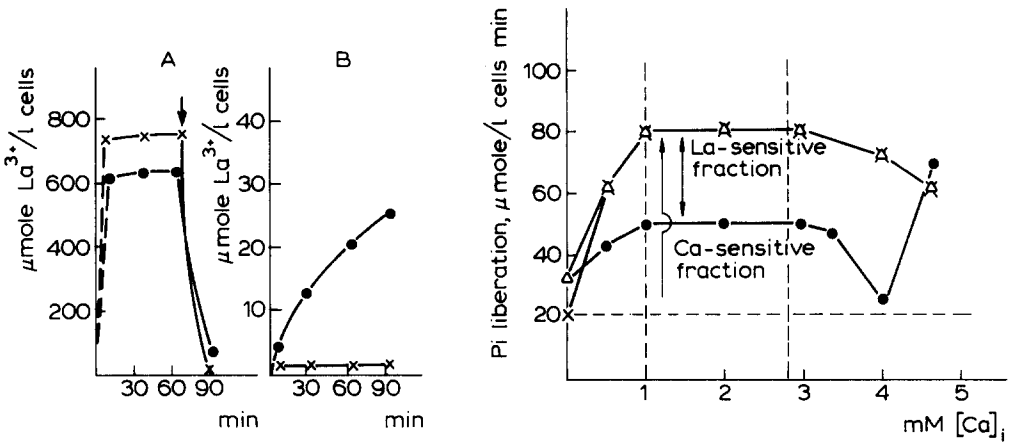

Fig. 1. Lanthanum binding to fresh and phosphate-ester-depleted red cells. $[La^{3+}]$ = 0.2 mM + 1 μCi/ml $^{140}LaCl_3$. Haematocrit 12%, pH 7.1, 37°C. x———x, fresh cells washed 3 times in 0.16 M NaCl; •———•, phosphate-ester-depleted cells. A, samples were taken into an equal volume of 0.16 M NaCl (0°C); B, samples were taken into an equal volume of 0.1 M EGTA (0°C). Red cells were rapidly separated by centrifugation in the cold through a 0.7 M sucrose cushion dissolved in 0.16 M NaCl. The arrow indicates the time when system A was supplemented with 1 mM EGTA. Data of a representative experiment.

Fig. 2. Effect of 0.2 mM lanthanum on the $(Ca^{2+} + Mg^{2+})$-ATPase activities of red cells containing various amounts of calcium. Medium: 120 mM KCl, 15 mM NaCl, 2.5 mM $CaCl_2$, 15 mM Tris-HCl. Haematocrit 15%, pH 7.1, 37°C. △———△, control; x———x, 0.1 mM ouabain; •———•, 0.2 mM $LaCl_3$. One of 5 similar experiments.

(no lanthanum penetration) maximum inhibition is achieved with 0.2—0.25 mM lanthanum.

On the basis of the above findings we studied the combined effects of lanthanum and calcium treatments under experimental conditions when the permeation of La^{3+} was negligible.

3. Effect of lanthanum on Ca^{2+} influx into red cells

Ca^{2+} influx into fresh, normal human red cells is less than 2—4 μmol/l of cells per h [15]. As shown in Fig. 3, addition of 0.15—0.25 mM $LaCl_3$ to the incubation medium significantly increases Ca^{2+} influx, 0.2—0.25 mM La^{3+} being the most effective in this resprect. There is a strong difference in the Ca^{2+} influx in different media: the maximum rate ± S.D. in an NaCl medium is 0.28 ± 0.08, whereas in KCl medium it is around 0.15 ± 0.04 μmol/l of cells per min (external Ca^{2+} concentration 2.5 mM, external La^{3+} concentration: 0.25 mM). In a LiCl medium, under the same conditions, the Ca^{2+} influx values fall between those found in NaCl and KCl, whereas in a choline/HCl medium they slightly exceed those found in NaCl. A significant part of the influx is dipyridamole (and SITS) sensitive in each medium except KCl.

In Mg^{2+}-depleted red cells, according to Ca^{2+} efflux measurements [17], the active Ca^{2+} extrusion is practically at a standstill. In these cells the initial rate of Ca^{2+} uptake, measured as the unidirectional flux of ^{45}Ca, is augmented up to 1—2 μmol/l of cells per min by an increase in the intracellular Ca^{2+} concentration. This increased Ca^{2+}-influx is strongly inhibited by 0.2 mM lanthanum in

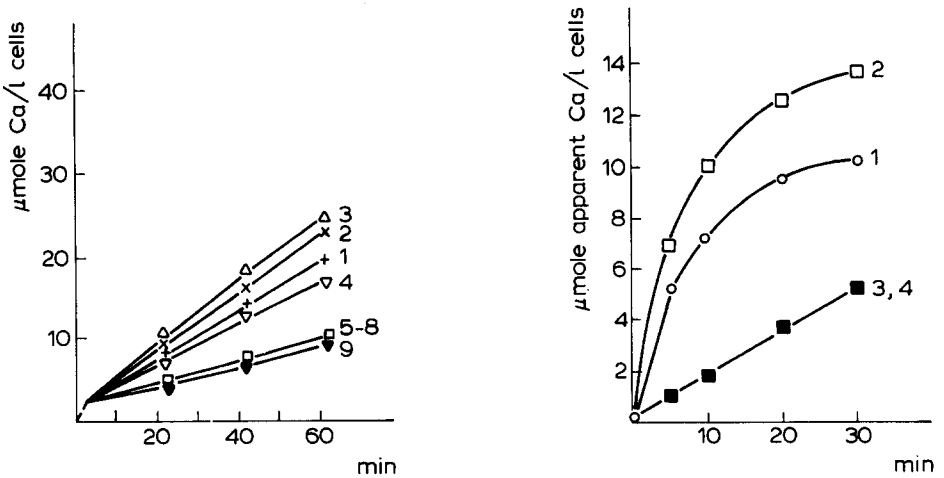

Fig. 3. Ca^{2+}-influx into red cells in the presence of lanthanum. Fresh cells were washed with 0.16 M NaCl 3 times. Medium: 15 mM Tris-HCl (pH 7.1), 2.5 mM $CaCl_2$ + ^{45}Ca (10 μCi/ml), 0.5% albumin in 135 mM NaCl (tubes 1—4 and 9) or in 135 mM KCl (tubes 5—8). Tube 9 contained 0.2 mM dipyridamole. Lanthanum concentration: 0.15 mM in tubes 1 and 5, 0.20 mM in tubes 2, 6 and 9, 0.25 mM in tubes 3 and 7, and 0.30 mM in tubes 4 and 9. Haematocrit 15%, 37°C. Ca^{2+}-influx without lanthanum was 1.8 μmol/l of cells per h in KCl, whereas it was 3.5 μmol/l of cells per h in NaCl medium. One of 10 similar experiments.

Fig. 4. Ca^{2+}-influx into Mg^{2+}-depleted red cells. Cells were Mg^{2+}-depleted as indicated in Materials and Methods and preincubated with 2.5 mM $^{40}CaCl_2$ for 15 min (tubes 1 and 3) and 30 min (tubes 2 and 4). Before adding ^{45}Ca tubes 3 and 4 were supplemented with 0.2 mM $LaCl_3$. Apparent $[Ca]_i$ was calculated from the data of ^{45}Ca uptake. Medium: 135 mM KCl, 15 mM Tris-HCl (pH 7.1). 2.5 mM $CaCl_2$ + ^{45}Ca (10 μCi/ml), 0.5% albumin. $[Mg^{2+}]_i$ was less than 5 μmol/l of cells. Haematocrit 15%, 37°C. One of 5 similar experiments.

the media (Fig. 4). In Mg^{2+}-depleted red cells the maximum rate of the lanthanum-sensitive Ca^{2+} influx was observed at 30—50 μM intracellular Ca^{2+} concentrations.

4. Effect of lanthanum on Ca^{2+}-induced K^+ transport

The K^+-permeability of propranolol-treated fresh red cells, as well as phosphate-ester depleted cells loaded with 20—200 μM of Ca^{2+}, exceeds the normal values by approx. 2 orders of magnitude. If fresh red cells are incubated with 0.2 mM $LaCl_3$ + 2.5 mM $CaCl_2$, intracellular Ca^{2+} concentration increases to about 20—30 μM within 1 h, but no rapid K^+ transport develops (Fig. 5A). Moreover, external lanthanum prevents increased K^+ movement in propranolol-treated and in Mg^{2+}-depleted red cells (Fig. 5, B and C), although propranolol induced Ca^{2+}-uptake is not inhibited by lanthanum (not shown). When the cells are loaded by the A23187 ionophore method up to millimolar concentrations of Ca^{2+}, the rate of rapid K^+ transport is unaffected by external lanthanum. The same is true for the acceleration of K^+-efflux caused by propranolol in these cells (Fig. 6).

The effect of propranolol on K^+ transport is most probably related to a Ca^{2+} release from the membrane lipids [15]. We compared the effects of increasing propranolol concentrations on Ca^{2+} and La^{3+} binding by isolated membrane

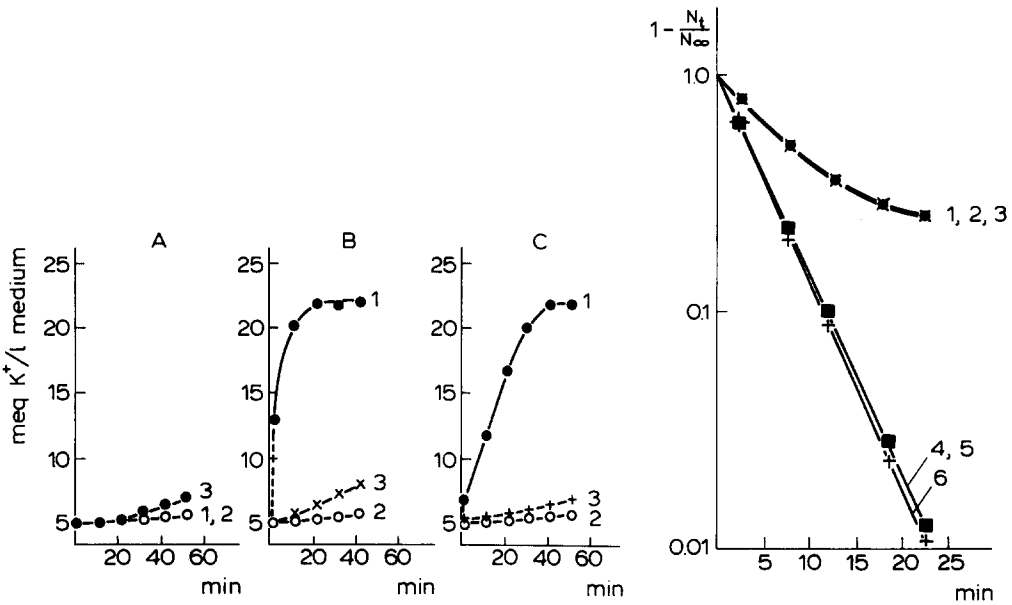

Fig. 5. Effect of 0.2 mM $LaCl_3$ on the K^+-efflux from red cells. A: Fresh cells, washed 3 times with 0.16 M NaCl B: Fresh cells, washed 3 times with 0.16 M NaCl + 1 mM propranolol C: Mg^{2+}-depleted red cells. Medium: 130 mM NaCl, 5 mM KCl, 15 mM Tris-HCl (pH 7.1), 2.5 mM $CaCl_2$. Haematocrit 30%, 37°C. 1, control; 2, 5 mM EGTA; 3, 0.2 mM $LaCl_3$. One of 6 similar experiments.

Fig. 6. ^{42}K-efflux from Ca^{2+}-loaded red cells under exchange conditions. $[Ca^{2+}]_i$: 2.4 mM. Medium: 120 mM KCl, 30 mM Tris-HCl (pH 7.1). 1, Control; 2, 0.2 mM $LaCl_3$; 3, 1 mM EGTA; 4, 1 mM propranolol; 5, 1 mM propranolol + 0.2 mM $LaCl_3$; 6, 1 mM propranolol + 1 mM EGTA. Haematocrit 5% 37°C. One of 4 similar experiments.

lipids and found the same type of decrease in the binding of both cations (Fig. 7).

5. Effects of lanthanum on the shape of red cells

Lanthanum does not alter the shape of fresh red cells. It does not affect the shape changes caused by ATP depletion either. On the other hand, the shape regeneration during the pumping out of Ca^{2+} from the microsphaerocytic Ca^{2+}-loaded cells can be blocked by lanthanum. Thus in media of appropriately chosen calcium + lanthanum concentrations the intracellular calcium levels can be stabilized. Fig. 8 demonstrates the effect of prolonged maintenance of calcium concentrations in the presence of lanthanum on the cell shape.

Both shape regeneration and the pumping out of Ca^{2+} are rapid processes, thus 'lanthanum stop' is essential for the simultaneous, accurate determination of the intracellular calcium concentration on the one hand and the distribution of shape forms on the other. Partial inhibition of the calcium pump by 0.03—0.06 mM lanthanum during incubation slows down the processes and makes the analysis easier. By fitting the data of the intracellular calcium and morphological distribution to a model (see Appendix) the transitions in cell shape could be quantitatively correlated to intracellular calcium concentrations. In the course of Ca^{2+}-pumping from red cells loaded with 0.4—0.6 mM calcium

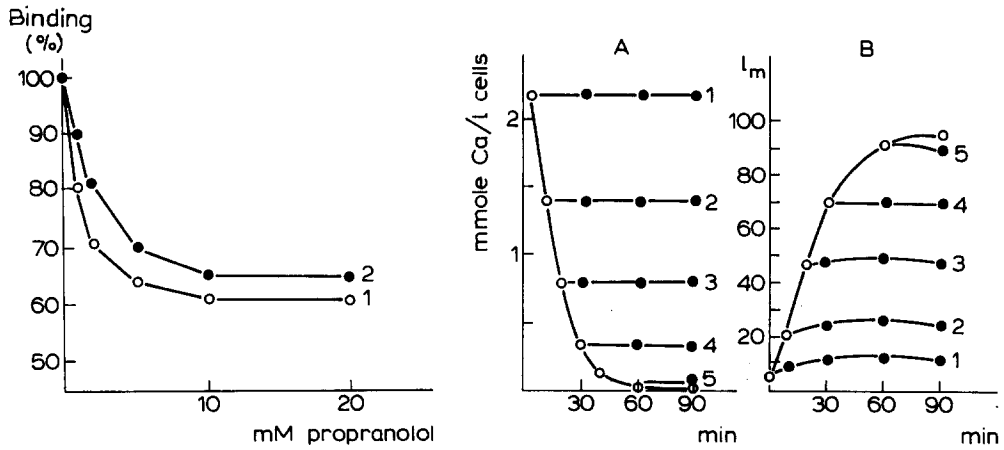

Fig. 7. Effect of propranolol concentration on the Ca^{2+} or La^{3+} binding of isolated membrane lipids. Tube 1: 2 mM $^{40}Ca^{2+}$ in the water phase + ^{45}Ca/l μCi/ml; Tube 2: 0.2 mM La^{3+} in the water phase + ^{140}La/ 0.2 μCi/ml. Propranolol concentrations in the water phase are shown on the abscissa. Binding of Ca^{2+} or La^{3+} to the lipids of the propranolol-free control was taken as 100% (18 nmol Ca^{2+}/μmol lipid-*P* in tube 1, and 21 nmol La^{3+}/μmol lipid-*P* in tube 2). Data are from a representative experiment.

Fig. 8. Ca^{2+}-content and morphological index of Ca^{2+}-loaded red cells. Medium: 120 mM KCl, 30 mM Tris-HCl (pH 7.1), 5 mM inosine. Haematocrit 15%, 37°C. At times indicated samples were taken for cell Ca^{2+} and I_m determination. o———o, control cells; •———•, cells suplemented with 0.2 mM lanthanum + Ca^{2+} corresponding to the intracellular levels. One of 3 similar experiments.

the transition between microsphaerocytes and transitory shape forms was found in the range of 48—94 (mean 73, $n = 18$) μmol calcium per l of cells, whereas between transitory shape forms and biconcave disks in the range of 23—63 (mean 49, $n = 18$) μmol calcium per l of cells. The relatively small differences between calcium concentrations corresponding to the two interconversion steps was conspicuous in every case and was in good agreement with the rapid transformation of the transitory cell shapes.

Discussion

Our experiments indicate that lanthanum, in concentrations below 0.25 mM in the medium, does not penetrate into fresh, intact cells. The same is true for propranolol-treated and Mg^{2+}-depleted cells, whereas there is a significant lanthanum influx (up to 20—30 μmol/l of cells per h) into phosphate-ester depleted, stored or Ca^{2+}-loaded (intracellular calcium >3 mM) red cells. Under experimental conditions when there is no lanthanum uptake, this non-permeable inhibitor can be safely used for investigating Ca^{2+}-dependent phenomena.

In fresh cells lanthanum arrests active Ca^{2+} extrusion; this allows studying passive Ca^{2+} permeation. The maximum Ca^{2+}-influx rates are obtained with 0.2—0.25 mM lanthanum, higher lanthanum concentrations are inhibitory. The maximum Ca^{2+} influx rates obtained with lanthanum are in good agreement with the rates observed in the presence of the lowest mercuric chloride concentration (0.2 mM) causing a complete arrest of the Ca^{2+} pump (unpublished

results). The rate of Ca^{2+} uptake is influenced by the composition of the incubation medium, that is external K^+ inhibits Ca^{2+} uptake. The same has been reported by Ferreira and Lew [18] for ATP-depleted red cells. In those cells increased Ca^{2+} uptake in low K^+ media can also be attributed to membrane hyperpolarization accompanying Ca^{2+}-induced rapid K^+ transport. In lanthanum-treated cells the effect of hyperpolarization can be excluded, since rapid K^+ transport is blocked by lanthanum (Fig. 5). The sensitivity of Ca^{2+}-uptake to anion transport inhibitors suggests an eventual Ca-anion cotransport mechanism.

The presence of Ca^{2+}-Ca^{2+} exchange in ATP-depleted red cell ghosts [19] and in ATP-depleted intact cells [18] has been reported. Ca^{2+} exchange was studied by us in Mg^{2+}-depleted cells, in which active Ca^{2+} transport is abolished. According to the data presented here, in these cells Ca^{2+} influx stimulated by internal Ca^{2+} can be inhibited by external lanthanum. Lanthanum thus seems to block a membrane-mediated Ca^{2+}-influx which requires Ca^{2+} on the trans side of the membrane. It would be premature to speculate on the role of the Ca^{2+} pump in this exchange process.

In propranolol-treated or ATP-depleted red cells external lanthanum inhibits the development of a rapid K^+ transport. In these cases intracellular $[Ca^{2+}]$ is low, and lanthanum may inhibit K^+ transport by binding to the external membrane surface and changing the Ca^{2+}-affinity of the receptors responsible for K^+ movement at the inside of the membrane. If red cells are loaded with high concentrations of Ca^{2+} by the A23187 ionophore method, lanthanum does not affect rapid K^+ transport. These observations make it probable that the protein structures involved in active Ca^{2+} extrusion and/or Ca^{2+}-Ca^{2+} exchange, and blocked by external lanthanum, are not identical with the molecular substance of the rapid K^+ transport.

Lanthanum does not alter the shape of normal red cells and does not influence the disk-sphere transformation that accompanies ATP depletion [20, 21]. These observations indicate that lanthanum in the concentrations applied does not stabilize the cell membrane. At the same time, lanthanum, if added during the course of the Ca^{2+} pumping from Ca^{2+}-loaded red cells, fixes the shape of the cells at a stage determined by the intracellular Ca^{2+}. Previous works on the activation of the Ca^{2+} pump when $[Ca]_i$ is increased by A23187 [22] or propranolol [15] refers to a heterogenous distribution of calcium among red cells. By fitting a model, however, to the measured (average) $[Ca]_i$ and the observed distribution of shape forms, the interconversion steps in the cell shape can be consistently related to a given intracellular calcium concentration. The dependence of this relationship on varying concentrations of intracellular Ca^{2+}-buffers and echinocytogenic membrane lipids, respectively, is reported elsewhere [23].

Appendix

The evaluation of the morphological data was based on the following assumptions:

1. The red cells may assume three well-distinguishable shapes: biconcave,

intermediate and spherical, depending on their intracellular Ca^{2+} concentrations.

2. Transitions from biconcave to intermediate, and from intermediate to spherical shapes occur at definite internal Ca^{2+} concentrations: at $[Ca]^{I}$ and $[Ca]^{II}$, respectively.

3. The values of $[Ca]^{I}$ and $[Ca]^{II}$ are the same in each single red cell in a given sample.

4. Cells with different shapes are present simultaneously because of the non-uniform distribution of Ca^{2+} among the cells.

5. The distribution of Ca^{2+} ions among the cells is logarithmic-normal, i.e. the logarithm of Ca^{2+} concentrations follows Gaussian distribution.

These assumptions enable us to calculate the values of $[Ca]^{I}$ and $[Ca]^{II}$ from experiments providing data for the mean value of Ca^{2+} concentration in the red cells and the corresponding ratios of the three different forms.

To this end we have to solve the following set of equations:

$$p_i = \phi\left[\frac{\ln[Ca]^{I} - \mu_i}{\sigma_i}\right];$$

$$p_i + q_i = \phi\left[\frac{\ln[Ca]^{II} - \mu_i}{\sigma_i}\right],$$

$$\ln\overline{[Ca]_i} = \mu_i + \frac{\sigma^2}{2}$$

for any 2 values of i, the number of experiments. Here p_i denotes the fraction of cells in biconcave shape, q_i the cells in intermediate form, $\ln [Ca]_i$ is the logarithm of the mean value of intracellular calcium concentration (which differs from the mean value μ_i of the logarithm of $[Ca]_i$ by $\sigma^2/2$), and $\phi((\xi - \mu)/\sigma)$ is the value of the standard normal distribution function with parameters μ and σ at the value ξ of the probability variable.

The validity of the above assumptions is supported by the similar values of $[Ca]^{I}$ and $[Ca]^{II}$ obtained by using several data points in the course of the calculations.

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