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Cholesterol depletion affects the Ca^{2+} influx but not the Ca^{2+} pump in human erythrocytes

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Control and cholesterol-depleted human erythrocytes were loaded with permeant Ca^{2+} chelators (Benz2-AM or Quin2-AM) in order to increase their exchangeable Ca^{2+} pool and to measure both Ca^{2+} fluxes and $[Ca]_i$ (free cytoplasmic calcium concentration). The fluxes were independent of the concentration and of the nature of the intracellular chelator. The ATP content was not decreased by more than 50% under our experimental conditions. Cholesterol depletion (up to 28%) induced a decrease in both Ca^{2+} fluxes and $[Ca]_i$ which was proportional to the extent of the depletion. It is shown that cholesterol depletion primarily altered the properties of the system responsible for Ca^{2+} entry causing a diminution of the $[Ca]_i$. This, in turn, induced a diminution of the activity of the Ca^{2+} pump without affecting the properties of this pump.

Introduction

In erythrocytes, as in other cells, the maintenance of a cytosolic Ca^{2+} concentration ($[Ca]_i$) at a very low level is controlled by a Ca^{2+}-ejecting ATPase or Ca^{2+} pump [1]. The influx of Ca^{2+} seems to be a carrier-mediated process because of the saturability of the Ca^{2+} uptake and of its inhibition verapamil [2] or nitrendipine [3] and was thus proposed to be similar or identical to the slow Ca^{2+} channel of excitable tissues [2]. In these cells, under physiological conditions, the total pool of exchangeable Ca^{2+} is very small (about 1 μmol/l cell). Thus, until recently, it was not possible to measure Ca^{2+} fluxes without artifically increasing this pool by ionophore addition [4], membrane resealing on Ca^{2+} buffers [5] or Ca^{2+} pump inhibition by ATP depletion [6] or vanadate addition [2,3]. These procedure alter either the membrane permeability or the physiological steady-state $[Ca]_i$. The use of permeant Ca^{2+} chelators [7,8] has made possible Ca^{2+} fluxes and $[Ca]_i$ measurements without these shortcomings [9]. These molecules, in their esterified form (Quin2-AM and Benz2-AM), cross the cell membrane, are hydrolysed by intracellular esterases, bind Ca^{2+} with high affinity and create an internal measurable pool of exchangeable Ca^{2+}.

Cholesterol, a major component of the plasma membrane of the erythrocyte is known to affect ionic permeabilities [10,11] and transport systems such as the Na^+/K^+ pump [12,13], the Na^+-K^+ cotransport [14] or the anion exchange [15]. The cholesterol content of the erythrocyte membrane is greatly altered under some pathological conditions [16]. It was thus of interest to examine whether cholesterol could also affect the systems

Abbreviations: $[Ca]_i$, free cytoplasmic calcium concentration; $[Ca]_o$, external calcium concentration; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol bis-(β-aminoethyl ether)*N*,*N*'-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid.

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involved in Ca^{2+} transport in erythrocytes. Recently the influence of cholesterol and other related sterols on the Ca^{2+} influx in erythrocytes has been reported [3,17]. However, in those studies, Ca^{2+} influx was measured in the presence of vanadate and thus under conditions of non-physiological $[Ca]_i$.

We have investigated the possible role of cholesterol on both the Ca^{2+} influx and efflux and the $[Ca]_i$ in erythrocytes, by using the chelator method. Chelator loading was carried out in the presence of 1 mM $^{40}CaCl_2$, a concentration which was used further during the ^{45}Ca flux experiments. Under these conditions, internal Ca^{2+} was in steady state and the true Ca^{2+} fluxes could be calculated from the equilibration kinetics of the cell Ca^{2+} with the tracer ^{45}Ca. Our results indicate that cholesterol depletion induces a decrease in the rate of Ca^{2+} influx that in turn reduces the $[Ca]_i$ level and the rate of Ca^{2+} efflux. A preliminary report of this work has alreadly been published [18].

Materials and Methods

Materials

Phosphatidylcholine from egg yolk (Fraction VE) was obtained from Sigma. Benz2-AM was a gift from Dr. V.L. Lew. Quin2-AM and [^{3}H]Quin2-AM were from Amersham (France). ^{45}Ca was from CEA-ORIS (France).

Preparation of erythrocytes and cholesterol depletion

Human blood was collected from healthy volunteers into heparin. One half of the cells (to be used as control) was washed three times in solution A (140 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 35 mM sucrose and 2.5 mM Na_2HPO_4/NaH_2PO_4 (pH = 7.4)). The other half of the cells (to be used as cholesterol-depleted) was washed in a similar way in solution B (20 mM NaCl, 130 mM KCl and the same concentration of the other constituents). Solution B was used in order to maintain normal Na^+ and K^+ concentrations in the cholesterol-depleted erythrocytes [19]. All the experiments were started with fresh erythrocytes.

Cholesterol depletion was carried out by an incubation of the cells (16 h, haematocrit 18%, 37°C) in solution B containing sonicated phosphatidylcholine vesicles as previously described [19,20]. During the last hour, adenine (2 mM) and inosine (10 mM) were added to the medium to replete the ATP pool. Cholesterol-maintained erythrocytes were incubated in solution A with cholesterol/phosphatidylcholine vesicles (molar ratio of the lipids = 0.85) under the same conditions and control erythrocytes as well but without vesicles. In all cases penicillin (5000 I.U./ml) was present during the 16 h incubation. At the end, cells were washed three times in their respective solution, A or B. Aliquots were taken for the measurements of cholesterol, phospholipid and ATP content.

Chelator loading, Ca^{2+} flux and $[Ca]_i$ measurements

The experimental protocol adapted from that of Lew et al. [9], was essentially as previously described [21] with minor modifications. Chelator loading was carried out in solution A for control and cholesterol-maintained cells, in solution B for cholesterol-depleted cells. In experiments in which $[Ca]_i$ was to be measured, the intracellular chelator content, $[chelator]_i$, was determined by one of the following methods as before [21]. Chelator-loaded, ATP-depleted cells were incubated in the presence of ionophore A 23187 and ^{45}Ca-EGTA buffers giving free Ca^{2+} concentrations in the nanomolar range ($[Ca]_o$). The pH of the medium, the pH and ^{45}Ca content of the washed cells were mesured after 90 min. $[Ca]_i$ at equilibrium was calculated using the relationship: $[Ca]_i/[Ca]_o = (|H^+|_i/|H^+|_o)^2$ [9]. From the plot of the ^{45}Ca content as a function of $[Ca]_i$, one can calculate $[chelator]_i$ and the dissociation constant of the Ca-chelator complex (K_d). K_d for Quin2 was about 100 nM and for Benz2 about 50 nM in agreement with previously reported values [7,9]. The second method implies the use of [^{3}H]Quin2-AM. The limitations of this method have been discussed in our previous paper [21]. In additional experiments, not described in this paper, $[chelator]_i$ was estimated from the Quin2 fluorescence after extraction and removal of haemoglobin. All these methods gave comparable results. After 75 min of chelator loading, all cells were washed three times in solution C (75 mM NaCl, 75 mM KCl and the

same concentration of the other constituents as that in solutions A and B). Solution C (containing ^{45}Ca) was used for Ca^{2+} influx experiments. At the times indicated, aliquots were taken, washed three times in solution D (150 mM NaCl, 35 mM sucrose, and 2.5 mM Na_2HPO_4/NaH_2PO_4, pH = 7.4). The cell pellet was lysed in 6% trichloroacetic acid, centrifuged and the ^{45}Ca activity was measured in an aliquot of the supernatant. ^{45}Ca-loaded cells (90 to 120 min load) were washed 3 times in solution C and reincubated in the same medium for Ca^{2+} efflux experiments.

Calculations

Since the chelator loading was carried out in the presence of 1 mM $CaCl_2$, at the beginning of the Ca^{2+} flux experiments internal Ca^{2+} was in steady state and the fluxes could be calculated from the equilibration kinetics of the cell Ca^{2+} with the ^{45}Ca tracer. Total exchangeable Ca^{2+} ($^{45}Ca_{eq}$, μmol/l cells) was calculated from the ^{45}Ca uptake at isotopic equilibrium (cpm/l cells) divided by the specific activity of ^{45}Ca in the medium (cpm/μmol). The values of ^{45}Ca uptake at each time, divided by the specific activity ^{45}Ca in the medium will be referred to as $^{45}Ca_t$. By plotting $\log(^{45}Ca_{eq} - {}^{45}Ca_t)$ as a function of time, one can determine k_i the rate constant for Ca^{2+} influx (h^{-1}). Ca^{2+} influx was calculated from $k_1 \cdot {}^{45}Ca_{eq}$. Similarly efflux kinetics were analyzed by plotting $\log {}^{45}Ca_t$ as a function of time to determine k_e, the rate constant for Ca^{2+} efflux. In some instances, as outlined in the legends of the figures, Ca^{2+} influx was calculated from the initial rate of ^{45}Ca uptake.

The free cytosolic Ca^{2+} concentration ($[Ca]_i$, nM) was calculated from

$$[Ca]_i = K_d \cdot {}^{45}Ca_{eq} / ([chelator]_i - {}^{45}Ca_{eq})$$

in which K_d(nM) is the dissociation constant of the Ca-chelator complex and $[chelator]_i$ (μmol/l cells) is the intracellular chelator concentration determined as described above. This calculation is valid since in chelator-loaded erythrocytes virtually all exchangeable Ca^{2+} will be bound to the chelator [9].

Other assays

Lipids were extracted with chloroform/isopropanol according to Ref. 22. Cholesterol was measured by the *o*-phthalaldehyde method [23] and phospholipids, after acid hydrolysis, by determination of inorganic phosphate [24]. Na^+ and K^+ content of the cells were measured, after three washes in isotonic choline chloride, by flame photometry. Cell volume was estimated from the haematocrit or from the haemoglobin content after lysis in water and absorbance reading at 540 nm. The extent of haemolysis was determined from the appearance of haemoglobin in the medium during the incubations. ATP content was determined after extraction with perchloric acid with an ultraviolet light enzymatic test (Boehringer-Mannheim GmbH Diagnostica, F.R.G.)

Results

In order to assess the validity of the chelator method to determine Ca^{2+} fluxes, ^{45}Ca uptake was measured in control erythrocytes loaded either with two different concentrations of Benz2-AM (Fig. 1a) or with the same concentration of Quin2-AM or Benz2-AM (Fig. 1b). In both cases, Ca^{2+} influx, measured from the initial rate of the uptake, was found to be the same. In six experiments using either Quin2-AM or Benz2-AM no significant difference in the values of Ca^{2+} influx was found (Table I).

The observation of control and chelator-loaded erythrocytes by dark field light microscopy did not reveal any difference in their morphology (results not shown). Cholesterol-depleted (chelator-loaded or unloaded) were mostly stomatocytes and spherostomatocytes as reported before [20].

We have measured also the ATP content of the cells under our experimental conditions since Tiffert et al. [25] reported that the incorporation of chelators induced substantial and irreversible loss of ATP. Both chelators did actually cause a diminution of the ATP level (Table I). However this diminution was higher with Quin2 than with Benz2. As reported previously, this was probably because the intracellular concentration of Benz2 was lower than that of Quin2 [21]. After washing out the chelator and reincubating the cells for 180 min, the ATP content was partially restored with Quin2 and totally with Benz2. The ATP level of the cholesterol-depleted cells was not significantly

TABLE I

THE EFFECT OF DIFFERENT Ca^{2+} CHELATORS ON THE ATP CONTENT AND ON THE Ca^{2+} INFLUX

Erythrocytes were incubated either with Benz2-AM or Quin2-AM (chelator loading), washed and reincubated for 180 min (Ca^{2+} influx measurement) as described under Materials and Methods. The ATP content is expressed as percentage of its value before chelator loading (A), after chelator loading (B) and at the end of the Ca^{2+} flux experiments (C). Internal chelator content was about 200–250 μmol/l cells for Quin2 and about 100 μmol/l cells for Benz2. Before chelator loading, the ATP content (mmol/l cells) was 0.86 ± 0.09 ($n = 9$) and 0.75 ± 0.08 ($n = 9$), respectively, in control and in cholesterol-depleted cells. Values are means ± S.E. of 3–6 experiments (number in parentheses) or means of two experiments. n.d., not determined.

	Erythrocytes:	ATP content (% of initial value)		Ca^{2+} influx (μmol/l cells per h)
		Control	Cholesterol-depleted	Control
	A	100	100	
Quin2	B	70.6 ± 8.3 (5)	73.1 ± 6.9 (6)	
	C	81.0 ± 7.7 (4)	78.2 ± 7.2 (5)	11.6 ± 1.6 (6)
Benz2	B	88.4 ± 3.8 (5)	82.2 ± 5.0 (5)	
	C	122 (2)	n.d.	14.9 ± 2.8 (6)

different from that in control cells either before or after chelator loading.

Ca^{2+} influx

Chelator-loaded control and cholesterol-depleted erythrocytes were incubated in a medium containing ^{45}Ca (1 mM) (Fig. 2a). The ^{45}Ca content of the cells was measured at different times ($^{45}Ca_t$). It increased over 180 min up to an equilibrium value ($^{45}Ca_{eq}$) of about 15–16 μmol/l

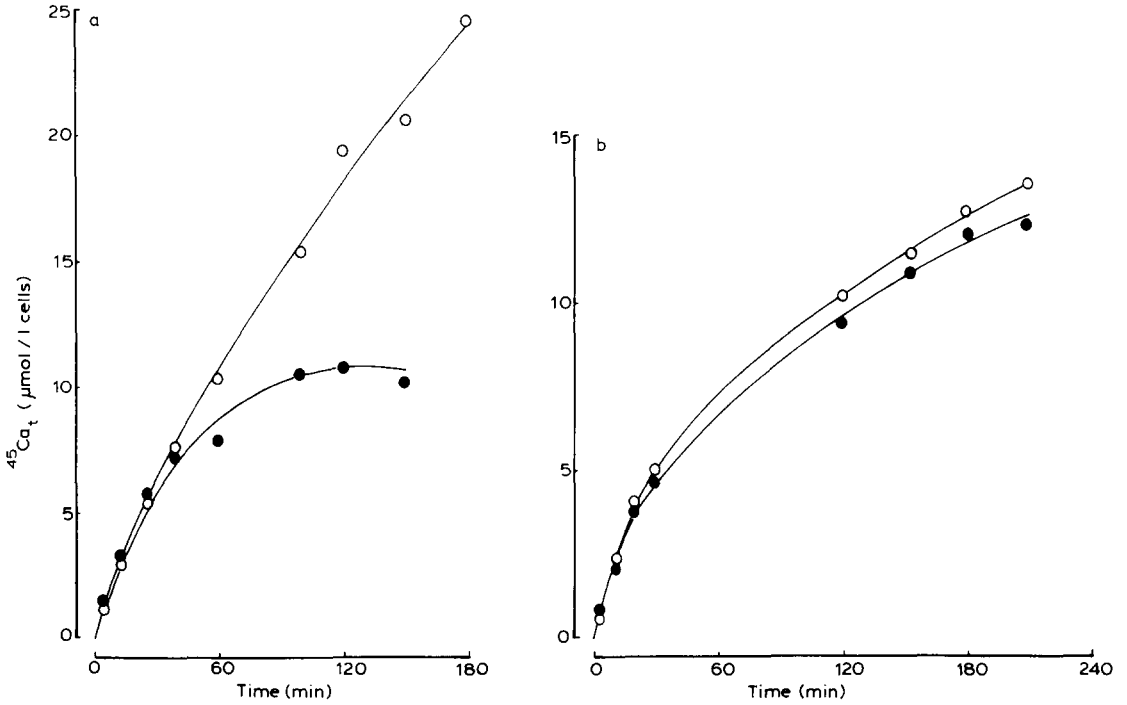

Fig. 1. ^{45}Ca uptake by chelator-loaded control erythrocytes. (a) Cells were incubated with Benz2-AM either 500 (●) or 1500 (○) μmol/l cells in solution C, washed and reincubated in the same solution containing ^{45}Ca. Ca^{2+} influx, determined from the initial rate of ^{45}Ca uptake, was 12.6 (●) and 12.0 (○) μmol/l cells per h. (b) Cells were incubated with either Benz2-AM (500 μmol/l cells) ○) or Quin2-AM (520 μmol/l cells) (●) and treated as in (a). Ca^{2+} influx determined as in (a) was 12.1 (○) and 11.8 (●) μmol/l cells per h.

cells in this experiment. The plot of $\log(^{45}Ca_{eq} - ^{45}Ca_t) = f(t)$ (Fig. 2b) was used to determined the rate constant for the influx (k_i). The semi-logarithmic plot was linear for control erythrocytes, indicating that exchangeable Ca^{2+} behaved as one homogeneous compartment in these cells. Ca^{2+} influx, calculated from $^{45}Ca_{eq} \cdot k_i$ was 13.7 μmol/l cells per h. In cholesterol-depleted cells, the plot was biphasic, revealing the existence of two compartments of exchangeable Ca^{2+}: the first one (rapid exchange) comprised 25% and the second one (slow exchange) 75% of the total exchangeable Ca^{2+}. In other experiments, these proportions were somewhat different (see for instance Fig. 3). Ca^{2+} influx in cholesterol-depleted cells was calculated from k_i estimated from the linear part of the curve of Fig. 2b (corresponding to the slow exchange). Justification of this procedure will be discussed below. Under these conditions, Ca^{2+} influx in cholesterol-depleted cells was 11 μmol/l cells per h.

Ca^{2+} efflux

Chelator-loaded control and cholesterol-depleted erythrocytes were incubated with ^{45}Ca (1 mM) for 120 min, washed and reincubated with ^{40}Ca (1 mM) (Fig. 2a). The ^{45}Ca content of the cells was measured at different times ($^{45}Ca_t$), for up to 80 min in this experiment. The plot of log ($^{45}Ca_t$) $= f(t)$ Fig. 2b) was used to determine the rate constant for the efflux (k_e). Ca^{2+} efflux was calculated from $^{45}Ca_{eq} \cdot k_e$ ($^{45}Ca_{eq}$ being the equilibrium value obtained from an influx experiment carried out with the same cells). The kinetics of Ca^{2+} efflux were the same as those found for Ca^{2+} influx, monophasic for control and biphasic for cholesterol-depleted cells. In this experiment, Ca^{2+} efflux was 13.2 μmol/l cells per h in control

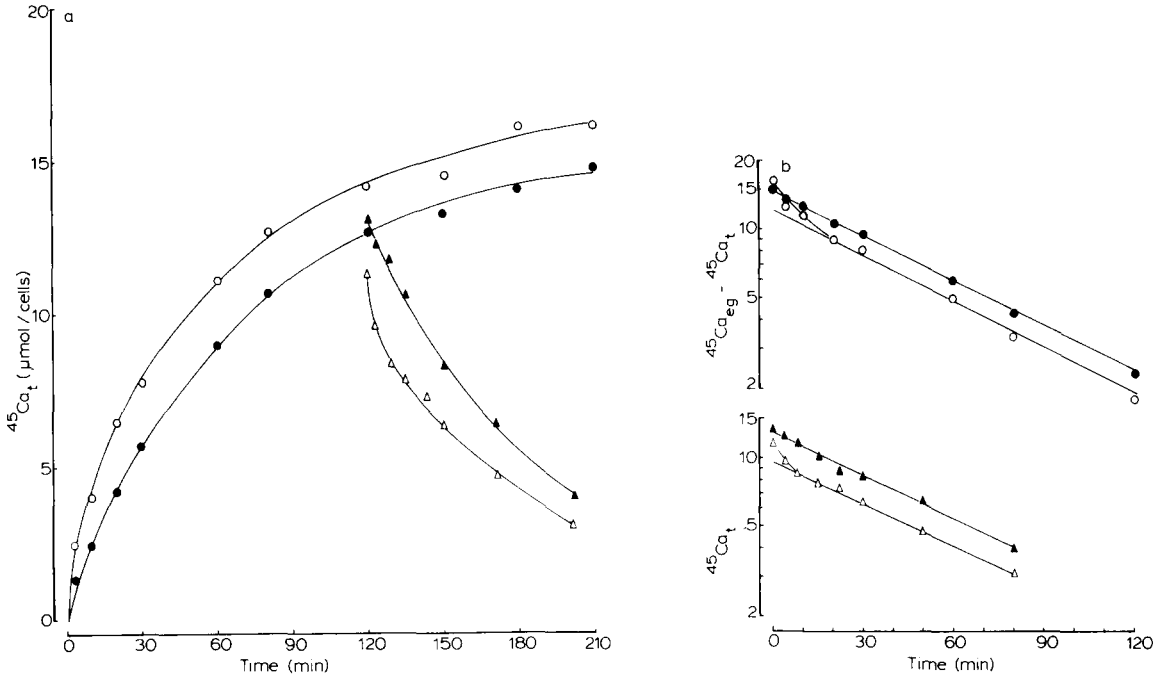

Fig. 2. ^{45}Ca content of cells during influx and efflux. Control and cholesterol-depleted cells were incubated with Benz2-AM (500 μmol/l cells), respectively, in solution A and B, washed in solution C and incubated in solution C (+^{45}Ca) for Ca^{2+} influx measurement (●, ○). An aliquot of the ^{45}Ca-loaded cells was washed in solution C and reincubated in the same solution for Ca^{2+} efflux measurement (▲, △). (a) ^{45}Ca content as a function of time ($^{45}Ca_t$) in control (●, ▲) and in cholesterol-depleted cells (○, △). (b) Semi-logarithmic plot of the difference between $^{45}Ca_{eq}$ (equilibrium value of $^{45}Ca_t$) and $^{45}Ca_t$ (influx) or of $^{45}Ca_t$ (efflux) as a function of time. Ca^{2+} fluxes were calculated from $^{45}Ca_{eq}$ and from the slopes of the linear part of these curves, as described under Materials and Methods. In this experiment, Ca^{2+} influx was 13.7 and 11.0 μmol/l cells per h, respectively, in control and in cholesterol-depleted cells and Ca^{2+} efflux 13.2 and 10.2 μmol/l cells per h. A typical experiment is presented. Six others (influx and efflux) and four others (influx only) gave similar results.

and 10.2 μmol/l cells per h in cholesterol-depleted cells (using the linear part of the curve of Fig. 2b to calculate k_e). In each experiment, both in control in control and in cholesterol-depleted cells, the value for Ca^{2+} efflux did not differ from that for Ca^{2+} influx by more than 10%.

Ca^{2+} fluxes were also measured in cholesterol-maintained erythrocytes (which have been incubated with cholesterol-phosphatidylcholine vesicles containing the two lipids in a molar ratio of 0.85 and therefore did not modify the cholesterol content of the erythrocytes). Both Ca^{2+} fluxes, in these cells, were not significantly different from those in control erythrocytes measured in the same experiments (results not shown).

Identification of the rapidly exchangeable Ca^{2+} pool in cholesterol-depleted cells

In order to determine whether the rapid pool in cholesterol-depleted cells could correspond to externally bound ^{45}Ca, their ^{45}Ca content was measured after washes either in D (isotonic phosphate saline) (Fig. 3a, curve A) or in D containing 100 μM EGTA (Fig. 3a, curve A') in the same experiment. The ^{45}Ca content (cpm/10 μl cells) was decresed by about 200 cpm in cholesterol-depleted cells with the second procedure whereas it was not changed in control cells (results not shown). The semi-logarithmic plot of curves A and A' (Fig. 3b) exhibited both a biphasic pattern. Therefore, the rapid pool could not be attributed only to ^{45}Ca bound at the external cell surface.

Since cholesterol-depleted erythrocytes exhibit stomatocytic shape with invaginations [20], a possibility was that this pool corresponded to ^{45}Ca entering the cells through endocytosis during the incubation. However, when chelator-unloaded cholesterol-depleted erythrocytes were incubated ^{45}Ca (1 mM) and EGTA (1 mM) (Fig. 3a, curve C'), no measurable radioactive uptake was detected, thus eliminating a simple resealing process to explain the rapidly exchangeable pool. Cholesterol depletion causes a slight haemolysis. Rapid resealing of some of these cells before the Ca^{2+} flux experiments could give rise to leaky cells with increased cationic permeability. To test this possibility, chelator-unloaded cholesterol-depleted cells were incubated with ^{45}Ca (1 mM) (Fig. 3a, curves B and B'). A measurable uptake of ^{45}Ca was observed, whereas in chelator-unloaded control cells, ^{45}Ca uptake was hardly detectable (results not shown). In addition this uptake was reversible

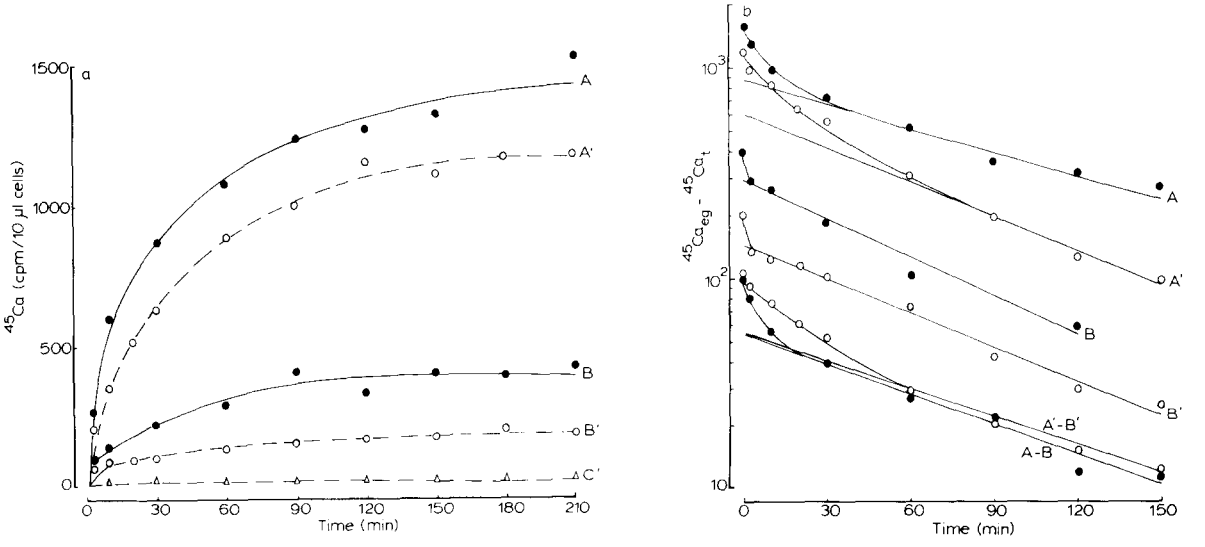

Fig. 3. ^{45}Ca uptake by Quin2-loaded (A, A') or unloaded (B, B', C') cholesterol-depleted cells. Cells were incubated without or with Quin2-AM in solution B, washed in solution C and incubated in solution C containing 1 mM ^{45}Ca (A, A', B, B') or 1 mM ^{45}Ca and 1 mM EGTA (C'). (a) ^{45}Ca content of the cells was measured after three washes either in solution D (A, B) or in solution D containing 0.1 mM EGTA (A', B', C'). (b) Semi-logarithmic plot of $^{45}Ca - ^{45}Ca_{eq}$ (see Fig. 2). A typical experiment is presented. Two others gave similar similar results.

upon reincubation in a ^{45}Ca-free medium (results not shown). All these observations indicate that the rapid pool could be attributed to exchangeable Ca^{2+} within leaky cells.

The semi-logarithmic plot of curve B and curve B′ were mostly monophasic (Fig. 3b). Therefore, it was expected that the difference between curves A and B (or between curves A′ and B′), by eliminating the leaky fraction, should represent ^{45}Ca uptake only in the impermeable chelator-loaded cholesterol-depleted cells. In fact, the semi-logarithmic plots of (A − B) or (A′ − B′) were again biphasic (Fig. 3b). The rapid pool observed under these conditions was attributed to the existence of an additional fraction of leaky cells created by the chelator loading procedure. This was shown by measuring the extent of haemolysis. Haemolysis was higher in cholesterol-depleted than in control cells without chelator, and enhanced in both types of cells by the chelator incorporation. It never exceeded 5% (results not shown).

In conclusion, only the slowly exchangeable Ca^{2+} pool had to be considered for flux or $[Ca]_i$ calculation in cholesterol-depleted erythrocytes, the rapidly exchangeable one corresponding to an artefact created by the presence of leaky cells.

Correlations between the diminution of the Ca^{2+} fluxes and of the $[Ca]_i$ and the cholesterol content

The Ca^{2+} fluxes, calculated as explained above in 11 experiments, were found to be decreased by cholesterol depletion. The values of the Ca^{2+} fluxes (either the influx or the mean value of both influx and efflux) were plotted as a function of the cholesterol content (Fig. 4a). A positive correlation was obtained between the two parameters ($r = 0.82$).

Knowing the concentration of the intracelluar chelator, $[chelator]_i$, the concentration of the total intracellular Ca^{2+} ($^{45}Ca_{eq}$) and the dissociation constant of the Ca-chelator complex (K_d), one can calculate $[Ca]_i$ (see Methods). $[chelator]_i$ was estimated either by [^{3}H]Quin 2-AM incorporation or from ^{45}Ca uptake in chelator-loaded ATP-depleted cells equilibrated with Ca-EGTA buffers and Ca^{2+} ionophore. As discussed before [21] the former method gave slightly overestimated value of $[chelator]_i$ relative to the latter one, leading to an underestimate of $[Ca]_i$. However, this should not distort the results to any considerable extent when one compared the values of $[Ca]_i$ in control to those in cholesterol-depleted cells. In eight experiments, $[Ca]_i$ was found to be lowered by

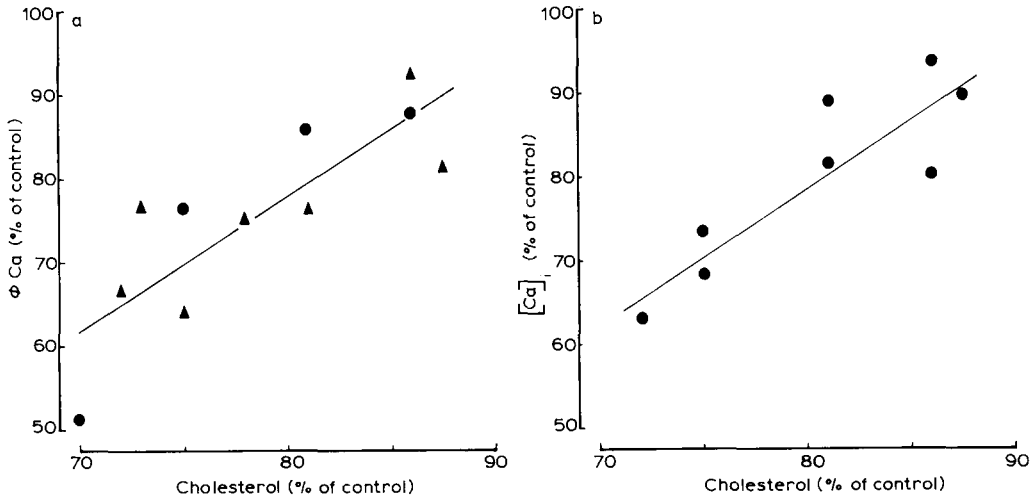

Fig. 4. Ca^{2+} fluxes and $[Ca]_i$ as a function of the cholesterol content. (a) Ca^{2+} influx (●) or Ca^{2+} influx and efflux (▲, mean value) (ϕ_{Ca}) and (b) $[Ca]_i$ were measured in chelator-loaded control and cholesterol-depleted cells as described under Materials and Methods. ϕ_{Ca} and $[Ca]_i$ (expressed as % of their values in control cells in the same experiment) are plotted as a function of the cholesterol content (expressed as % of its value in control cells). The lines were obtained by linear regression analysis: (a) $r = 0.82$; (b) $r = 0.88$.

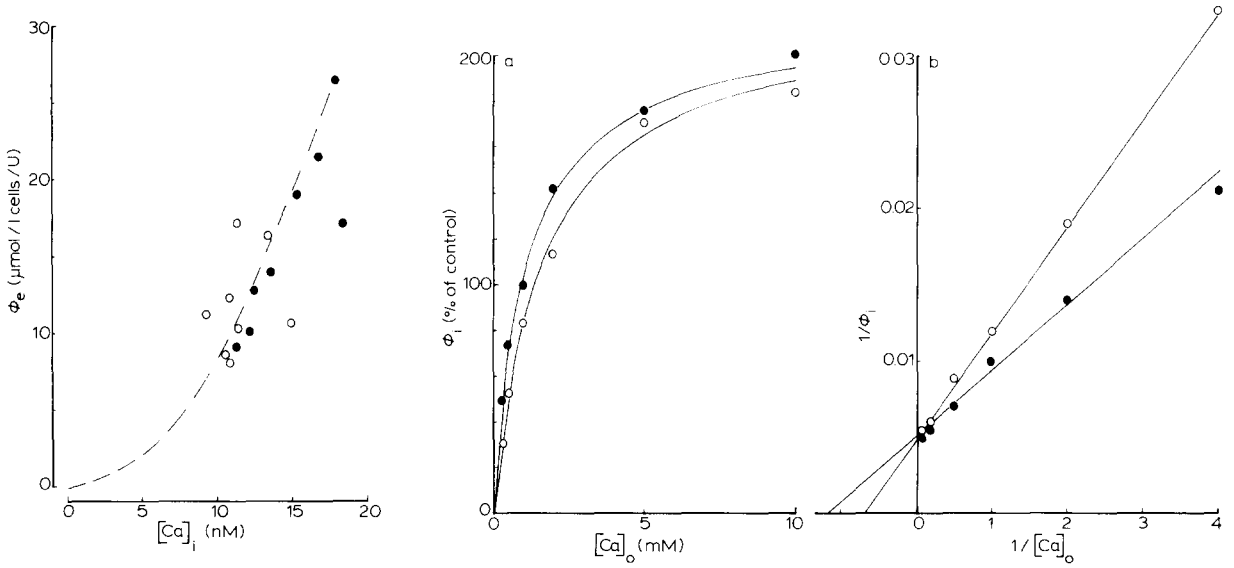

Fig. 5. (Left). Ca^{2+} efflux as a function of $[Ca]_i$. Ca efflux (ϕ_e) and $[Ca]_i$ were measured in chelator-loaded control (●) and cholesterol-depleted cells (○) as described under Materials and methods. The dotted line corresponds to an equation of the form: $\phi_e = A[Ca]_i^2$ in which A is expressed in μmol/l cells per h per nM^2 (see Ref. 9). The value of A calculated for control cells (0.073 ± 0.004) was not significantly different from that corresponding to cholesterol-depleted cells (0.090 ± 0.011). The mean value of A (0.081) was thus used to draw the dotted line on the figure.

Fig. 6. (Right). Ca^{2+} influx as a function of $[Ca]_o$. Control (●) and cholesterol-depleted cells (○) were loaded with Quin2-AM in solutions A or B containing EGTA (0.1 mM) in place of $CaCl_2$. They were wshed in solution C, also containing EGTA (0.1 mM) in place of $CaCl_2$, and incubated in solution C containing the indicated concentrations of $^{45}CaCl_2$. Ca^{2+} influx was determined from the initial rate of ^{45}Ca uptake in control cells and from the rate of ^{45}Ca uptake between 20 and 60 min in cholesterol-depleted cells, a period chosen to eliminate the rapidly exchangeable Ca^{2+} pool in these cells (see Figs. 2b and 3b). (a) Ca^{2+} influx (ϕ_i, expressed as % of its value in control cells at $[Ca]_o = 1$ mM) as a function of $[Ca]_o$; (b) double-reciprocal plots ($1/\phi_i = f(1/[Ca]_o)$). The lines were obtained by linear regression analysis ($r = 0.99$ in both cases). Maximal influx was 194 and 190%, respectively, in control and in cholesterol depleted cells and K_{Ca} (dissociation constant of the Ca-transporter complex) was 0.79 and 1.21 mM. A typical experiment is presented. Two others gave similar results.

cholesterol depletion (Fig. 4b). A positive correlation was obtained between $[Ca]_i$ and the cholesterol content ($r = 0.88$).

Cholesterol affects the mechanism of Ca^{2+} entry

The results presented above indicate that cholesterol depletion leads to a diminution of the turnover rate of intracellular Ca^{2+} in erythrocytes, together with a reduction of the steady-state $[Ca]_i$ both being proportional to the extent of cholesterol depletion. Because the mechanism of Ca^{2+} entry (simple diffusion, carrier-mediated or Ca^{2+} channel) is independent of that of Ca^{2+} exit (Ca^{2+} pump), it was investigated whether one, the other, or both mechanisms were affected.

To analyze the characteristics of the Ca^{2+} pump, Ca^{2+} efflux (ϕ_e) was plotted as a function of the $[Ca]_i$ for both control and cholesterol-depleted cells in eight experiments (Fig. 5). It was assumed, as shown before by Lew et al. [9], that the dependence of the pump rate on $[Ca]_i$ in the nanomolar range, is described by $\phi_e = A[Ca]_i^2$. The points corresponding to control cells could satisfactorily fit a parabolic curve with a mean value for A equal to 0.073 ± 0.004 μmol/l cells per h per nM^2. The points corresponding to cholesterol-depleted cells presented a larger scatter but the mean value of A (0.090 ± 0.011 μmol/l cells per h per nM^2) was not significantly different from that in control cells. It thus appears that the Ca^{2+}-pump characteristics were not modified by cholesterol depletion and that the relative inactivation of the Ca^{2+} pump was the result of a decrease in the $[Ca]_i$.

To explore the possible modifications of the mechanism of Ca^{2+} entry, Ca^{2+} influx (ϕ_i) was measured in chelator-loaded control and cholesterol-depleted erythrocytes as a function of the external Ca^{2+} concentration ($[Ca]_o$) (Fig. 6a). Ca^{2+} influx approached a plateau value at about 5 mM $[Ca]_o$ in both cases. Assuming that Ca^{2+} influx is a carrier-mediated transport, the maximal flux (ϕ_m) and the apparent dissociation constant of the Ca-transporter complex (K_m) can be calculated from the linear regression analysis of the double-reciprocal plot: $1/\phi_i = \phi_m/K_m + [Ca]_o$ (Fig. 6b). Cholesterol depletion did not change ϕ_m but decreased the apparent affinity of the transporter for $[Ca]_o$ (K_m was 0.79 mM in control and 1.21 mM in cholesterol-depleted cells).

Discussion

In this study, Ca^{2+} fluxes have been measured in erythrocyte loaded with membrane-permeant Ca^{2+} chelator. The incorporation of the chleator ester is followed by its hydrolysis and by the entry of a certain amount of Ca^{2+} which binds to the chelator. This extra Ca^{2+} behaves as an exchangeable pool and thus permits the measurements of the Ca^{2+} fluxes and the determination of the $[Ca]_i$ which becomes an easily defined function of this pool and of the chelator concentration [9].

Ca^{2+} influx measured using two different chelators (Benz2 or Quin2) or two different concentrations of the same chelator were equal. This indicates that neither the level nor the nature of the intracellular chelator affects the permeability of the membrane. The former conclusion is in agreement with that of Lew et al. [9]. However, these authors reported a 10-fold stimulation of Ca^{2+} influx in Quin2-loaded cells relatively to Benz2-loaded cells [25], an effect that we were not able to detect. Morphological observation of chelator-loaded erythrocytes did not reveal any difference realtively to unloaded erythrocytes, which suggests that the $[Ca]_i$, a critical determinant of the erythrocyte shape, was not modified by the presence of the chelator.

A disadvantage of the use of the intracellular chelators is the formation of formaldehyde resulting in ATP depletion [25]. In our experiments, this latter was mimized by pretreating the cells with adenine and inosine and by using low concentration of chelators. Under these conditions, the ATP content of the erythrocytes was maintained around 0.6–0.8 mmol/l, a range compatible with normal activity of the Ca^{2+}-pump ATPase.

Cholesterol is known to restrict the motion of the disordered fatty acid chains of phospholipids in artificial or biological membranes [26,27]. Thus, cholesterol addition results in a decrease in membrane fluidity at temperatures above the phase transition temperature and cholesterol removal, in an increase in membrane fluidity [28]. It has been often suggested that these fluidity changes could explain the changes in the activities of membrane-bound enzymes induced by the manipulation of the cholesterol content. However, conflicting results were obtained about the effect of cholesterol on the Ca^{2+}-ATPase. In some studies, cholesterol was reported not to affect [29–32] and in others to modulate this enzyme activity [33–35]. The only work concerning the erythrocyte membrane indicates that cholesterol removal had no effect on the Ca^{2+}-ATPase activity [31]. The effect of cholesterol on carrier-mediated transports has been studied less. The transport of anions [15,36] and the furosemide-sensitive Na^+-K^+ cotransport [14] are both inhibited in cholesterol-loaded erythrocytes, whereas the carrier systems for monocarboxylates and monosaccharides are activated by cholesterol and inhibited by its removal [36].

The main conclusion drawn from this study is that the primary effect cholesterol depletion in erythrocytes is on the system responsible for Ca^{2+} entry. When Ca^{2+} influx was measured as a function of the external concentration of Ca^{2+}, a curve saturating at about 5 mM was obtained. This observation is in agreement with previous work carried out with ATP-depleted [37], vanadate-treated [2] or Benz2-loaded erythrocytes [25]. The saturation of Ca^{2+} influx and its inhibition by nitrendipine in erythrocytes at concentrations similar to those used in heart and smooth muscle cells had led to the suggestion that erythrocytes possess a Ca^{2+} channel [3]. We have observed that cholesterol depletion did not change the maximal velocity across this transport system but decreased its affinity for Ca^{2+}. Interestingly similar effects of cholesterol manipulations on the Ca^{2+} influx in

erythrocytes have been reported [3,17]. However, in these studies ^{45}Ca uptake was measured in the presence of vanadate, which inhibits the ATPases and thus leads to Ca^{2+} accumulation within the cells. Nevertheless, the agreement between the results obtained by these different methods strengthen the idea that cholesterol interferes with the system responsible for Ca^{2+} entry, whether it is a channel or not.

The reduction of the Ca^{2+} influx in cholesterol-depleted erythrocytes leads to a decrease in the $[Ca]_i$ and this, in turn, to a diminution of the rate of Ca^{2+} efflux through the Ca^{2+} pump. Our results show that this diminution occurs without affecting the characteristics of the pump and thus are in agreement with a previous study in erythrocyte membranes indicating that cholesterol removal had no effect on the Ca^{2+}-ATPase activity [31].

Erythrocytes can be regarded as a usefull model of plasma membranes of other cells if one considers, for instance, the possible involvement of cholesterol in cardiovascular diseases in general or atherosclerosis in particular. In rabbits fed a cholesterol-rich diet, the development of atherosclerosis is associated with hypercholesterolemia, accumulation of cholesterol in nearly all organs as intracellular lipid droplets and incorporation into cell membranes and endothelial cell damage [38]. Interestingly, it has been shown that lanthanum which competes with calcium and nifedipine and verapamil, both drugs known as calcium antogonists or calcium channel blockers, were effective in preventing atherosclerosis in rabbits [39–41]. This suggests that a stimulation of the Ca^{2+} influx and an increase in the intracellular calcium concentration could be involved in the genesis of the disease. The correlation between the diminution of the cholesterol content and the diminution of the Ca^{2+} influx in erythrocytes reported in this study supports this hypothesis.

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