Biochimica et Biophysica Acta 903 (1987) 257–264
Elsevier

BBA 73686

Transport and control of Ca^{2+} by pigeon erythrocytes. III. A 'paradoxical' expulsion of Ca^{2+} induced by a low dose of A23187 at 0 °C

Jean W. Lee and George A. Vidaver †

Department of Chemistry, University of Nebraska, Lincoln, NE 68588-0304 (U.S.A.)

(Received 6 March 1987)

Key words: Calcium ion transport; Calcium ion expulsion; Ionophore A23187; Lysophosphatidylcholine; (Pigeon erythrocyte)

Pigeon erythrocytes expelled preloaded $^{45}Ca^{2+}$ in response to a low dose of A23187 at 0 °C. We call this phenomenon 'paradoxical' expulsion. Within the first minute, 1.85 ± 0.38 μmol/l cell water was expelled; after that the internal $^{45}Ca^{2+}$ began to rise. The rises in Ca^{2+} uptake with and without A23187 addition were essentially paralleled. No premonitory rise of $^{45}Ca^{2+}$ upon the addition of A23187 was observed. Expulsion of $^{45}Ca^{2+}$ in response to A23187 was probably by the action of the Ca^{2+} pump and not by Na^+–Ca^{2+} exchange since vanadate inhibited, but K^+ replacement of Na^+ in the medium had no effect. Lysophosphatidylcholine (lysoPC) caused an abrupt increase in $^{45}Ca^{2+}$ influx by cells at 0 °C and was dose dependent. However, a very low dose of lysoPC induced expulsion of preloaded $^{45}Ca^{2+}$ similar to that by A23187, the response was fast and transitory, without any premonitory rise in $^{45}Ca^{2+}$ uptake. The results lend support to the suggestion that the signal to which cells respond may be a sudden change in Ca^{2+} influx per se rather than a change in internal Ca^{2+} concentration. These features of 'paradoxical' $^{45}Ca^{2+}$ expulsion induced by A23187 and lysoPC are not expected from mass-action equilibria but, instead, agree with the characteristics of an energy-dissipating control mechanism.

Introduction

Cell Ca^{2+} is involved in metabolic control as a 'second messenger' like cAMP [1–4] and as a 'third messenger' elicited by inositol 1,4,5-trisphosphate from the hydrolysis of phosphatidylinositol 4,5-diphosphate [3,5]. Cytoplasmic Ca^{2+} concentration is controlled by processes distributing Ca^{2+} among various cell compartments and by entry and exit processes of the Ca^{2+} pump, leak, and the Na^+–Ca^{2+} exchanger.

We have studied the control of internal Ca^{2+} concentration by intact pigeon erythrocytes and found that they do not control their Ca^{2+} by a simple feedback mechanism regulated by mass-action equilibria of calcium-calmodulin association [6]. Among other features, the steady-state cell Ca^{2+} was approximately proportional to Ca^{2+} influx and was affected by external as well as internal Ca^{2+}. Also, trifluoperazine did not increase cell Ca^{2+} in the presence of divalent cation ionophore A23187 [7]. The failure of simple explanations by feedback control mechanisms raised questions about how cells actually sensed, classified and processed the information carried by Ca^{2+} influxes and levels. In the most elementary re-

† Deceased.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid.

Correspondence (present address): J.W. Lee, Harris Laboratories, Inc. P.O. Box 80837, Lincoln, NE 68501, U.S.A.

sponses, are signal sensing, processing and responses already discrete separate packages of operations? A related question is whether the central control mechanisms are mass-action equilibria or are continually energy-dissipating. The usual allosteric mechanisms [8] are examples of the first, while connected cycles and cascades of phosphorylation and dephosphorylation [8,9] are examples of the second.

When a low dose of A23187 was added to pigeon erytrocytes at 0 °C, instead of an increase in $^{45}Ca^{2+}$ uptake, we observed an expulsion of preloaded $^{45}Ca^{2+}$. We call this unexpected phenomenon 'paradoxical' expulsion. Another entirely different agent, lysophosphatidylcholine (lysoPC), caused an abrupt rise in $^{45}Ca^{2+}$ influx at 0 °C. However, a very low dose of lysoPC induced a similar 'paradoxical' expulsion of $^{45}Ca^{2+}$. We described herein this unusual behaviour of the cells in response to a signal of calcium change and the significance it implied: The behaviour observed parallels characteristic features of energy-dissipating control mechanisms rather than mass-action equilibria.

Materials and Methods

Materials were as previously described [6,7].

Pigeon erythrocytes were collected, washed and resuspended in an isoosmotic medium containing amino acids at the concentrations they occurred in chicken plasma [6]. The cells were kept at 0 °C in 33% cell suspension in this isotonic amino acid medium plus 184 μM $^{45}Ca^{2+}$. Loading of $^{45}Ca^{2+}$ was carried out by increasing $^{45}Ca^{2+}$ to 2.0 mM. Specific radioactivity of $^{45}Ca^{2+}$ was the same at 14.8 GBq/mol. After 20 min 3 μmol A23187 per kg cells (the stock solution was 0.2 mM in ethylene glycol/dimethylsulfoxide (2 : 1, v : v), the final concentration of A23187 in aqueous medium was 1.08 μM) or solvent blank was added. Aliquots (300 μl) of the cell suspension were withdrawn and processed by two different protocols. In the first (Fig. 1A) cells were diluted into 9 ml diluent at 0 °C containing 149 mM NaCl, 0.15 mM MgEGTA, 5 mM K^+-Tes, (pH 7.4) and 5 μM [^{3}H]maltitol which served as a marker for entrained medium. The cells were centrifuged within 4 min at 17370 × *g* for 2 min and washed once more with 140 mM NaCl and 5 mM K^+-Tes (pH 7.4). The total processing time was approx. 20 min. In the second method (Fig. 1B) cells were diluted into 9 ml diluent plus 2% (w/v) polyvinylpyrrolidone. After mixing, 3.3 ml 5.75% (w/v) sucrose, 74.5 mM NaCl, 5 mM K^+-Tes (pH 7.4) and 0.15 mM MgEGTA was carefully underlaid to form a density gradient to separate cells from incubation medium. The polyvinylpyrrolidone provided a viscous medium for better separation and less centrifugal damage. Cells were centrifuged promptly (within 2.5 min) in a swinging bucket rotor at 23500 × *g* for 2 min. The exact lag time of centrifugation from dilution was recorded to correct for the $^{45}Ca^{2+}$ loss during this interval (Fig. 2). After centrifugation, the supernatant was withdrawn by aspiration to about the last 0.3 ml which was removed carefully by a swab.

Cell pellets were extracted and $^{45}Ca^{2+}$ cpm (corrected for cpm from entrained medium) determined as described in Ref. 6. With the first protocol correction was minimal and many samples could be processed in the 8 place SS-34 rotor. For the second method approx. 0.04% of the incubation medium remained in the cell pellet and could amount to about 50% correction in samples of very small $^{45}Ca^{2+}$ cpm. These samples are the initial time points of $^{45}Ca^{2+}$ influx experiments; however, since $^{45}Ca^{2+}$ influx time-course curves are largely linear (Fig. 3), their accuracies are not crucial for slope estimation. The second method allows one to extrapolate $^{45}Ca^{2+}$ loss from zero time to include $^{45}Ca^{2+}$ both in the inaccessible pool and the accessible pool which was lost in the first method.

Concentration of $^{45}Ca^{2+}$ uptake was expressed as μM which is μmol $^{45}Ca^{2+}$/l cell water. Previously determined values of 0.675 ml cell water/g fresh cell pellet and 12% extracellular water were used for converting μmol/pellet to μmol/l cell water.

In some experiments cells were preincubated at 39 °C for 15 min. Cell suspensions, 20% in isotonic amino acid medium with 24.2 mM $NaHCO_3$ replacing the equivalent amount of NaCl, were incubated in a 25 ml flask with a side-arm in a 39 °C water bath with shaking. CO_2, 5% in air from a compressed-gas tank, was bubbled through water, and continuously flushed through the side-

arm opening into the flask atmosphere to form a pH 7.4 CO_2-HCO_3^- buffer system mimicking in vivo conditions. The top opening of the flask allowed easy access for additions and withdrawals. After incubation cells were diluted, centrifuged, and resuspended in isotonic amino acid medium and kept at 0 °C in 33% suspension. In order to decrease the basal $^{45}Ca^{2+}$, 50 μM 45 Ca^{2+} was used during the preincubation step.

For experiments where lysoPC was used, lysoPC dose was expressed as % of membrane phospholipid [7]. A stock solution was made by sonic dispersion of 2 mg lysoPC/ml cell-medium until clear. Instead of A23187, the aqueous lysoPC solution or cell medium (0% control) was added to the cell suspension.

Results

(1) A low dose of A23187-induced expulsion of preloaded $^{45}Ca^{2+}$

When 3 μM A23187 was added to cells incubated in 2 mM $^{45}Ca^{2+}$ at 0 °C, instead of a rise in $^{45}Ca^{2+}$ uptake, we observed an abrupt expulsion of preloaded $^{45}Ca^{2+}$ from these cells. This unexpected observation has been repeated many times in two different protocols (Figs. 1A and B). We call this phenomenon the 'paradoxical' expulsion of $^{45}Ca^{2+}$ induced by a low dose of A23187. In Fig. 1A the cells were washed twice and therefore exposed for approx. 20 min to low $^{45}Ca^{2+}$ during processing, so the $^{45}Ca^{2+}$ expulsion apparently occurring in the first 0.5–1 min might actually take approx. 20 min during processing.

A different protocol shows $^{45}Ca^{2+}$ expulsion occurs within about 1 min after A23187 addition (Fig. 1B). The loss is apparently limited in time since the subsequent solvent blank and A23187 curves are almost parallel (Fig. 1B, also seen in Fig. 1A). With the protocol employed for Fig. 1B, cells are still exposed for 1.5–2.5 min to low $^{45}Ca^{2+}$ dilution medium, but the data of Fig. 1B are corrected for $^{45}Ca^{2+}$ loss during this 1.5 to 2.5 min interval by using the $^{45}Ca^{2+}$ loss vs. time curve of Fig. 2 (see Methods). Note, there is still statistically significant $^{45}Ca^{2+}$ expulsion (0.73 ± 0.28 μM, $P < 0.025$, $n = 14$) without this correction. With this correction, $^{45}Ca^{2+}$ expelled in 1

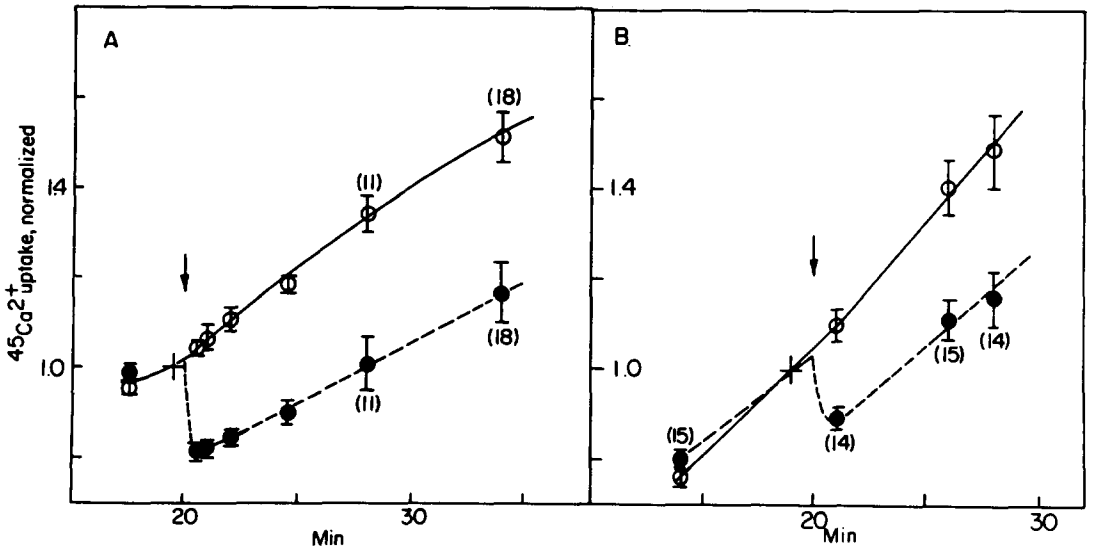

Fig. 1. Expulsion of $^{45}Ca^{2+}$ after the addition of a low dose of A23187 at 0 °C. Cells were preloaded with 2 mM $^{45}Ca^{2+}$ at 0 °C for 20 min, solvent blank (open circle, solid line) or 3 μmol A23187/kg cells (solid circle, broken line) was added (arrow at 20 min). $^{45}Ca^{2+}$ uptake was cellular concentration expressed as μM which is μmol $^{45}Ca^{2+}$/liter cell water. $^{45}Ca^{2+}$ uptake values are normalized against the uptake value before A23187 or solvent addition (+). This was 8.22 ± 0.62 μM ($n = 38$) and 9.00 ± 0.81 ($n = 30$) for (A) and (B), respectively. (A) Cells were washed twice according to the first method of processing, $n = 19$, except as note in parenthesis. (B) Cells were washed once according to the second method of processing, the loss of $^{45}Ca^{2+}$ during processing was corrected by using the curve of $^{45}Ca^{2+}$ loss vs. time of Fig. 2, n in parenthesis.

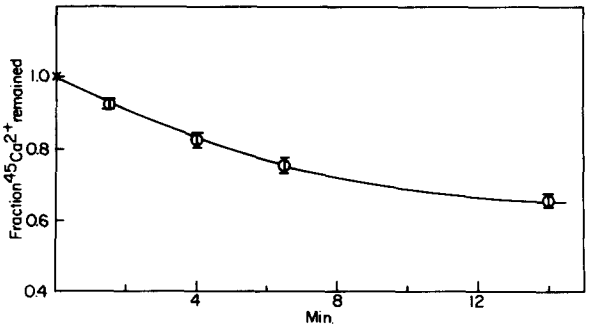

Fig. 2. $^{45}Ca^{2+}$ loss vs. time at 0 °C. Cells were loaded with 2 mM $^{45}Ca^{2+}$ at 0 °C. In some experiments they were preincubated at 39 °C at 50 μM $^{45}Ca^{2+}$ for 15 min (see Methods). Several aliquots were withdrawn from the same cell suspension at the same time, diluted, and held for various times at 0 °C before centrifugation. The diluting medium and processing is of the second method. The exact time of aliquot dilution and the start of centrifugation were recorded to give the exact processing time (*x*-axis). Each individual $^{45}Ca^{2+}$ uptake vs. time curve was plotted and $^{45}Ca^{2+}$ uptake at zero time was extrapolated, it was then used as the basal number of zero loss (1.0) to calculate the fraction $^{45}Ca^{2+}$ remained. Bars are standard deviation of the mean, $n = 8$. The mean was from results of duplicate experiments of four different preincubation conditions: (1) Na^+ medium, 2 mM Ca^{2+}, 0 °C for 15 min. (2) Na^+ medium, 2 mM Ca^{2+}, 48 μM A23187, 0 °C for 15 min. (3) K^+ medium, 50 μM Ca^{2+}, 39 °C for 15 min. (4) K^+ medium, 6 mM Vanadate, 50 μM Ca^{2+}, 39 °C for 15 min.

min is 1.85 ± 0.38 μM ($n = 14$), $P < 0.01$, from zero loss.

The foregoing data indicate addition of A23187 at 0 °C produces a rapid loss of preloaded $^{45}Ca^{2+}$ with no premonitory $^{45}Ca^{2+}$ rise. This loss is against a very large Ca^{2+} concentration gradient and it appears to last only a few minutes.

Fig. 2 shows that there is a rapid $^{45}Ca^{2+}$ loss into low Ca^{2+} dilution medium. The total $^{45}Ca^{2+}$ taken up was 11.00 ± 2.23 μM ($n = 8$). About 35% of this occupies an accessible pool of approx. 3.8 μM, most of which ws lost within the first 5 min. The remaining $^{45}Ca^{2+}$ is in a relatively inaccessible pool.

In Fig. 1A $^{45}Ca^{2+}$ loss occurs both before dilution into a low Ca^{2+} medium and for approx. 20 min thereafter. Ca^{2+} uptake depicted in Fig. 1A represents the $^{45}Ca^{2+}$ in inaccessible pools. The curves in Fig. 1B reflect $^{45}Ca^{2+}$ in both accessible and inaccessible pools. The two figures are very similar except that the average $^{45}Ca^{2+}$ uptake at 19 min in Fig. 1A is lower than that in Fig. 1B. In experiments where both protocols were used, the average 19-min uptake value was 5.31 ± 0.24 μM for the two-wash protocol and 7.43 ± 1.14 μM for the one-wash protocol ($n = 16$). This difference approximates the size of the accessible pool of Fig. 2.

Apparently 'paradoxical' expulsion affects the accessible pool and empties most of it. The $^{45}Ca^{2+}$ rise, resuming in both Figs. 1A and 1B a few minutes after A23187 or solvent blank addition, presumably represents movement of $^{45}Ca^{2+}$ from the accessible into the inaccessible pool(s).

We assume 3 μM A23187 acts because it increases Ca^{2+} influx at 0 °C. If so, only a modest increase in Ca^{2+} entry triggers the expulsion. This is inferred from the actions of a much larger dose of ionophore, 48 μM (Fig. 3). Cells were poisoned with 6 mM vanadate at 39 °C in K^+ medium and then kept at 0 °C in the same medium to minimize Ca^{2+} pump [10] and Na^+–Ca^{2+} exchange activities [11]. After 10 min at 0 °C with 2 mM Ca^{2+}, adding tracer $^{45}Ca^{2+}$ (arrow at 10 min) gave a linear $^{45}Ca^{2+}$ uptake rate of approx. 0.82 ± 0.15 μM/min ($n = 6$). At 20 min 48 μM A23187 was added. The influx rate increased to 1.97 ± 0.34 μM/min ($n = 6$) at 2 min after A23187 addition and to 1.90 ± 0.27 μM/min ($n = 6$) at 15 min after A23187 adition. The onset of A23187 action was

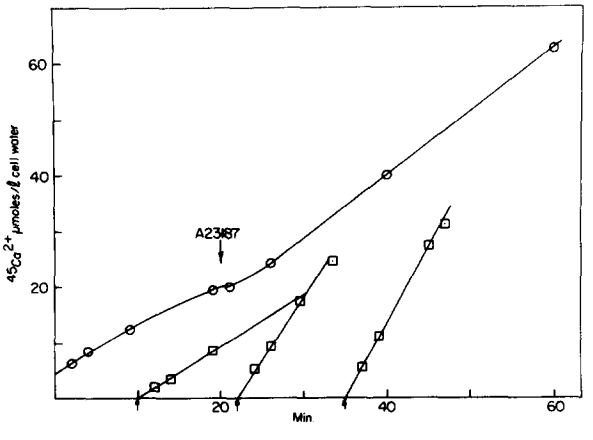

Fig. 3. Increase of $^{45}Ca^{2+}$ influx by the addition of 48 μM A23187 at 0 °C. Cells were preincubated in 6 mM vanadate and 127 mM KCl instead of NaCl. Tracer $^{45}Ca^{2+}$ was added at zero time to measure net uptake (○) or at later times (arrows) to other aliquots to measure $^{45}Ca^{2+}$ influx (□). A23187 (48 μmol/kg cells) was added at 20 min. This figure is representative of six experiments.

fairly rapid at 0 °C. It was detectable with tracer added at 2 min after A23187 addition (Fig. 3). Lower A23187 doses (24 and 12 μM) gave less than proportional increases in $^{45}Ca^{2+}$ uptake (not shown). Possible reasons included are: (i) Ca^{2+} influx is proportional to [A23187]^n with $n > 1$; (ii) the background Ca^{2+} entry changes to compensate for A23187-mediated entry; and (iii) active expulsion is not fully abolished.

We estimate Ca^{2+} uptake caused by 3 μM A23187 is $\leqslant 3/48$ times the incremental uptake with 48 μM A23187, or $\leqslant 0.07$ μM/min. The background $^{45}Ca^{2+}$ influx into unpoisoned cells in Na^+ medium at 0 °C was 0.69 ± 0.12 μM/min ($n = 12$). An approximate 10% increase in Ca^{2+} influx could be caused by the addition of 3 μM A23187.

A modest percentage increase in Ca^{2+} influx (approx. 10%) appears to trigger expulsion. It is also possible that the ratio of d(Ca^{2+} influx)/dt before and after A23187 is the signal; this ratio is not small.

(2) $^{45}Ca^{2+}$ was probably expelled by the action of the calcium pump

The preloaded $^{45}Ca^{2+}$ could be expelled by the Ca^{2+} pump or by Na^+–Ca^{2+} exchange. Cells were preincubated at 39 °C for 15 min with or without 6 mM vanadate and then brought to 0 °C for the $^{45}Ca^{2+}$ uptake study (see Methods). Preincubation

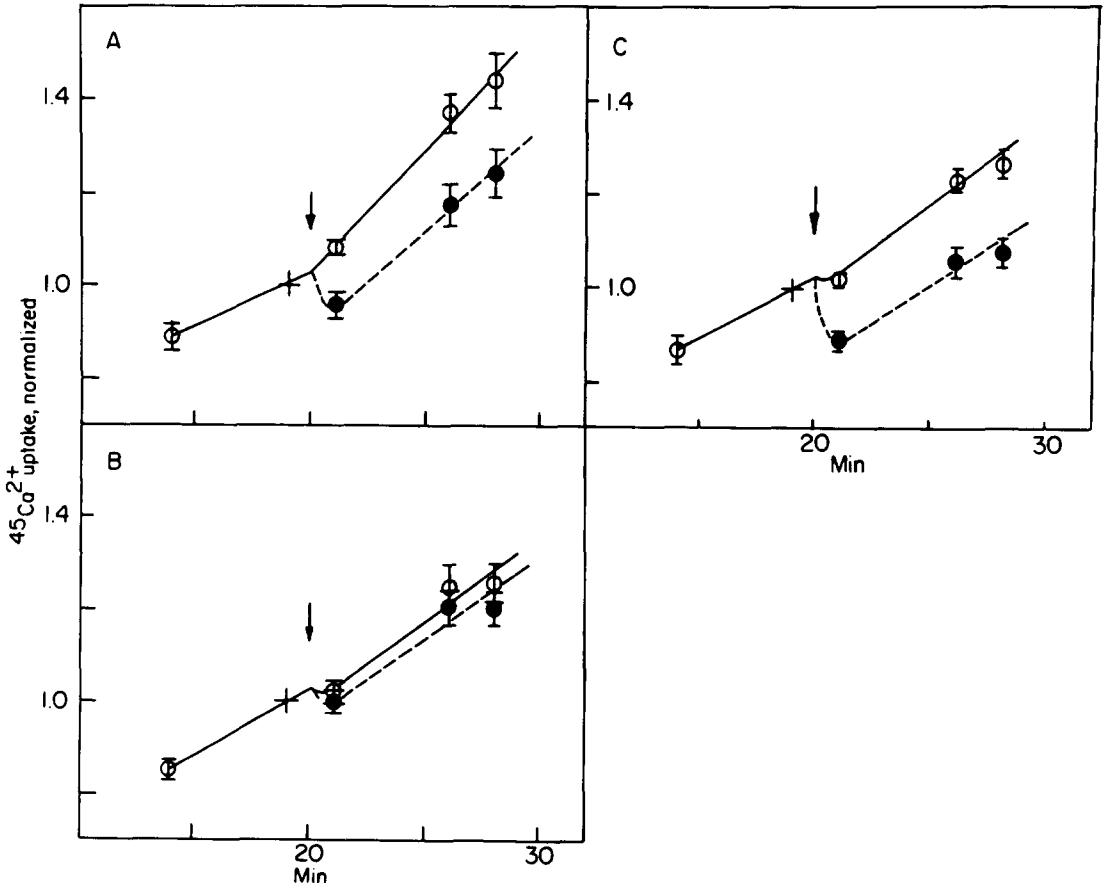

Fig. 4. Effect of vanadate and K^+ replacement of Na^+ on 'paradoxical' expulsion of $^{45}Ca^{2+}$ induced by A32187. Protocols were the same as those of Fig. 1B except that cells were preincubated at 39 °C for 15 min as described in Methods. Cells were preincubated, washed and resuspended in Na^+ medium (A), with 6 mM vanadate added to the Na^+ medium (B) or Na^+ was replaced by K^+ (C). The 19 min $^{45}Ca^{2+}$ uptake was 14.06 ± 0.48 μM for (A), 21.56 ± 1.06 μM for (B) and 13.05 ± 0.59 μM for (C), $n = 16$.

did not affect the ability of cells to respond to A23187, i.e., a similar 'paradoxical' expulsion of $^{45}Ca^{2+}$ was seen upon addition of A23187 (Fig. 4A). The amount of $^{45}Ca^{2+}$ expelled at one min after the A23187 addition ws 1.80 ± 0.37 μM ($n = 8$) $P < 0.005$. Pretreatment of cells with 6 mM vanadate resulted in much high $^{45}Ca^{2+}$ uptake (21.56 ± 1.06 μM at 19 min) than that of the control (14.06 ± 0.48 μM) probably due to the inhibition of Ca^{2+} pump by vanadate [10]. Under this condition $^{45}Ca^{2+}$ expulsion in response to A23187 became insignificant (Fig. 4B). When Na^+ in the cell medium was replaced by K^+ to inhibit the action of the Na^+–Ca^{2+} exchanger [11], cells expelled $^{45}Ca^{2+}$ in response to 3 μM A23187 identically to those in Na^+ medium. (Fig. 4C vs. 4A). The amount expelled in one minute was 1.77 ± 0.24 μM ($n = 8$) $P < 0.005$. These results indicate that $^{45}Ca^{2+}$ was expelled by the Ca^{2+} pump and not by the Na^+–Ca^{2+} exchanger.

(3) A low dose of lysoPC also induced a rapid 'paradoxical' expulsion of $^{45}Ca^{2+}$ somewhat similar to that of A23187

We have recently reported that lysoPC induced a rapid but transitory increase in $^{45}Ca^{2+}$ influx and an abrupt $^{45}Ca^{2+}$ net uptake by pigeon red cells at 39° C [7,12]. If a small percent increase in $^{45}Ca^{2+}$ influx triggers a 'paradoxical' expulsion of preloaded $^{45}Ca^{2+}$, then a low dose of lysoPC might also induce such a phenomenon.

First we addressed whether lysoPC could induce a rapid $^{45}Ca^{2+}$ influx at 0° C. Fig. 5 shows that lysoPC did, indeed, cause a rapid increase in $^{45}Ca^{2+}$ uptake at low doses (in our 33% cell suspension, the highest dose used, 4% of membrane phospholipid, is only 0.045 mg lysoPC/ml cell medium). The increase in $^{45}Ca^{2+}$ influx is dose-dependent (Fig. 6), with a shallow increase at very low doses (< 2%, broken line), which becomes steep and linear at higher doses (> 2%, solid line). When 0.5% lysoPC was added, instead of an abrupt increase in $^{45}Ca^{2+}$ uptake, we observed a 'paradoxical' expulsion of previously loaded $^{45}Ca^{2+}$ (Fig. 7). The magnitude is smaller than that induced by 3 μM A23187, only 0.81 ± 0.21 μM ($n = 9$) expelled after one min, however, the effect was significant ($P < 0.005$). The rapid, but transitory features of this lysoPC-induced expulsion are

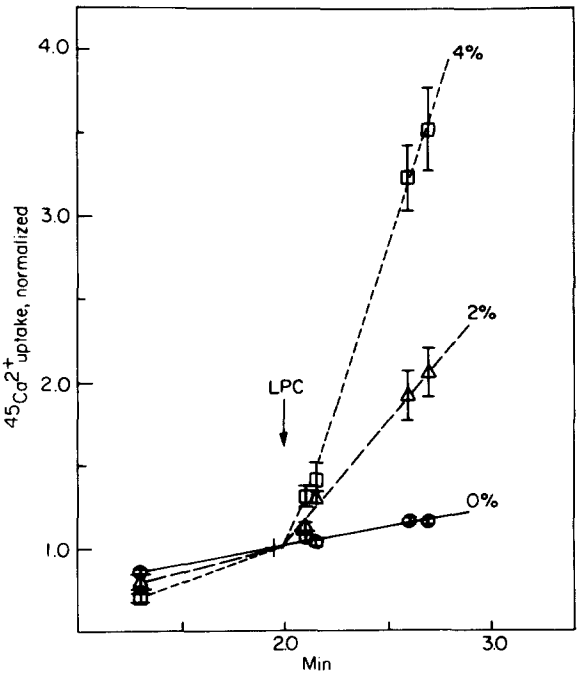

Fig. 5. Increase of $^{45}Ca^{2+}$ uptake by lysoPC (LPC) at 0° C. The method of Fig. 1B was used. The lysoPC dose was expressed as % of membrane phospholipid [7]. $^{45}Ca^{2+}$ uptake at 19.5 min was 6.80 ± 0.91 μM ($n = 4$), 7.12 ± 0.84 μM ($n = 4$) and 7.68 ± 0.93 μM ($n = 3$) for 0 (○), 2 (△) and 4% (□) lysoPC, respectively.

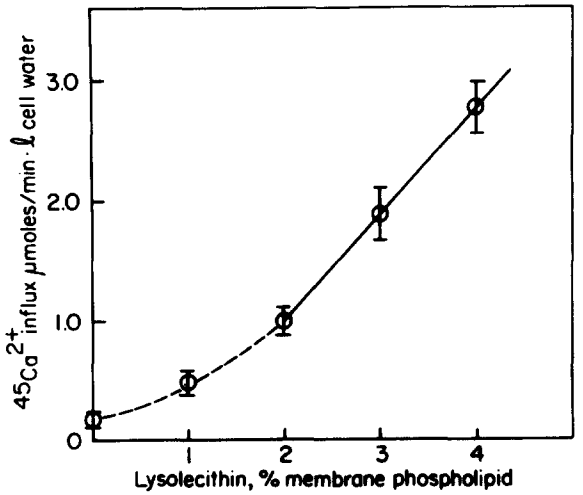

Fig. 6. Dependence of $^{45}Ca^{2+}$ influx on the lysoPC dose. $^{45}Ca^{2+}$ was calculated from the linear lines after lysoPC addition. Bars are standard deviations of the mean, $n = 4$, except for 4% lysoPC, $n = 3$.

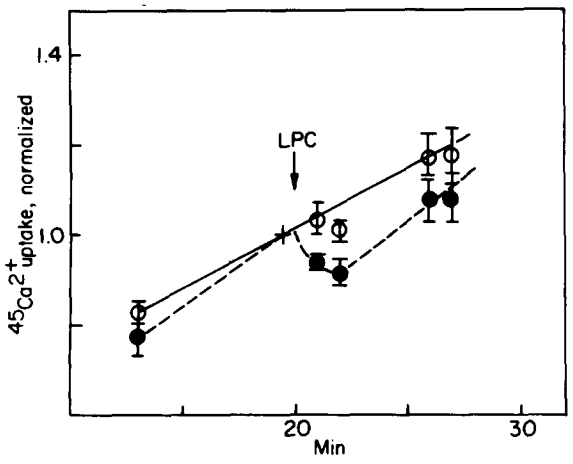

Fig. 7. Expulsion of $^{45}Ca^{2+}$ after the addition of a very low dose of lysoPC at 0 °C. The method of Fig. 1B was used except 0.5% lysoPC was added instead of 3 μM A23187 (solid circles, broken line), aqueous medium was used for the control (open circles, solid line). $^{45}Ca^{2+}$ uptake at 19.5 min was 6.44 ± 0.53 μM for the control and 6.58 ± 0.56 μM for lysoPC cells, $n = 9$.

also similar to that triggered by A23187.

After the transitory expulsion, $^{45}Ca^{2+}$ uptake by lysoPC-treated cells followed a steeper rise than the uptake of these without lysoPC. This feature differs from the almost parallel rise of both curves from cells with and without the A23187 addition (Fig. 1B). An obvious explanation is that lysoPC acts differently from A23187. Besides causing a sudden increase in $^{45}Ca^{2+}$ influx, lysoPC could also inhibit Ca^{2+} pump activity as has been shown in certain membrane vesicle preparations [12]. The inhibition of Ca^{2+} pump action is indicated by the linearity of $^{45}Ca^{2+}$ uptake curves when larger doses of lysoPC were used (Fig. 5). However, inhibition of the Ca^{2+} pump by lysoPC may take some time, so that a small and brief $^{45}Ca^{2+}$ expulsion triggered by the sudden increase of $^{45}Ca^{2+}$ influx could be observed. The steepr rise in $^{45}Ca^{2+}$ uptake by lysoPC cells than that of the control occurred later, indicating that the inhibition of the Ca^{2+} pump lags behind the rapid reponse of the 'paradoxical' expulsion.

Discussion

The first feature attracting our attention was expulsion of $^{45}Ca^{2+}$ at 0 °C in response to A23187, rather than increased uptake (or no change, i.e. perfect resistance to the uptake load). This suggested that at 0 °C, A23187-mediated influx into a peripheral pool acted as a discrete 'expel' signal. Perhaps at 0 °C subsequent steps accurately balancing influx and efflux did not occur, and thus allow this phenomenon to be detected. We did observe similar 'paradoxial' expulsion of $^{45}Ca^{2+}$ at higher temperatures under certain conditions but without very good reproducibilities. Therefore, only studies at 0 °C were persued further.

$^{45}Ca^{2+}$ was apparently expelled by the action of the Ca^{2+} pump and not by the Na^{+}–Ca^{2+} exchanger since preincubation with vanadate abolished the 'paradoxical' expulsion while incubation in a K^{+} medium had no effect. The maximum rate of Ca^{2+} pump activity in human red cells was reported to be 226 μmol/l cells per min [13]. The temperature dependence of the Ca^{2+} pump varies, with a steeper drop in activity below 27 °C [14]. Taking this into consideration, the Ca^{2+} pump is able to expel about 2 μmol/l per min at 0 °C, approximately the amount of the 'paradoxical' expulsion observed in our study.

A possible artifact producing apparent expulsion might be extraction of preloaded $^{45}Ca^{2+}$ from cells by the added A23187 and redeposit of $A23187_2 \cdot {}^{45}Ca^{2+}$ back into the medium at the subsequent dilution step. However, the A23187 enters cells from a medium with 2 mM $^{45}Ca^{2+}$ of at least as high specific radioactivity as that already in the cells. Therefore, almost no $^{45}Ca^{2+}$ from the medium could be carried into the cell membrane by the A23187. The concentration of 3 μmol A23187/kg cell corresponds to a concentration of 0.45 μM A23187 in the aqueous medium of 33% cell suspensions since 60% of A23187 was estimated to be partitioned in cell membranes [16]. A23187 would need to form a 1 : 1 complex with Ca^{2+} (it is reported to form a 2 : 1 complex with a $K_d = 3.7 \cdot 10^{-7}$ [15]) in order to extract as much $^{45}Ca^{2+}$ as appears to be expelled. Thus, the artifactual mechanism would need to be too efficient to be even plausible.

Pigeon red cells contain a small amount of mitochondria [17] which can store Ca^{2+}. Another artifactual source of $^{45}Ca^{2+}$ expulsion could be induced by A23187 releasing the stored Ca^{2+} from mitochondria. However, the external Ca^{2+} con-

centration (2 mM) is still higher than the total internal Ca^{2+} (50 μM [6]), which would maintain a concentration gradient against $^{45}Ca^{2+}$ efflux even if all the compartmentalized Ca^{2+} was equilibrated by A23187 action. We cannot rule out the possibility of an uneven distribution of internal cell free Ca^{2+} (an intracellular Ca^{2+} gradient has been reported in certain cells [18,19]). In that case, A23187 could cause a sudden release of internal Ca^{2+} near the surface membrane resulting in a local Ca^{2+} concentration in, (i) mM range to reverse the concentration gradient, or (ii) μM range to activate the Ca^{2+} pump, causing apparent $^{45}Ca^{2+}$ expulsion without showing a premonitory rise of $^{45}Ca^{2+}$. In case (ii), there is an actual increase in $[Ca^{2+}]_i$ to cause the activation of the Ca^{2+} pump and an apparent 'paradoxical' Ca^{2+} expulsion. The signal is $d[Ca^{2+}]_i$. However, an uneven distribution of intracellular free calcium is not a feature of the simple mechanism of mass-action equilibria.

If we do not make the assumption of uneven internal Ca^{2+} distribution then A23187 did cause a 'paradoxical' expulsion of $^{45}Ca^{2+}$ without a premonitory rise. The lack of a premonitory $^{45}Ca^{2+}$ rise suggested that a change (increase) in Ca^{2+} influx per se might be the signal for expulsion rather than the resultant increase in cell Ca^{2+}. A similar 'paradoxical' expulsion induced by a completely different agent, lysoPC, supports this suggestion. This is analogous to the chemotactic proteins in *Escherichia coli* where the changes in protein methylation level, not the methylation levels themselves, are the 'signals' [20].

The abrupt onset of the response suggests a system poised, awaiting a trigger to utilize large potentially available energy fluxes. If the stimulus for 'paradoxical' expulsion is a approx. 10% increase in Ca^{2+} influx, and the accessible pool is (nearly) emptied in response, this is a large 'signal amplification' and implies an energy-dissipating control mechanism.

The short duration of Ca^{2+} expulsion is not expected from displacement of mass-action equilibria; these would be expected to eventually return to near their original state. Such time-limited responses are shown by the energy-dissipating chemotactic system of *E. coli*. Note that here, signal sensing, processing and responses are discrete 'packaged' sets of operations. As a prokaryotic signal processing system it suggests that complex, computer-like information processing evolved early [21]. For eukaryotes, the energy-dissipating control mechanism may also represent the standard, commonly encountered mode, with Ca^{2+} control by pigeon erythrocytes as a not atypical case.

Acknowledgement

We thank Cynthia Stryker for her excellent technical assistance.

References

1 Gennaro, R., Pozzan, T. and Romeo, D. (1984) Proc. Natl. Acad. Sci. 81, 1416–1420
2 Rasmussen, H. (1981) Calcium and cAMP as Synarchic Messengers, John Wiley and Sons, New York
3 Marx, J.L. (1984) Science 224, 271–274
4 Nishizuka, Y. (1984) Trends Biochem. 9, 163–166
5 Berridge, M.J. (1985) Nature 312, 315–321
6 Lee, J.W. and Vidaver, G.A. (1984) Cell Calcium 5, 501–524
7 Lee, J.W. and Vidaver, G.A. (1984) Cell Calcium 5, 525–536
8 Goldbeter, A. and Koshland, D.E. Jr. (1982) Q. Rev. Biophys. 15, 555–591
9 Chock, P.B., Rhee, S.G. and Stadtman, E.R. (1980) Annu. Rev. Biochem. 49, 813–843
10 Varecka, L. and Carafoli, E. (1982) J. Biol. Chem. 257, 7414–7421
11 Reuter, H. and Seitz, N. (1968) J. Physiol. 195, 451–470
12 Lee, J.W., Ting, A. and Vidaver, G.A. (1986) Life Sci. 38, 1013–1019
13 Muallem, S. and Karlish, S.J.D. (1982) Biochim. Biophys. Acta 687, 329–332
14 Mollman, J.E. and Pleasure, D.E. (1980) J. Biol. Chem. 255, 569–574
15 Lew, V.L. and Simonsen, O. (1980) J. Physiol. (London) 308, 60p
16 Pheiffer, D.R. and Lardy, H.A. (1976) Biochemistry 15, 935–943
17 Lee, J.W. Beygu-Farber, S. and Vidaver, G.A. (1973) Biochim. Biophys. Acta 293, 446–459
18 Williams, D.A., Fogarty, K.E., Tsien, R.Y. and Fay, F.S. (1986) Nature 318, 558–561
19 Keith, C.H., Ratan, R., Maxfield, F.R., Bajer, A. and Shelanski, M.L. (1986) Nature 316, 848–850
20 Koshland, D.E. Jr. (1981) Annu. Rev. Biochem. 50, 765–782
21 Schmiz, A. and Hildebrand, E. (1985) Nature 317, 641–643