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Mobilization of intracellular calcium by extracellular ATP and by calcium ionophores in the Ehrlich ascites-tumour cell

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We have studied the changes of the intracellular free calcium concentration ($[Ca^{2+}]_i$) effected by external ATP, which induces formation of inositol trisphosphate, and by the divalent cation ionophores ionomycin and A23187. Both, ATP (40 μM) and ionophores (1–80 μmol/l cells ionomycin; 20–400 μmol/l cells A23187), produced a transient rise of $[Ca^{2+}]_i$ which reached its maximum within 15–30 s and declined near resting values (about 200 nM) within 1–3 min. When the $[Ca^{2+}]_i$ peak surpassed 500 nM a transient cell shrinkage due to simultaneous activation of Ca^{2+}-dependent K^+ and Cl^- channels was also observed. The cell response was similar in medium containing 1 mM Ca^{2+} and in Ca^{2+}-free medium, suggesting that the Ca mobilized to the cytosol comes preferently from the intracellular stores. Treatment with low doses of ionophore (1 μmol/l cells for ionomycin; 20 μmol/l cells for A23187) depressed the response to a subsequent treatment, either with ionophore or with ATP. Treatment with ATP did also inhibit the subsequent response to ionophore, but in this case the inhibition was dependent on time, the stronger the shorter the interval between both treatments. This result suggests that the permeabilization of Ca stores by ATP is transient and that Ca can be taken up again by the intracellular stores. Refill was most efficient when Ca^{2+} was present in the incubation medium. Addition of either ATP or ionomycin (1–25 μmol/l cells) to cells incubated in medium containing 1 mM Ca^{2+} decreased drastically the total cell Ca content during the following 3 min of incubation. In the case of ATP the total cell levels of Ca returned to the initial values after 7–15 min, whereas in the case of the ionophore they remained decreased during the whole incubation period. These results indicate that Ca released from the intracellular stores by either ATP or ionophores is quickly extruded by active mechanisms located at the plasma membrane. They also suggest that, under the conditions studied here, with 1 mM Ca^{2+} outside, the Ca-mobilizing effect of ionophores is stronger in endomembranes than in the plasma membrane.

Introduction

It is often assumed that Ca ionophores act primarily by increasing the permeability of plasma membrane to Ca^{2+}. Under this assumption treatment of animal cells with Ca ionophores in Ca^{2+}-containing media should invariably result in an

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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increase of the Ca content of the cells. However, a 'paradoxical' decrease of the cell Ca content on treatment with Ca ionophores has been reported in several cell lines [1–4]. This behaviour could be explained if the effect of the ionophore were stronger on endomembranes than in plasma membrane. Under this view the primary effect of the ionophore would be to release Ca from the intracellular stores to the cytoplasm. This released Ca would be rapidly extruded by plasma membrane Ca^{2+} pumps into the medium producing a net loss of cellular Ca. This has been reported to be the case for GH_4C_1 strain of rat pituitary cells [3].

Treatment of the Ehrlich cell with the ionophore A23187 in Ca^{2+}-containing medium has been reported to increase transitorily the cytoplasmic free Ca concentration ($[Ca^{2+}]_i$) [5], even though the total cell Ca content is little modified or even decreased [1,2]. Incubation with micromolar concentrations of ATP has also been reported to increase $[Ca^{2+}]_i$ [6]. The mechanism includes phosphodiesterasic cleavage of phosphatidylinositol bisphosphate at the plasma membrane with production of inositol trisphosphate, which releases Ca from intracellular stores [7]. Both treatments, ionophores [8] and ATP [6], produce a transient increase of K^+ permeability by activation of Ca^{2+}-dependent K^+ channels. The simultaneous activation of Ca^{2+}-sensitive Cl^- channels conduces to cell shrinkage by KCl loss [9]. The cell shrinkage is fast, and it is followed by a slower return to the initial volume by salt gain mediated by a Na^+/Cl^--cotransport system, which is activated by cell shrinkage [9].

We study here the changes of $[Ca^{2+}]_i$, the net ^{45}Ca movements and the volume responses effected by ATP and by the divalent cation ionophores ionomycin and A23187 in the Ehrlich cell with the aim of adding new insights to the dynamics of the Ca redistribution induced by these treatments.

Methods

Methods for propagation and handling of the Ehrlich cells were as described previously [11]. Cells were used within 4 to 7 days after injection. The standard incubation medium contained (in mM); NaCl, 140; $CaCl_2$, 1; $MgCl_2$, 1; sodium pyruvate, 5; K-Hepes, 10 (pH 7.4). Ca^{2+}-free medium was achieved by addition of 2 mM EGTA to the standard medium. $[Ca^{2+}]_i$ was estimated from specific fluorescence measurements in cells loaded with fura-2 [12], and cell volume changes were followed by light scattering measurements [13]. The cells were loaded with fura-2 by incubation at 1% cytocrit with 1 μM fura-2/AM during 20 min at 37° C. The cells were then washed twice with standard medium, resuspended at 2.5% and stored at room temperature with stirring. Measurements of both volume and $[Ca^{2+}]_i$ were performed at room temperature and under magnetic stirring in aliquots of this cell suspension within 30–180 min of preparation. This cell concentration and temperature were chosen in order to obtain reliable measurements of both, changes of cell volume and $[Ca^{2+}]_i$, under exactly the same experimental conditions. In preliminary experiments changes in cell density did not substantially modify the results and the increase of temperature to 37° C made somewhat faster both the volume response and the $[Ca^{2+}]_i$ transients. $[Ca^{2+}]_i$ was estimated from fluorescence measurements of the cell suspension at 340 nm excitation and 500 nm emission in a Hitachi 605-S spectrofluorimeter. Calibration of the fura-2 signal was performed by lysing the cells with digitonin and quenching with $MnCl_2$, as described before [7]. Cell volume changes were followed by transmittance measurements of the cell suspension in a double-beam spectrophotometer at 650 nm, as described previously [14].

Net fluxes of Ca were measured using ^{45}Ca. In preliminary experiments we found that cells took up externally-added ^{45}Ca very fast, a steady intracellular level being attained within 20–30 min, which remained unchanged for hours. To a 2.5% cell suspension, prepared as described above, $^{45}CaCl_2$ (specific activity, about 10^{13} cpm/mol) was added to give $2 \cdot 10^9$ cpm/l in the cell suspension. After 60 min incubation at room temperature this cell suspension was used for light scattering and net fluxes experiments (see Fig. 6 legend). Samples of the cell suspension were taken just before and at different times after the opportune drug additions for determination of the cell-associated radioactivity. For these purposes the cells

were separated from the incubation medium using two different procedures. In the first procedure a 100-μl aliquot of the cell suspension was quickly mixed with 1 ml of ice-cold standard medium containing 5 mM EGTA which had been pipetted on top of 0.4 ml of an oil cushion (di-*n*-butyl phthalate and di-*n*-butyl sebacetate oils mixed to give a specific gravity 1.015 g/ml) in a 1.5-ml Eppendorf tube. Immediately the tube was centrifuged for 15 s at $12000 \times g$. The supernatant solution and most of the oil were removed by aspiration, the walls of the tube were carefully cleaned with cotton swabs and the cell pellet was extracted with 1 ml of 0.6 M perchloric acid and counted for radioactivity by scintillation counting. In the second procedure a 100-μl aliquot of the cell suspension was passed through a 0.8-ml Dowex 50-X-100 column mounted in Pasteur pipette [15] and eluted with 1.6 ml of a solution containing 300 mM mannitol, 5 mM K-Hepes (pH, 7.4) and 2 mg/ml bovine serum albumin, and the eluent was directly counted for radioactivity. Each procedure has special advantages in different conditions for measuring influx or efflux of ^{45}Ca in short time intervals, but they gave similar results for the net flux measurements performed here. Only data obtained using the first procedure are shown in Fig. 6.

$^{45}CaCl_2$ was purchased from Amersham international, plc. Fura-2/AM and fura-2, free acid, were obtained from Molecular Probes, Junction City, OR, U.S.A. Ionophore A23187 and ionomycin were obtained from Boehringer Mannheim GmbH and Calbiochem, respectively. Other chemicals were obtained either from Sigma London, BDH Chemicals or E. Merck, Darmstadt.

Results

Fig. 1 compares the $[Ca^{2+}]_i$ and volume responses to ionomycin and ATP in cells incubated in Ca^{2+}-free medium. Both treatments produced a transient increase of $[Ca^{2+}]_i$ which reached its maximum within 15–30 s and declined near the resting values (about 200 nM) after 1–3 min. When $[Ca^{2+}]_i$ peak surpassed 500 nM a volume response was also observed, its extent depending on $[Ca^{2+}]_i$ within the range of 500–2000 nM (see also Figs. 2 and 3). The presence of Ca (1 mM) in

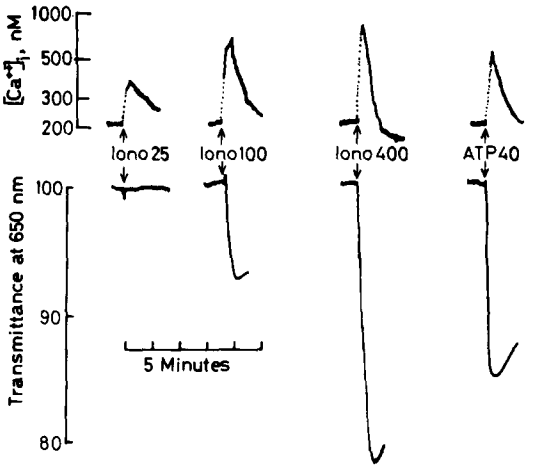

Fig. 1. Effects of several doses of ionomycin (25, 100 and 400 nM) and ATP (40 μM) on $[Ca^{2+}]_i$ (upper records) and cell volume (lower records) in Ehrlich ascites tumour cells. EGTA (final concentration 2 mM in the cell suspension) was added 1 min before ionophore or ATP, whose additions are marked by arrows.

the incubation medium did not modify significantly the $[Ca^{2+}]_i$ nor the volume response (see below). Shrinkage of the cells was completed within 15–30 s and was followed by a slower swelling which returned cell volume to about the initial value within 4–8 min. Characterization of these volume responses has been performed before (regulatory volume decrease and regulatory volume increase, see Refs. 9 and 10). When ATP was the agonist the $[Ca^{2+}]_i$ and volume responses could also be graded by using concentrations within the 0.5–4 μM range (data not shown). Maximal doses of ATP had always smaller effect, both in $[Ca^{2+}]_i$ and volume response, than maximal doses of ionomycin. The records shown in Fig. 1 would seem to suggest that ATP could induce a somewhat larger volume response than ionomycin for similar $[Ca^{2+}]_i$ peaks (compare the second and the fourth record), but this was not systematically observed. Note that both the $[Ca^{2+}]_i$ and the volume response became sharper the larger was the dose of ionomycin used. In some cell batches the response to ATP, both for $[Ca^{2+}]_i$ and for cell volume, was weak. That was generally associated to a poor response to Ca ionophores as well suggesting that the failure was due to a reduced

content of Ca in the intracellular stores. These cell batches were discarded.

Fig. 2 shows the $[Ca^{2+}]_i$ and volume responses to successive additions of ionomycin or ATP. Pretreatment with ATP prevented the volume response to the treatment with ionomycin, added 2 min later (second record). This was accompanied by a decrease of the $[Ca^{2+}]_i$ peak effected by ionomycin (1150 nM in the firt record and 491 nM in the second), which was below the 500 nM threshold necessary for the volume response. Pretreatment with low doses of ionophore, which produced $[Ca^{2+}]_i$ peaks below the threshold for volume response, prevented the volume responses to the subsequent application of maximal doses of ATP (third record) or ionomycin (fourth record in Fig. 2). This was always accompanied by a decrease of the size of the $[Ca^{2+}]_i$ transients. Ionophore A23187 behaved similarly to ionomycin, although doses about 10-times larger were required to obtained similar responses (Fig. 3). Pretreatment for 2 min with 500 nM A23187, a subthreshold dose for the volume response, prevented the response to the subsequent application of maximal doses of ionomycin (Fig. 3), A23187 or ATP (data not shown), even in the presence of 1 mM external Ca^{2+}. We must remark now that the extent of the shrinkage induced by ATP or, even more surprisingly, the Ca ionophores, was similar in the absence (excess EGTA) or in the presence of 1 mM Ca^{2+} in the incubation medium (see, for example, Fig. 5). The size of the $[Ca^{2+}]_i$ peaks was also similar (less than 15% difference in repeated experiments), the level reached 1–3 min after the agonist application tending to be somewhat higher in the presence of 1 mM Ca^{2+} (data not shown).

The effect of ATP preventing the response to subsequently added ionophore depended strongly on the time interval between both additions. Fig. 4 shows that no volume response to the ionophore was obtained 1 min after ATP addition (second record), but sensitivity to the ionophore was restored partly after 5 min (third record) and fully after 14 min (fourth record). These times varied somewhat from batch to batch of cells and also with the temperature of incubation. The presence of external Ca^{2+} during the interval between ATP and ionophore additions was required for full restoration of the sensitivity to the ionophore, as documented in Fig. 5. Records 1 and 2 compare the volume responses obtained for sequential ATP

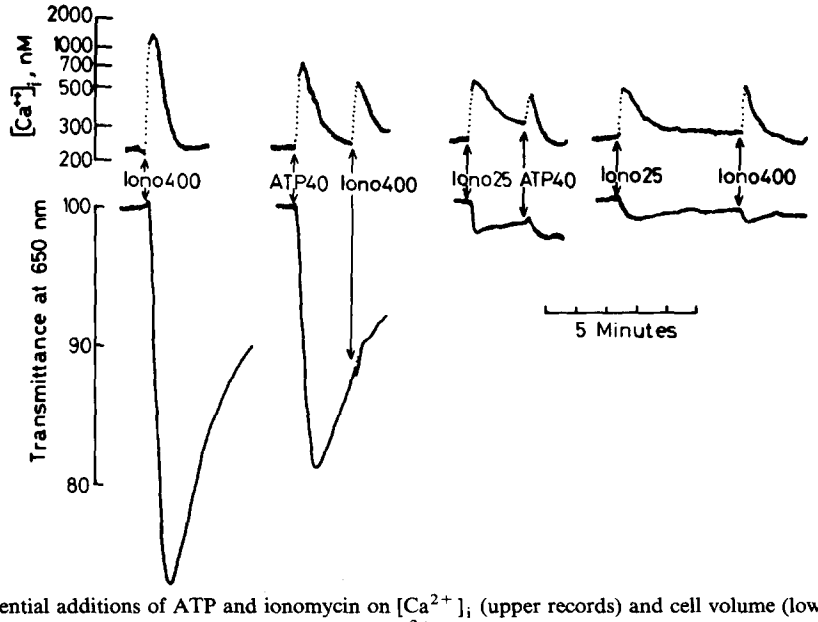

Fig. 2. Effects of sequential additions of ATP and ionomycin on $[Ca^{2+}]_i$ (upper records) and cell volume (lower records) in Ehrlich ascites tumour cells. The incubation medium contained 1 mM Ca^{2+}. Concentrations for ionomycin and ATP are given in nM and μM, respectively.

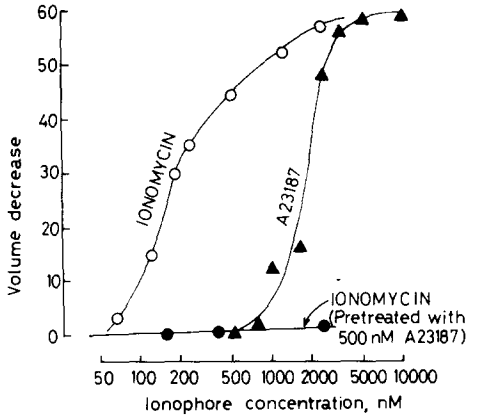

Fig. 3. Shrinkage of Ehrlich ascites-tumour cells obtained with different concentrations of ionomycin and A23187. Volume decrease is expressed in arbitrary units. Curves labelled 'ionomycin' and 'A23187' were obtained in Ca^{2+}-free medium, but addition of 1 mM Ca^{2+} did not make differences in the results. For the lower curve (filled circles) the cells were incubated in Ca^{2+}-containing medium and 500 nM A23187 followed 2 min later by different doses of ionomycin as shown. The first addition (500 nM A23187) did not give any volume response. All the experiments were performed with the same batch of cells.

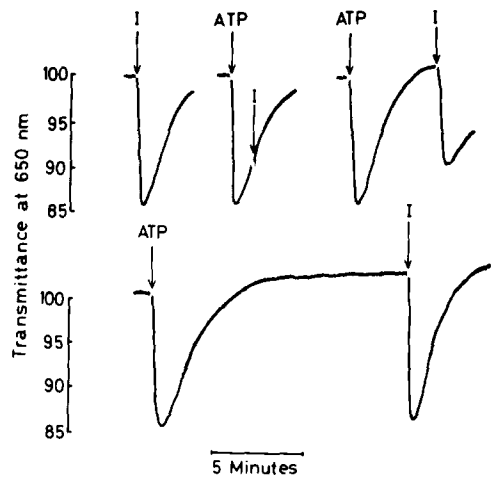

Fig. 4. Effects of sequential additions of ATP (40 μM) and A23187 (10 μM, marked 'I' in the figure) on cell volume of Ehrlich ascites tumour cells. The incubation medium contained 1 mM Ca^{2+}.

and ionophore additions, in Ca^{2+}-containing and Ca^{2+}-free media, respectively. Whereas the effect of ATP was similar in both cases, the effect of the ionophore was much weaker in the second. The third record demonstrates that the effect of external Ca^{2+} is not due to its presence during the ionophore addition, since addition of Ca^{2+} just before the ionophore did not restore its effect on the volume response. The presence of extracellular Ca^{2+} seems then necessary during the whole interval between ATP and ionophore additions. The effect of subthreshold doses of ionophore preventing the volume response induced by a subsequent treatment with either ionophore or ATP was not decreased by increasing the time interval between both additions (data not shown).

The effects of ATP and ionomycin on the net Ca movements through the plasma membrane were studied in cells first incubated with ^{45}Ca. After 15–30 minutes of incubation cell ^{44}Ca content remains steady for hours (not shown). Fig. 6 shows the results of a typical experiment in which either ATP or ionomycin were added to the cell suspension after 60-min preincubation with 1 mM ^{44}Ca. The experiments were performed in such a way that allowed simultaneous measurement of transmittance changes (upper records) and withdrawal of samples for determination of cell-associated radioactivity (lower records). All the three treatments shown in Fig. 6 (30 and 120 nM ionomycin and 40μm ATP) decreased the total cell Ca content. After 3 min 94% of the exchangeable Ca had been extruded with the highest dose of ionomycin whereas the extrusion with the smaller dose of ionophore or with ATP amounted 67–70% of the exchangeable Ca by the same incubation period. The total cell Ca content returned to near the

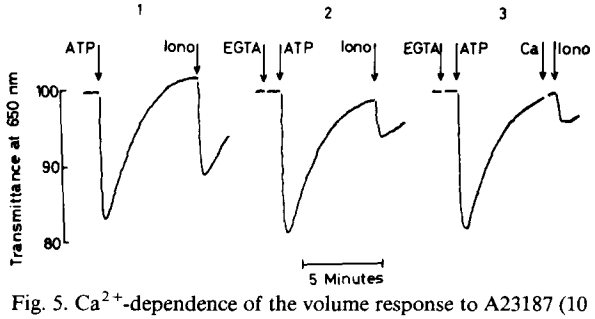

Fig. 5. Ca^{2+}-dependence of the volume response to A23187 (10 μM, marked 'iono' in the figure) added 6 min after a first ATP addition (40μM). Cells were initially suspended in medium containing 1 mM Ca^{2+}. The final concentration of EGTA after addition (records 2 and 3) was 2 mM. The last addition of Ca^{2+} in record 3 increased Ca concentration in the suspension by 2 mM (concentration of Ca^{2+}, 1 mM in the suspension).

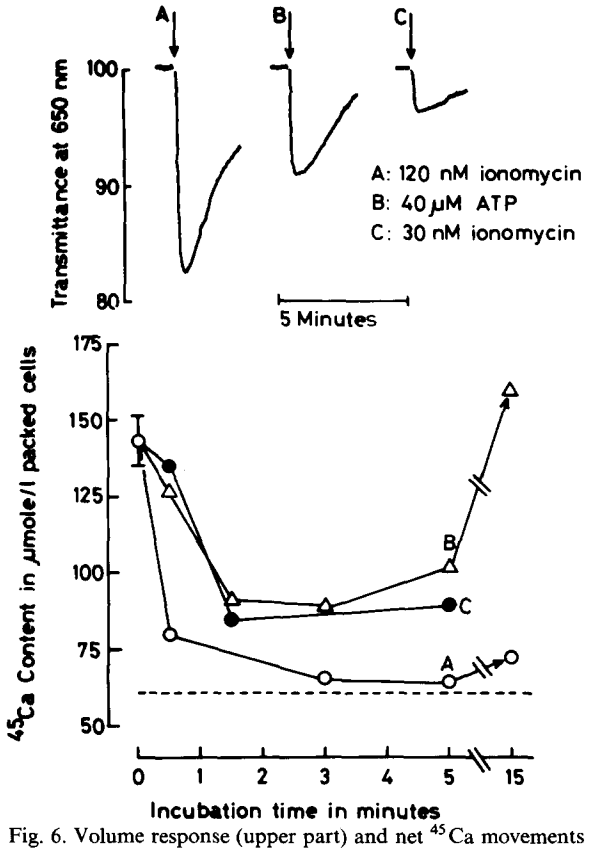

Fig. 6. Volume response (upper part) and net ^{45}Ca movements in Ehrlich ascites tumour cells treated with either ionomycin (120 and 30 nM) or ATP (40 μM). Cells were first incubated during 60–120 minutes in medium containing 1 mM ^{45}Ca. Then aliquots of the cell suspension were transferred to a spectrophotometer cuvette and the agonist was added at the time shown (arrows in upper part, $t = 0$ in the lower part). Samples were taken from the cuvette after different incubation periods, as shown, for analysis of cell-associated radioactivity. Cells were separated from the incubation medium by centrifugation through an oil mixture (see Methods). The dotted line represents the size of the non-exchangeable cell ^{45}Ca pool, measured after addition of 5 mM EGTA and 2 μM ionomycin to an aliquot of the cell suspension and incubation during 15 min. The size of this pool is likely to be overestimated in the figure since it includes the blank of external ^{45}Ca carried out by the cells through the oil on centrifugation. The bar represents the standard error of the measurements. Note the interruption of the time scale between 5 and 15 min.

initial level within 15 min after treatment with ATP but remained low by that time after treatment with ionophore. Similar results were obtained in another three experiments in which the cells were separated from the incubation medium using a different procedure (see Methods, results not shown). Using high concentrations of ionomycin (2–3 μM) we found no changes or slight increases of total Ca content of the cells.

Discussion

The responses of the Ehrlich cell to Ca ionophores and to ATP have been studied in some detail previously (see Introduction). Both treatments induce a transient increase of $[Ca^{2+}]_i$, which results on activation of ionic channels conducing to shrinkage of the cells by loss of KCl. Our results add new insights on the Ca^{2+}-sensitivity of these channels and on the dynamics of Ca redistribution induced by ATP and by ionophores. The net loss of KCl requires the simultaneous activation of Ca^{2+}-dependent K^+ and Cl-channels. The less sensitive of these channels will determine the overall Ca^{2+}-sensitivity of the process. We show here that the threshold for volume response lies at 500 nM $[Ca^{2+}]_i$, a concentration which is about 2.5-times the resting value. The activation curve is very sharp, maximal effect being obtained at 1–2 μM Ca^{2+}. Under the conditions in which our experiments were performed $[Ca^{2+}]_i$ seemed the only factor determining the shrinkage of the cells. For these reasons the volume response is even more sensitive than direct measurements to detect $[Ca^{2+}]_i$ differences in the range of 0.5–1 μM.

ATP and ionophores seemed to release Ca from the same intracellular stores since the application of one of them decreased the response to the subsequent application of the other (Fig. 2). The Ca^{2+}-permeabilizing effect of both, ATP and ionophores, was stronger in endomembranes than in the plasma membrane, since: (i) the responses were similar in Ca^{2+}-containing and Ca^{2+}-free media (Fig. 5), and (ii) both treatments produced a net decrease of cell Ca content (Fig. 6). This preferential effect on endomembranes was expected for ATP, whose action has been documented to be mediated by inositol trisphosphate [7], a Ca^{2+}-permeabilizing agent specific for intracellular stores [16]. In the case of the Ca ionophores the outcome was striking at first sight since both membranes should show similar sensitivity. Although asymmetrical distribution of the ionophore could be postulated, a simpler explana-

tion arises by taking into account that the electrochemical gradient for Ca^{2+} to the cytoplasm is likely to be much larger from the cellular stores than from the extracellular medium. In that case, even if the concentrations of the ionophore were similar in both membranes, the fluxes from the cellular stores would be larger as far as its Ca content is maintained. This interpretation offers also a simple explanation for the transient nature of the ionophore effect, which has also been reported in other cell preparations [17]. These observations should be taken into account when interpreting the effects of Ca ionophores. A similar preferential effect of Ca ionophores in endomembranes has been demonstrated for ionomycin in GH_4C_1 rat pituitary cells [3] and inferred for A23187 in smooth muscle cells [18].

The Ca released from the cellular stores by either ATP or ionophores is quickly extruded to the medium by active mechanisms located at the plasma membrane. It should be remarked that, even though the total cell Ca content decreases (Fig. 6), $[Ca^{2+}]_i$ remains above resting levels, at least during the first minute after the addition of the agonist (Figs. 1 and 2). In the case of ATP the cellular stores are refilled within a few minutes of incubation in Ca^{2+}-containing medium (Fig. 4) and the total Ca content of the cells returns near the resting level (Fig. 6). This is consistent with the ephemeral nature of the inositol trisphosphate rise induced by ATP [7] and with observation that the sensitivity to this agent, which is lost immediately after a first application, is recovered within a few minutes of incubation in Ca^{2+}-containing medium [6]. The presence of external Ca^{2+} is an absolute requirement for full restoration of the volume response after ATP addition (Fig. 5), suggesting that most of the Ca released from the cellular stores is extruded to the external medium and must enter again through the plasma membrane before the cellular stores can be replenished once the permeabilizing effect of ATP has elapsed. This interpretation is also consistent with the net Ca movements observed (Fig. 6). In the case of the ionophore the decrease of total cell Ca content was maintained during the whole incubation period, as it was expected from the permanent nature of its permeabilizing effect. The results shown in this paper help to explain the reported 'paradoxical' decrease of the Ca content in Ehrlich cells treated with ionophores in Ca^{2+}-containing medium without the need of postulating insensitivity to ionophores in tumoral cells [1,2]. A 'paradoxical' expulsion of Ca induced by low doses of A23187 reported recently in pigeon erythrocytes [4] might be explained similarly.

Acknowledgements

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