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EFFECT OF Na, METABOLIC INHIBITORS AND ATP ON Ca MOVEMENTS IN L CELLS

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SUMMARY

1. The Ca movements in normal and 'ghost' L cells have been examined; all measurements were made using ^{45}Ca.

2. Normal cells have a Ca concentration of about 1 m-mole/l. of cell volume, and exchange Ca in a complex way but with great rapidity; the time taken for the initial Ca* content to fall to half was less than 2 min.

3. Poisoning normal cells with DNP 10^{-3} M + IAA 10^{-4} M causes a marked reduction in the Ca efflux, no change in Ca influx and an increase in total Ca.

4. Variation in internal or external Na concentration does not alter the Ca fluxes or concentrations. Application of cyanide or ouabain and alteration of external K concentration had no effect on the Ca fluxes.

5. The sulphydryl reagents, ethacrynic acid and N-ethylmaleimide (NEM), have a rapid and marked effect on reducing the Ca efflux.

6. L cell ghosts previously poisoned with DNP + IAA have a low Ca efflux. When ATP or CTP is incorporated into such cells the Ca efflux becomes normal.

7. An extra amount of phosphate is produced by L cell ghosts when pumping Ca. This is equivalent to the splitting of 1·8 moles of ATP per mole of Ca pumped.

8. It is concluded that L cells have a Ca pump driven by ATP, and that Na has no effect on Ca movements in these cells.

INTRODUCTION

In most cells the free intracellular Ca concentration is probably very low. It has been shown recently that in R.B.C.S this is achieved by means of a Ca pump driven by ATP (Schatzmann & Vincenzi, 1969) whereas in squid axon it has been suggested that Ca extrusion is coupled to an inward

downhill movement of Na (Baker, Blaustein, Hodgkin & Steinhardt, 1969; Blaustein & Hodgkin, 1969).

From these experiments the question arises of whether Ca pumping in other non-excitable cells is by direct ATP splitting as in R.B.C.S or by coupling with Na movements as in squid axon or by a combination of both mechanisms. Evidence by Borle (1969*a*, *b*) suggests that in Hela cell fibroblasts, Ca extrusion is dependent on metabolism, but whether directly due to ATP coupling or a concomitant movement with Na could not be decided.

Recently we have developed a method of lysing and refilling cultured cells (MacKinnon, 1969), similar in esssence to that used to prepare R.B.C. ghosts. The present paper uses this method to examine the Ca movements in L cells in greater detail than could hitherto have been done. The results show that Ca extrusion in these cells uses ATP and is not affected by Na movements.

METHODS

L cells (Clone, 929; Sanford, Earle & Likely, 1948) were obtained from Flow Laboratories, Irvine, and cultured as monolayers on plastic Petri dishes ('plates') in Basal Eagles Medium or MEM as previously described (Lamb & MacKinnon, 1971*a*). Most experiments were conducted in Krebs buffered with a Tris-maleate buffer, containing 2·8 mM-Ca at room temperature (20° C). The Ca movements and contents were measured by means of ^{45}Ca, obtained from Amersham as sterile $CaCl_2$. In order to check that the radioactivity was due to ^{45}Ca, several samples were kept over 6 month periods and their $t_{\frac{1}{2}}$ of decay verified as 165 days. The over-all experimental technique is outlined below and detailed in the captions of the Results section. In preliminary experiments it was found that a substantial amount of Ca* adhered to the vessels containing the cells; for this reason blanks (i.e. plates with no cells) were always included in each experimental run. Also, it was found impossible to use glass bottles of cells rather than plastic Petri dishes for Ca* experiments, partly because glass takes up more Ca* than plastic and partly because of the higher ratio of vessel surface area/cell numbers of bottles.

Experimental procedure. Cells grown in BME or in MEM at 37° C were generally equilibrated in Krebs at 20° C for 1 hr and then used for influx or efflux experiments. To wash the extracellular radioactivity from the cells six cold washes with Krebs were used. The various procedures used are shown in Table 1. The solid arrows indicate points at which the fluid was drained from the plates. This was done by pouring off the fluid and sucking the last few drops out with a Pasteur pipette. The × 3 20° C washes (step 3) were used to heat up the cells and plate to room temperature from 2° C before starting an efflux. Comparison of cells so treated with others not so treated showed there was no significant Ca* loss as a result of this procedure. The cell counts were made either on a Vickers M 6 cell counter on a Coulter Counter, or by using a haemocytometer. The radioactivity was measured by adding the 1 ml. sample to 10 ml. of Bray's mixture (Bray, 1960) and counted in a Tricarb 3000 Scintillation Counter against similarly prepared standards.

Technique for refilling L cells. Plates of cells were grown until less than a full monolayer was formed. They were then equilibrated in Krebs for at least 30 min. After draining, 10 ml. hypotonic solution A (see below) was added and left for 10 min.

This was drained off and solution B added and left for 3 min. For a Ca* efflux, step 2 in Table 1 was then started. Observation of the cell changes in a phase-contrast microscope showed that (1) the cells (especially the ends of the processes) swelled and shrank almost instantaneously when the new fluid reached them, (2) during the lysis 'debris' was observed to leave the cells, (3) the 'ghosts' had a similar volume to the original cell, (4) Brownian movement could often be observed within the 'ghost' cells, and (5) many cells became detached during this procedure.

Phosphate measurements. These were all made on L cell ghosts filled either with 7 mM-ATP and 1·4 mM-Ca or only with ATP. (Measurement with a Ca electrode showed that this amount of ATP bound about 0·2 mM-Ca.)

TABLE 1. Outline of the procedures involved in measuring the influx, efflux and total Ca using ^{45}Ca on plates of L cells. The arrows indicate where the plates were drained. Most experiments were done on 9 cm Petri dishes containing some 3×10^6 cells, requiring 10 ml. Krebs. In all influx and efflux experiments the plates were agitated by placing them on top of a slowly rotating kymograph drum set at an angle of about 15° to the horizontal

Step	Procedure	Influx	Efflux	Total Ca
1	Ca* Soak	1–30 min Krebs (20° C)	4–18 hr BME (37° C) or Krebs (20° C)	10 days BME (37° C)
2	× 6 2° C wash Krebs	½ min	½ min	½ min
3	× 3 20° C wash Krebs	—	½ min	—
4	Krebs efflux into 10 ml. at 20° C 1 ml. sample for Ca* counting at end	—	1–30 min	—
5	Trypsin 2 ml. 37° C + Krebs 3 ml. 37° C	1 min 5–10 min	1 min 5–10 min	1 min 5–10 min
6		Cell count (4 ml.)	Isotope count (1 ml.)	

After incubation under the specific conditions the ghosts were lysed in distilled water at 2° C, and 13 % (w/v) TCA was added to precipitate protein. After centrifugation the P_i content of the supernatant was estimated by the method of Berenblum & Chain (1938). In these experiments the cell numbers were measured on separate plates of cells treated in a similar way to the plates used for P_i measurement.

Experimental design and statistical analysis. In all the 'ghost' experiments only one variable was altered at a time, and the results analysed by the *t* test. In some experiments a factorial design was adopted to allow up to three variables and their interactions to be tested (Fisher, 1947; Brownlee, 1957, p. 19). Experimentally it was convenient to work with 3 variables at 2 levels, requiring eight experiments (2^3) for no replication or sixteen experiments (2×2^3) with one replication. Within each group the experiments were randomized. An analysis of variance was applied to the results. The significances quoted refer to the appropriate comparisons (Brownlee, 1957, chap. XI).

Poisons. DNP, IAA and KCN were all made up as × 10 concentrated solutions, the pH adjusted to that of Krebs and then an aliquot added to the experimental solutions.

Solutions. The Krebs contained (m-mole/l.) Na^+ 136·58; K^+ 5·65; Ca^{2+} 2·8; Mg^{2+} 1·17; Cl^- 146·96; PO_4^{2-} 0·58; SO_4^{2-} 1·17; glucose 10·98; plus phenol red 0·0002% and calf serum 5%.

The hypotonic solution (A) contained NaCl 10 mM, $MgCl_2$ 1·2 mM and cysteine HCl 2·5%; when loading with Ca* the solution contained 1·4 mM-Ca* Cl; in the experiments to incorporate ATP, 7 mM-ATP was added. The reconstitution solution (B) was made by adding 1·6 ml. M-KCl to 10 ml. of A.

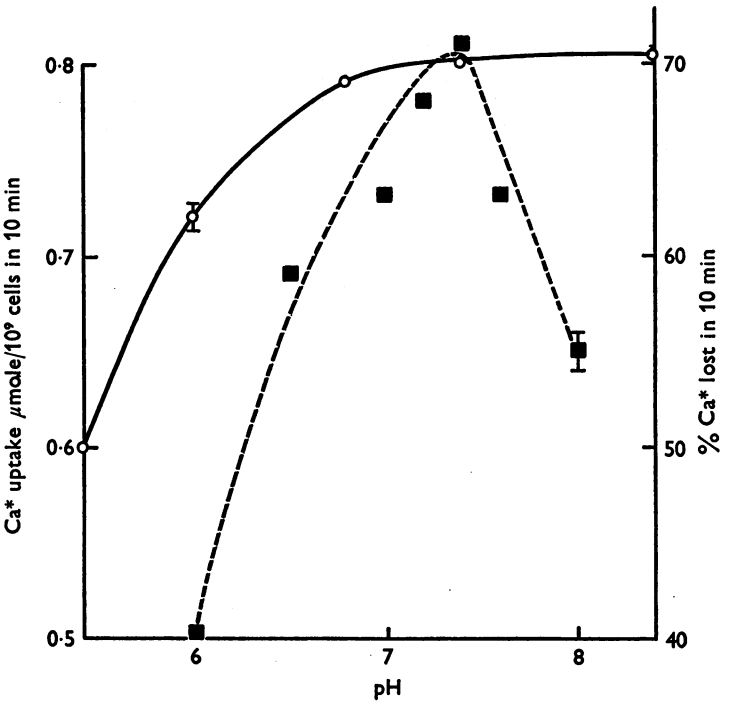

Fig. 1. The effect of pH on the Ca fluxes in L cells. Abscissa: pH. Ordinate: left, uptake as μmole/10^9 cells per 10 min shown as circles with a continous line; right, efflux as % lost in 10 min shown as squares with a dashed line. Note that the efflux over 10 min has a maximum value around a pH of about 7·4. The efflux over 1 min had a similar pH optimum. n = 10 per point. Typical errors (2 × S.E.) shown. Lines fitted by eye.

pH control. It is not easy to control the pH of plates of cells in air with a bicarbonate buffer and therefore most experimental solutions were buffered with Tris-maleate (16 and 5 mM respectively). For a few experiments a Tris-HCl buffer was used. Even using these buffers the pH of the solutions did not remain constant and so a series of preliminary experiments were done to test the effect of pH on Ca movements. Fig. 1 shows that the efflux is much more sensitive to pH alteration than is the influx but that around the normal pH used (7·2–7·4) small variations in pH had only small effects. It was concluded that none of the effects observed were likely to be due to pH changes.

RESULTS

Ca efflux from fresh cells

Plates of cells loaded with Ca* in Krebs or growth medium for some hours and then washed with inactive Krebs at 2° C showed a very rapid and large loss of Ca* over the first 10–20 sec followed by a slow, almost exponential (over the range measured) loss (Fig. 2 upper). Blank plates

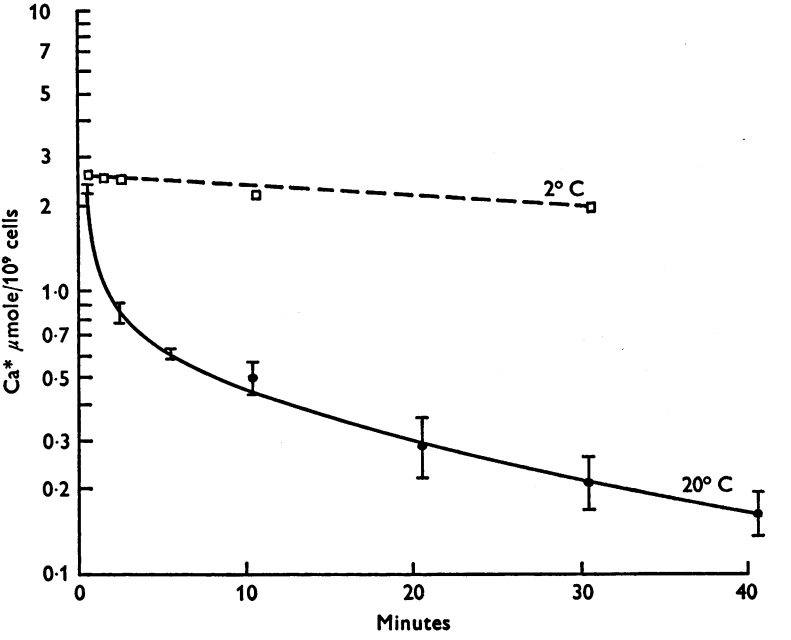

Fig. 2. Ca* washout from L cells. Cells loaded with Ca* in BME for 18 hr at 37° C. All cells given an initial × 6 wash at 2° C in Krebs and then efflux continued at 2° C (upper curve □) or 20° C (lower curve ●). Points represent mean Ca contents of plates analysed at the times shown. Bars are ± 2 S.E., last three points at 20° only two observations, rest 4–20, errors at 2° C < size of symbols. During the first 30 sec of cold wash (not shown) the Ca content of blank plates and plates with cells fell from several hundred μM to the values shown. It was assumed this was due to extracellular Ca being washed off. Cold efflux can be fitted by a single exponential, warm efflux complex and much faster. [K_1] of these cells = 167 m-mole/l. intracellular water.

similarly treated, showed an equally rapid and large loss initially which fell to the background level within a minute or two. Thus it was assumed that the initial loss was 'extracellular' and was largely over within the first 30 sec at 2° C. After exposure to Ca* solution all plates were therefore washed with six cold washes to remove this extracellular activity, either

before analysis or before an efflux experiment (see Table 1). Fig. 2 (lower) shows the average Ca* efflux at 20° C of plates treated in this way. It can be seen (1) that the efflux is very temperature sensitive (see later) and is non-exponential; (2) that the Ca content of the cells is 2·3 μmole/10^9 cells (about 0·8 m-mole/l. of cell), similar to that in the cold efflux experiments; (3) the over-all $t_{\frac{1}{2}}$ is 1·5 min, with a slow 'tail' with a $t_{\frac{1}{2}}$ of 20–30 min.

Effects of the metabolic inhibitors DNP and IAA. Borle (1969*b*) found that these agents used separately reduced the Ca* efflux from Hela cells. In preliminary experiments we found no effect of DNP by itself and only a small effect of IAA but very pronounced effects when DNP + IAA were used together. The reasons for these differences are unclear but may be related to the fact that L cells can derive energy both from anaerobic and aerobic pathways depending on the pH (Danes & Paul, 1961).

Fig. 3 shows a typical experiment of the effect of DNP + IAA on the fraction of Ca remaining in L cells after various times. It is clear that unpoisoned cells lost a great deal more Ca* than poisoned cells over the 30 min period. Similar results were obtained in three other experiments. It will be noticed that the Ca* loss during the first minute is greatly decreased by DNP + IAA, suggesting that the initial as well as the later part of the efflux curve is being poisoned. Statistically the effect of DNP + IAA did not vary over the efflux period. This result needs to be interpreted with caution as the latter contents are greatly determined by the initial rate of loss. Additional support for this idea that the initial efflux is active was given in Fig. 1 in that the pH dependence of the efflux over the first minute was similar to the 10 min efflux but quite unlike that of the influx. We found that this effect of DNP + IAA was largely irreversible, suggesting damage to the ATP producing systems or the cell membrane or to both. In an attempt to circumvent this, experiments with KCN, 10^{-4} M, in glucose-free solutions and of hypoxia ($P_{O_2} < 5$ torr) in glucose-free solutions were carried out. Neither of these combinations had any discernible effects on Ca movements.

Effects of Na ethacrynate and N-ethylmaleimide. Both these substances affect SH groups (Skou, 1965). Fig. 4 shows that both also produce a rapid inhibition of the Ca efflux, the maximum effect being similar to that of DNP + IAA inhibition ($P > 5\%$). The ethacrynate results are similar to those described by Schatzmann & Vincenzi (1969) for R.B.C.S but the concentrations required are greater. This may be due to a species variation in sensitivity (Davis, 1970).

Other agents. In several experiments the effects of Na on the Ca efflux was studied. Neither external Na reduction (replaced by sucrose or choline), nor internal Na elevation (by soaking in K-free solutions or ouabain) had any effect either at normal or reduced (0·1 mM) external Ca

concentration. These cells are therefore like R.B.C.s rather than squid axon in showing no coupling between Na and Ca. In other experiments it was found that ouabain (up to 2×10^{-3} M), external K (up to 100 mM) or Ca concentration (Fig. 5) had no effects on Ca efflux.

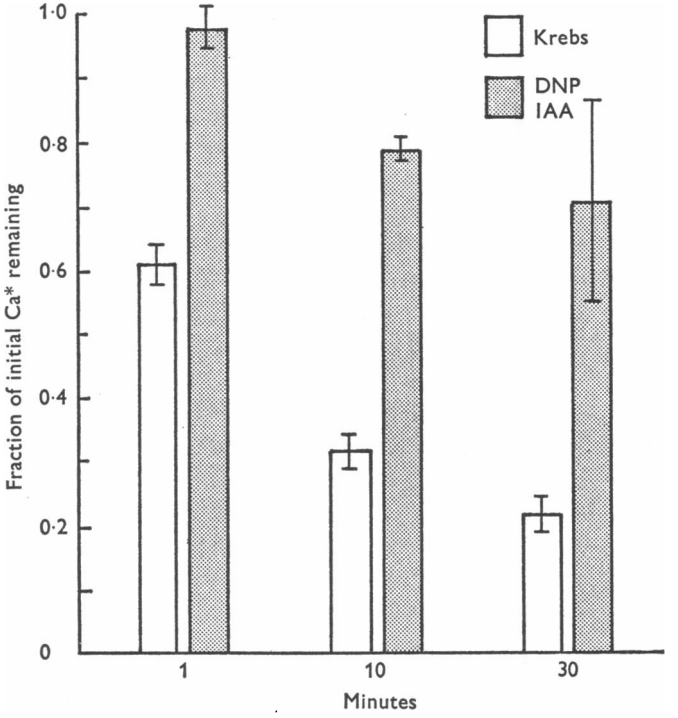

Fig. 3. Effect of DNP 10^{-3} M + IAA 10^{-4} M on Ca* efflux in L cells. The ordinate shows the fraction of the original Ca* remaining at the various times shown on the abscissa. Cells loaded overnight in BME, efflux in Krebs at 20° C. The efflux from each plate and the Ca* remaining measured at times shown. Each column represents the average of two separate plates. DNP + IAA reduced the Ca reflux to 27 % ($P < 0{\cdot}1$ %), the effect not being significantly different at the various times. The fraction remaining decreases with time as expected ($P < 0{\cdot}1$ %). Analysis of variance applied to all results (errors are ± S.E.).

Ca influx in fresh cells

The results so far show that poisoning with DNP + IAA, ethacrynate or NEM reduces the Ca efflux. Fig. 6 presents experiments illustrating the Ca influx at 2 and 20° C under various conditions. The results show that neither Na removal (replaced by choline at 2·8 or 0·1 mM external Ca), nor DNP + IAA have any effect on the Ca influx. Again temperature has a marked effect on the Ca exchanges. A detailed analysis of this shows (1)

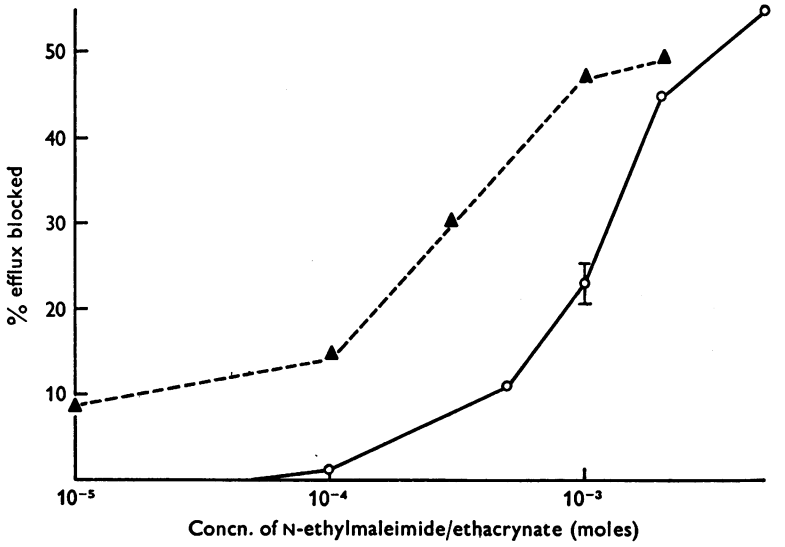

Fig. 4. Dose–response curves for ethacrynate and N-ethylmaleimide (NEM) on Ca efflux. Abscissa: drug concentration. Ordinate: the % of the 10 min efflux blocked by ethacrynate (circles) and NEM (triangles). Cells loaded with Ca* for several hours, drugs added at start of efflux. $n = 10$ for each point. Typical error (± 2 S.E.) shown.

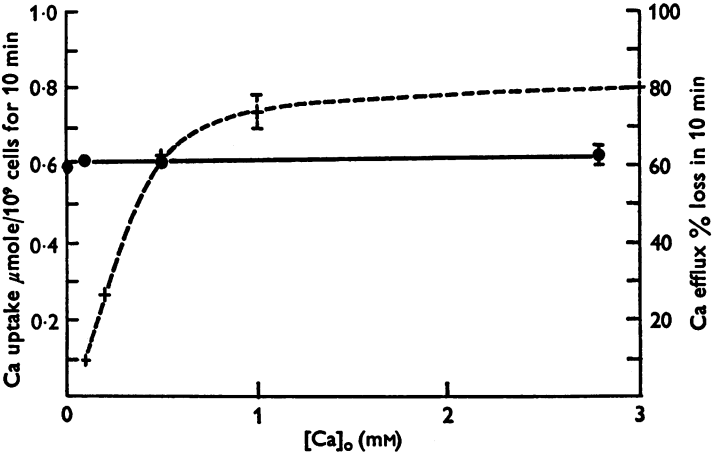

Fig. 5. The effect of external Ca concentration on Ca fluxes. Abscissa: Ca concentration. Ordinate: left, Ca uptake over 10 min (crosses, dashed line); right, efflux as % of initial content lost over 10 min (circles, continuous line). Cells kept in 2·8 mM-Ca normally and put into test Ca concentration containing Ca* at start of influx or efflux. Efflux $n = 10$, influx $n = 5$. Typical errors are ± 2 S.E.

that the Q_{10} of the Ca* influx is less than that of the Ca* efflux, and (2) that the Q_{10} of the Ca* efflux varies throughout an efflux; so that the activation energy over 0–1 min is 4300 cals/mole.degree but rises to 15,300 cals/mole.degree later.

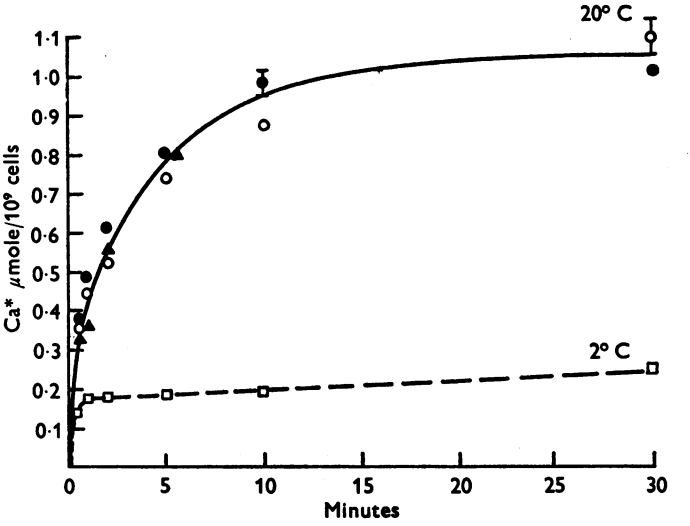

Fig. 6. Ca* influx (upper) at 20° C in Krebs (○), Krebs +DNP and IAA (●) or Na-free choline (▲). Each point an average of two plates in Na-free Krebs, four plates at 30 min, and seven plates at other times and conditions. DNP + IAA or low Na does not alter the Ca* influx. Representative errors (± 2 S.E.) are indicated by bars. Lower points (□) are at 2° C in Krebs. Each point average of three to five plates. Errors (± 2 S.E.) < size of symbols. Lines fitted by eye. Cells kept for ½–1 hr in Krebs before influx measurement made.

In further experiments in cells kept for some hours in Tris-maleate Krebs, a large difference between the Ca influx into normal and Na-loaded cells was noticed. Investigation of this point showed that it was due to the presence of maleate as the weak acid of the buffer. Fig. 7 shows that cells kept in Tris-HCl buffer have the same Ca uptake whether they have a normal or a rising intracellular Na; in Tris-maleate buffer, however, normal cells show a progressive decline in their Ca uptake, whereas cells with a rising intracellular Na remain at or above their initial uptake. A similar decline in the Ca efflux occurs in Tris-maleate buffered normal cells. Recently Kramer & Gonick (1970) have shown that maleate blocks Na/K ATPase and reduces the ATP content of renal cortex cells. Such an effect in our cells might explain the efflux but not the influx reduction. At the moment it is probably best to suppose that the prolonged presence of maleate reduces the calcium permeability and that in this condition raised internal Na (but not reduced external Na) does interact with Ca permeability.

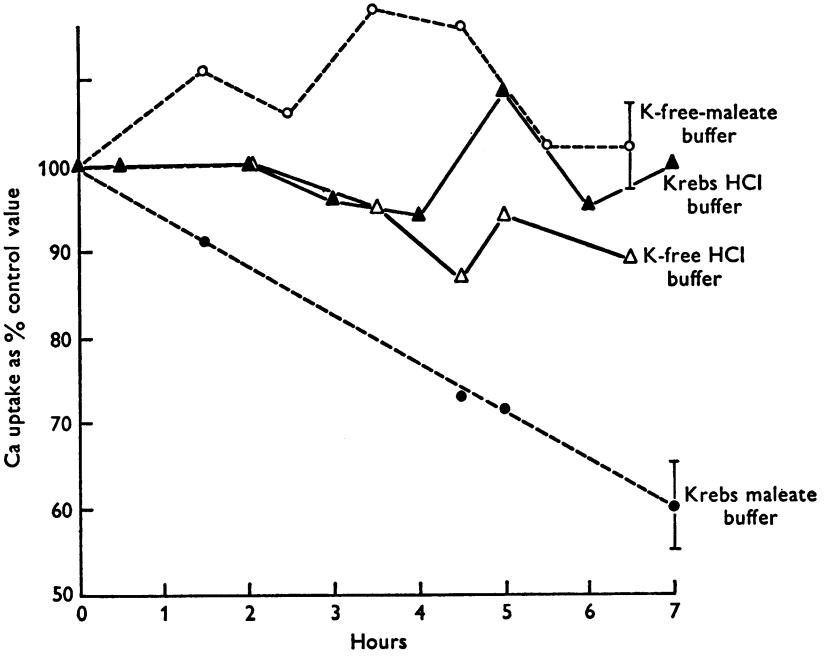

Fig. 7. The effect of maleate and HCl buffers on the Ca uptake. Abscissa: time the cells were incubated in the solutions shown. Ordinate: Ca uptake over 10 min as a % of the control value. Cells incubated in Krebs buffered with Tris-maleate (circles, dashed line) or Tris-HCl (triangles, continuous line) containing 5 mM-K (filled symbols) or zero K (open symbols) and then a 10 min uptake in the same solution containing Ca*. The K-free Krebs causes $[Na]_i$ to rise by about 12 m-mole/l. intracellular water per hour. Krebs with a maleate buffer causes a progressive decline in the Ca influx over the 7 hr studied, the other solutions produce no change. n = 5–10 per point (typical errors are ± 2 S.E.).

Ca contents in fresh cells

The results therefore show that DNP + IAA reduces the efflux of Ca but does not affect the influx. So the cells should gain Ca. In three preliminary experiments, Krebs containing DNP + IAA produced an average increase in Ca* content of 175% in 4 hr ($P < 1\%$ in each experiment by analysis of variance). The average rate of Ca gain was about 0·003 p-mole/cm^2.sec calculated on the basis that DNP + IAA takes 1 hr to decrease the cellular levels of ATP. DNP + IAA also consistently decreased the cell numbers over this time (to 68%, $P < 5\%$).

These experiments have the disadvantage that probably not all the cell Ca has exchanged with Ca* in the 4 hr of the experiment. To get round this difficulty this experiment was repeated in cells initially labelled with Ca*

by growing them for 10 days in a Ca* growth medium Table 2 shows that these cells contained rather more total Ca* than those treated in Krebs but that DNP+IAA produced a similar rise in total Ca (a net flux of 0·004 p-mole/cm^2.sec). It will also be noticed that poisoning produced an initial drop in the Ca content of the cells. This effect was noticed in other experiments in growth medium and in Krebs. As no unidirectional fluxes were done in growth medium no firm explanation can be offered for this. Perhaps it is a reflexion of a decreased Ca binding as the ATP levels run down in the cells (Palek, Curby & Lionetti, 1971). Other experiments with ethacrynate gave similar net fluxes.

TABLE 2. Effect of DNP+IAA on total calcium in L cells. Cells grown for 10 days in Ca* growth medium during which their number increased × 13, then equilibrated in Ca* Krebs with same specific activity and treated for 3 or 6 hr with DNP+IAA. Each number is the Ca content for one plate of cells expressed as μ-mole/10^9 cells. It can be seen that DNP+IAA initially causes a reduction in total Ca (to 80 %; P = 2–5 %) followed by an increase (to 120 %; P = 2–5 %). The difference between the early and late DNP+IAA effects was significant at $P < 1$ %. The control Ca content did not change with time (by analysis of variance applied to results). Another experiment gave similar results

	Time treated with DNP+IAA	
Treatment	3 hr	6 hr
DNP 10^{-3} M	2·91	4·34
+	2·74	4·51
IAA 10^{-4} M		
Krebs	3·61	3·59
	3·42	3·78

Experiments with L cell ghosts

The results so far show that the efflux of Ca from L cells differs in several respects from the influx. Thus the efflux is markedly affected by pH, has a higher Q_{10} than the influx, is unaffected by external Ca concentration, is markedly reduced by metabolic inhibition and by ethacrynate and NEM. These results are consistent with the hypothesis that the L cell has a Ca pump at its membrane driven by ATP, but do not provide very direct evidence for such a hypothesis. To provide more direct evidence we extended these experiments by using L cell 'ghosts', with which we could directly manipulate the internal as well as the external environment of the cells.

In preliminary experiments L cells were loaded with various Ca* concentrations and then analysed. It was found that the cell Ca, as μmole/10^9 cells, was approximately equal to the Ca concentration of the lysing solution (up to 15 m-mole/l.). To produce cells with internal Ca concentrations

in the normal range a lysing concentration of 1·4 m-mole/l. was used in the following experiments.

Effect of ATP on Ca efflux. Fig. 8 shows a preliminary experiment in which normal cells or cells poisoned with DNP + IAA were lysed and then refilled with either Ca* and 7 mM-ATP or only with Ca*. The main point

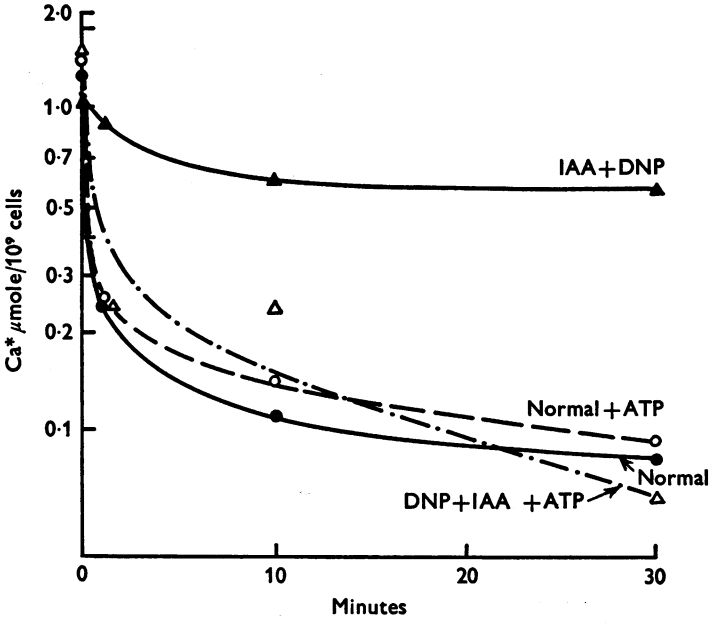

Fig. 8. Effect of ATP on Ca* efflux in ghost L cells. Each point represents the Ca content of a plate of L cells plotted semi-logarithmically. The circles are normal cells, the triangles cells previously poisoned with DNP 10^{-3} M + IAA 10^{-4} M for 4 hr. Each plate was then lysed and reconstituted at time zero; and the efflux followed until the time shown on the abscissa. The open symbols show plates which were reconstituted in a solution containing 7 mM-ATP in addition to the other constituents. It can be seen that incorporated ATP speeded up the Ca efflux in poisoned cells ($P < 2·5\%$, by analysis of variance) but had no effect in unpoisoned cells whose efflux was already fast. (△ at 1 min displaced to right as coincident with ●, △ at 10 min uncertain as cell count low.) Lines drawn by eye.

illustrated is that poisoned cells refilled with ATP lose Ca* at a similar rate to normal cells, whereas poisoned cells not so treated have a slow loss of Ca*. Other points are that ATP added to normal ghosts has no effect on the Ca* loss, indicating that the ghosting technique itself does not destroy endogenous ATP, a result similar to that in R.B.C.S (Schatzmann & Vincenzi, 1969). Fig. 9 shows the average results from a further two experiments. The general conclusions are similar to the results of Fig. 8. In

addition a comparison with the results of Fig. 3 shows that the cells are more leaky to Ca than normal cells, a result expected from similar experiments with R.B.C.S. From Fig. 9, an active Ca flux can be calculated on the assumption that the efflux over the first minute most closely resembles a unidirectional flux. This gives a figure of 0·17 p-mole/cm².sec, a flux which is probably the maximum the cells can produce, but some 20 times

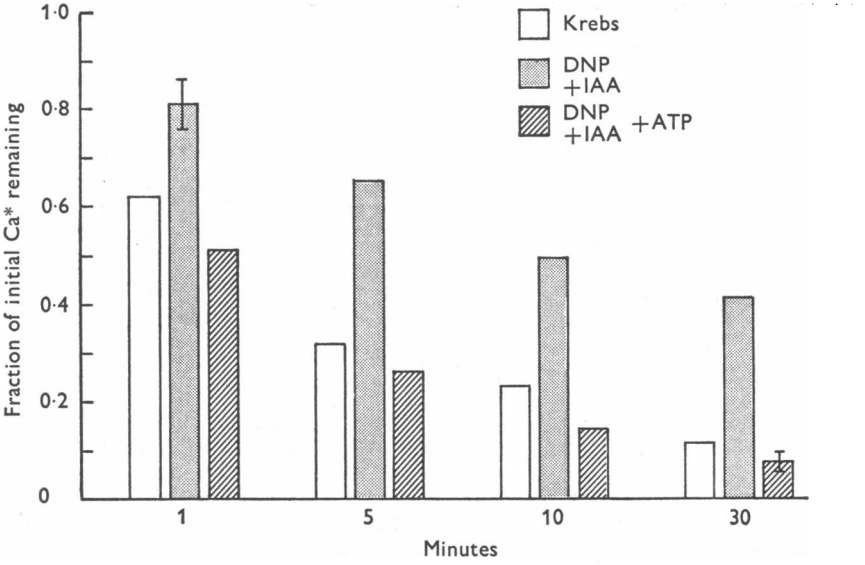

Fig. 9. Effect of ATP on Ca* efflux in ghost cells poisoned with DNP + IAA. The ordinate shows the fraction of the original Ca* remaining in the cells after the various times shown on the abscissa. Other details of experiment as Fig. 8. Results are averages of two separate experiments, each column containing two to six observations. Representative maximum and minimum errors (± 2 S.E.) are shown. DNP + IAA causes a reduction in the Ca efflux which is restored by ATP added in the lysing solution. ($P < 1\%$ for all times.) Note however the increased leakiness of the membranes compared to unlysed cells (Fig. 3). The normal cells contained 1·18 ± 0·07 (S.E., $n = 20$), DNP + IAA treated cells 0·81 ± 0·05 ($n = 16$) and the DNP + IAA + ATP cells 0·94 ± 0·08 ($n = 26$) μM-Ca/10^9 cells respectively at the start of the efflux. The active efflux over the first minute was 0·17 p-mole/cm². sec.

greater than the net flux which occurs when otherwise normal cells are poisoned.

These results show that the diminution of Ca efflux produced by poisoning cells can be reversed by the incorporation of ATP into the cells. They therefore give good evidence for the hypothesis that Ca efflux is dependent on ATP.

In a further series of experiments various other triphosphates were

incorporated into L cell 'ghosts'. Fig. 10 shows that cytidine triphosphate can also restore Ca pumping as well as adenosine triphosphate. It is unclear whether CTP is being used by the ATPase system or whether it is converting ADP to ATP.

Phosphate production during Ca pumping. It was possible to measure the amount of ATP splitting required for calcium pumping in these cells. To do so the phosphate produced by poisoned ghosts reloaded with ATP in

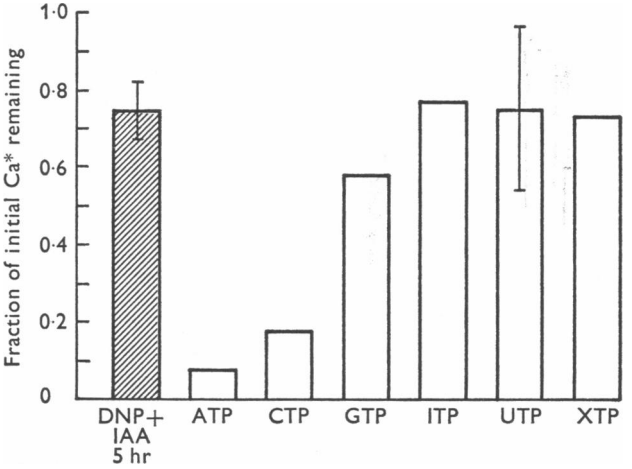

Fig. 10. Effect of various triphosphates on Ca* efflux in ghosts. All cells treated with DNP + IAA for 4–6 hr, then lysed and reconstituted with Ca* and either with no triphosphates (hatched column) or with 7 mM of the triphosphates shown (other columns). Results are fraction of original Ca* remaining after a 10 min efflux.

the presence or absence of internal Ca was measured. Fig. 11 gives the extra phosphate produced by ghosts when Ca is being pumped. It can be seen that this amounts to 1 μmole/10^9 cells over the first minute. In ten separate experiments (some shown in Fig. 9) 0·55 μmole/10^9 cells of Ca was pumped over the first minute. Combining these results gives a value of 1·8 mole ATP split per 1 mole Ca pumped, over the first minute of efflux; over the next 4 min the value is similar. This evidence shows that Ca pumping is associated with the splitting of ATP. It provides further evidence for the view that the Ca efflux is driven by ATP.

It will be noted that after the first minute there is a linear, much slower rate of phosphate production. A similar result was shown by Schatzmann & Vincenzi (1969) in R.B.C.S. Its cause is not clear, for in general the intracellular composition of the cells will no longer remain constant once the cells are heated up. One possibility is that the Ca-free cells do not remain so for long, due to the inward Ca leak from the washing solution. This can-

not be the whole explanation, however, as the total phosphate production also falls (not shown in Fig. 11). A contributory factor may be an inhibition of the Na/K pump as the level of ADP rises in the cell.

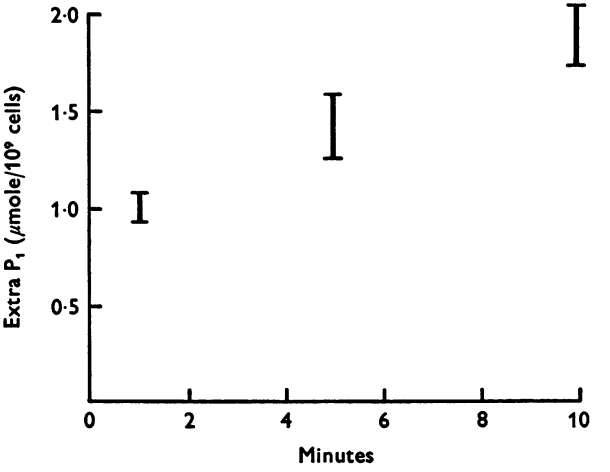

Fig. 11. Phosphate released by ghost L cells while pumping Ca. Poisoned cells lysed and then refilled with either 7 mM-ATP and Ca* or only with 7 mM-ATP in addition to the other constituents. Results shown are the extra P_i released when Ca is present. Results are averages of five to eight observations, with ±2 S.E.

DISCUSSION

These experiments indicate that the Ca efflux from L cells has different properties from the Ca influx into these cells, and in particular is markedly decreased by poisoning with DNP+IAA, suggesting that this efflux requires ATP. The 'ghost' experiments show that when poisoned cells are refilled with ATP then Ca efflux returns to normal. When so replaced the ATP is split as Ca is pumped out, confirming that energy is required for this process. These various facts can best be fitted by supposing that L cells have a Ca pump at their membranes which splits ATP.

The ghost experiments also show that 1·8 molecules of ATP are required to pump 1 molecule of Ca^{2+}. This figure is obtained by measuring the phosphate release in cells with and without Ca present but with normal $[Na]_i$ and $[K]_i$ and containing 7 mM-ATP. It is assumed that the presence or absence of Ca only affects the Ca pumping and nothing else. This is perhaps unjustified, but was also made by Schatzmann & Vincenzi (1969). The estimate of the cost of Ca pumping obtained by them came to 1·1 and 1·4 moles of ATP per mole of Ca. So our figure is rather larger than that obtained in R.B.C.S.

This calculation assumes that all the P_i released from ATP is used to pump Ca out of the cell, but it is possible that some of the ATP is used to sequester Ca within the cell. Whether or not this occurs depends on whether there are functional mitochondria and other bodies left in the ghost cells. Although there is no direct evidence on this point it was shown in Fig. 8 that unpoisoned ghost cells are still capable of pumping Ca and can therefore presumably still produce ATP in functioning mitochondria. It is therefore likely that some sequestering of Ca is occurring in these cells so that the cost of pumping Ca out of the cell is overestimated.

The energy necessary to pump Ca out of the cell against an electrochemical gradient may be calculated from the relation, Energy (kcals) = $ZF\ (E_m - E_{Ca})$ where the energy is in kcals/equivalent, F is 23 kcals per volt-equivalent and the membrane potential is in volts (Hodgkin, 1951). Using an E_m of -15 mV (Lamb & MacKinnon, 1971*b*), a $[Ca]_o$ of 2·8 mM and assuming a $[Ca]_i$ of 10^{-7} M (Portzehl, Caldwell & Ruegg, 1964; Baker, Hodgkin & Ridgeway, 1970) then the energy necessary to pump 1 mole Ca is about 6·5 kcal. If the energy available from the reaction ATP $\rightarrow$ ADP is 8 kcal/mole (H. Portzehl, personal communication) then the over-all efficiency of the reaction is about 45% (assuming no Ca is sequestered). With a membrane potential of -90 mV the cost of Ca pumping in an otherwise similar cell would increase by 50%.

It is difficult to be sure of the normal rate of Ca extrusion in these cells. Calculations from the initial rate of Ca extrusion in reloaded ghosts gives a unidirectional flux of 0·17 p-mole/cm^2.sec, probably a maximum pumping rate for these cells. On the other hand cells which have been blocked with DNP + IAA or ethacrynate show a Ca gain equivalent to a net flux of only 0·004 p-mole/cm^2.sec, a value about 1/20 of that of the maximum. The reason for this discrepancy is not clear. In order to try to decide between these figures we have calculated the cost (in terms of O_2 consumption) of these fluxes to see if this would exclude the high figure.

38 mole ATP can be produced by the break-down of 1 mole glucose, a reaction which requires 149 l. O_2 and 1 mole Ca^{2+} is pumped by 3 mole ATP. Therefore to pump Ca at a flux of 0·17 p-mole/cm^2.sec, a cell would use $0{\cdot}24 \times 10^{-6}$ μl. O_2/hr. The total O_2 consumption of an L cell is normally about 5×10^{-6} μl. O_2/hr (Danes & Paul, 1961) so that even this high rate of Ca pumping would only account for 5% of the total O_2 consumption. Clearly then it is possible (energetically) for these cells to pump Ca at the higher rate. In earlier work (Lamb & MacKinnon, 1971*a*) the Na pumping rate was estimated at 0·68 p-mole/cm^2.sec in L cells. An approximate measurement of the number of molecules of ATP split per molecule of Na transported was also obtained (MacKinnon, 1969). This came to 3 mole Na/mole ATP. From this and the above data the cost of the Na/K pumping

in these cells also comes to a figure of $0{\cdot}24 \times 10^{-6}$ μl. O_2/hr. It is perhaps curious that the cost of Ca and of Na/K should be so similar.

Resting potential and active flux. Since these cells have an active Ca efflux which may be electrogenic, and asymmetrical Na and K fluxes (three Na ions expelled for two K ions taken up (Lamb & MacKinnon, 1971*a*), it seemed worth while to calculate the maximum contribution such ion movements could make to the membrane potential. The Ca contribution was calculated by using equation (1.6) from Hodgkin & Rushton (1946) and gave a current of 3×10^{-8} A/cm^2. The size of the membrane potential this gives depends on the membrane resistance, which has not been measured directly but may be estimated from the chloride movements (Hodgkin, 1951). As the chloride flux in these cells is 6 p-mole/cm^2.sec (Lamb & MacKinnon, 1971*b*) a resistance of 4×10^4 Ω cm^2 is obtained. Using this value the maximum contribution of Ca efflux to the membrane potential would be -1 mV. A similar calculation for Na gives a contribution of -1 mV. It can therefore be concluded that neither ion would contribute much to the observed membrane potential.

It was found that Na ethacrynate and NEM blocked Ca extrusion. This effect came on with great rapidity and so is probably a surface effect rather than an effect on the mitochondria.

Other agents tried (K, Ca, Na, cyanide and ouabain) had no effect on the Ca movements. Of particular interest is the finding that the Ca movements or levels were not affected by the internal or external Na concentration. (Similar results have been obtained by us in two other lines of cultured cells.) This means that there is no coupling of transport between the Na and Ca movements in these cells as was recently suggested in squid axon (Baker *et al.* 1969; Blaustein & Hodgkin, 1969). It appears therefore that the Ca pump in these cells is similar to R.B.C.s but quite unlike that in squid axon and perhaps in heart muscle (Reuter & Seitz, 1968). From the examples of Ca pumping so far investigated it would appear that in excitable cells the extrusion of Ca is linked to Na influx, but in inexcitable cells it is not.

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