Biochimica et Biophysica Acta, 468 (1977) 146—156

BBA 77746

THE EFFECT OF A23187 UPON CALCIUM METABOLISM IN THE HUMAN LYMPHOCYTE

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(Received December 7th, 1976)

Summary

Treatment of human peripheral lymphocytes with mitogenic concentrations of the divalent cation ionophore A23187 led to an initial marked increase in the uptake of calcium by these cells, but the amount of accumulated calcium retained decreased with time so that after 8—12 h of culture, the calcium content of treated cells was only 1.5—2.0-fold higher than that of control cells. Three possible explanations for the biphasic nature of ionophore-induced calcium uptake were considered: (1) the ionophore underwent chemical or metabolic inactivation upon prolonged incubation; (2) massive accumulation of calcium caused irreversible uncoupling of mitochondria in these cells with consequent loss of accumulated calcium; or (3) with time there was a redistribution of ionophore within the cell, and sufficient ionophore was taken up by internal, most likely mitochondrial, membranes to cause an efflux of calcium from internal stores. By developing a bioassay for ionophore and examining the time-dependent effects of ionophore in the presence and absence of calcium, it was concluded that the third explanation was the most likely. The general implications of these results are discussed.

Introduction

The divalent cation ionophore A23187, which has been shown to increase the permeability of plasma membranes, endoplasmic reticulum, and mitochondrial membranes to Ca^{2+} and Mg^{2+} [1—4], is a potent mitogen for peripheral lymphocytes [5—8]. Its mitogenic effects are critically dependent upon the

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Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

external calcium concentration [5,8], but minimally affected by the external magnesium concentration [6]. These facts suggest that A23187 acts by increasing the uptake of calcium into lymphocytes but numerous questions remain regarding the role of calcium in A23187-induced proliferation. We have made a study of the effect of A23187 upon calcium metabolism in the human peripheral lymphocyte. Our results indicate that the time course of A23187 action is complex; an initial marked increase in calcium content of the lymphocytes is followed by a decline in calcium levels over a period of many hours after A23187 addition. Experimental exploration of this time-dependent biphasic effect of the ionophore indicates that the most likely explanation of the phenomenon is that during the initial period of ionophore action its effects are predominantly on the plasma membrane, but at later times the ionophore has a major effect upon the calcium permeability of the mitochondrial membranes as well.

Methods

Lymphocyte preparation. Lymphocytes were isolated from freshly drawn human blood as described previously [8]; the final cell population consisted of 95% lymphocytes with red blood cells and platelets accounting for most of the 5% contamination. Unless otherwise specified, cells were suspended in 1-ml cultures, containing modified Eagle medium with a calcium concentration of 1.0 mM and 10% human AB serum.

Total cellular calcium determination. Three sets, each of ten replicate cultures, of isolated human lymphocytes were incubated at 37° C in 1-ml cultures in modified Eagle medium with 1.0 mM Ca^{2+} and 10% AB serum at a concentration of $8 \cdot 10^6$ cells/ml. One set was given ethanol (10 μl) and two sets were given A23187 in ethanol (4 μM). The ethanol control and one A23187-treated set were incubated for an additional hour, and the second A23187-treated set was incubated for 17 h. 0.5 h before the end of each incubation period, [^{14}C]-dextran (0.25 μCi) was added to each culture. At the end of each incubation period, the ten replicate cultures in each set were combined and the cells centrifuged at 800 × *g* for 2 min at room temperature in an International Centrifuge. The supernatant was decanted, the inside of the tubes wiped, and the cell pellet extracted in 1 ml of 10% trichloroacetic acid plus 1% $LaCl_3$. The trichloroacetic acid pellets were centrifuged at 800 × *g* for 10 min and the supernatant saved. The pellets were washed two times in trichloroacetic acid/$LaCl_3$ and the washes combined with the supernatant to give a final volume of 2 ml. Calcium content of the extracts was determined by atomic absorption spectrophotometry. To determine the contribution of extracellular calcium to the extract total, extracellular space was calculated by measuring the radioactivity both in the trichloroacetic acid supernatant and trichloroacetic acid pellet. The pellet was dissolved in NCS before counting. Extracellular calcium accounted for 7—39% of the total calcium measured in the various samples.

^{45}Ca uptake. Isolated human lymphocytes were suspended at $1 \cdot 10^6$ cells/ml. ^{45}Ca (5 μCi) was added to each tube, followed immediately by either A23187 (in 10 μl ethanol) or ethanol (10 μl). At the times indicated in the figure legends, duplicate tubes were harvested by centrifuging the cultures at 800 × *g*

for 2 min at room temperature, decanting the supernatant, and washing the cell pellets twice in cold isotonic NaCl, pH 7.2. Pellets were then dissolved in NCS and counted in a scintillation counter.

ATP assay on FCCP and/or A23187-treated lymphocytes. ATP was assayed in perchloric acid extracts of human lymphocytes with a minor modification of the luciferase enzyme method of Stanley and Williams [9]. The reaction mixture consisted of 2 ml of 0.1 M Na_2HAsO_4 plus 20 mM $MgSO_4$, pH 7.4; and either 10 μl perchlorate extract or ATP standard plus 10 μl 1% $HClO_4$. The reaction was initiated by addition of firefly extract and assayed in a scintillation counter by counting for 0.1 min.

Oxygen consumption. Oxygen consumption in control and FCCP (20 μM)-treated lymphocytes was measured polarographically with a Clark-type oxygen electrode. A cell suspension of $2 \cdot 10^6$ cells/ml in modified Eagle medium with 1.0 mM Ca^{2+} and 10% serum was continuously stirred in a water-jacketed incubation chamber with a capacity of approx. 1.1 ml, maintained at 30° C.

Materials. Modified Eagle medium with Earle's salts was made up from its components, purchased from Grand Island Biological Co. $^{45}CaCl_2$ and [*carboxy*-^{14}C] dextran-carboxyl were purchased from New England Nuclear. NCS solubiliser was purchased from Amersham/Searle. A23187 was a gift from Dr. Robert Hamill of the Eli Lilly Co.; it was stored at −20° C in ethanol as a 2 mM solution. FCCP was a gift from P. Heytler (du Pont). Firefly lantern extract was purchased from Sigma.

Results

Effect of A23187 on total cell calcium

Calcium in lymphocyte pellets was measured by atomic absorption with a correction for extracellular space determined with [^{14}C]dextran. Calcium content of untreated lymphocytes ranged from 880 (experiment 1) to 580 (experiment 2) pmol of calcium per 10^6 cells. After 1 h of incubation with 4 μM A23187, the lymphocyte calcium content increased to 2600 (experiment 1) or 2500 (experiment 2) pmol of calcium per 10^6 cells. However, the high levels of cellular calcium induced by the ionophore were not maintained. After 17 h of incubation with A23187, cellular calcium content had declined to 800 (experiment 1) or 1200 (experiment 2) pmol of calcium per 10^6 cells.

Effect of A23187 on ^{45}Ca uptake

Fig. 1 shows that an analogous transient A23187-induced uptake of calcium could be demonstrated by following the uptake of ^{45}Ca into lymphocytes. When the concentration of A23187 was either 1 or 2 μM, an initial period of calcium uptake was followed by a plateau phase and then a decline in the accumulated ^{45}Ca to levels approaching control values. It must be emphasized that there was a great deal of variability in the response to ionophore of cells from different individuals on different days. Although curves qualitatively similar to Fig. 1 were reproducibly obtained, both the absolute magnitude of the response to A23187 and the length of time required for peak calcium uptake were variable from donor to donor and from day to day.

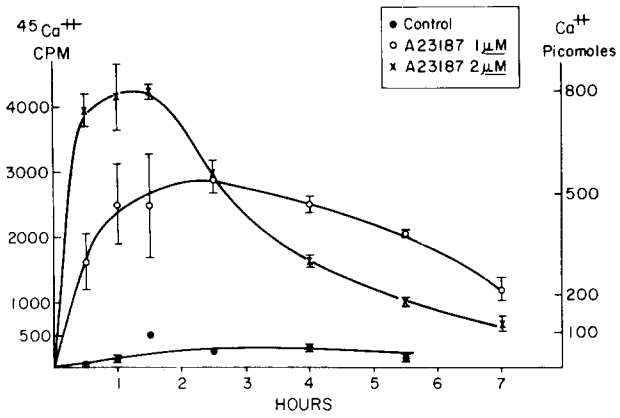

Fig. 1. The time course of accumulation of ^{45}Ca into human peripheral lymphocytes after the addition of the divalent cation ionophore A23187. Isolated human lymphocytes were suspended in modified Eagle medium with 1.0 mM Ca^{2+} plus 10% AB serum at $1 \cdot 10^6$ cells/ml. ^{45}Ca (5 μCi) was added to each tube, followed immediately by either A23187 (1 μM (○———○) or 2 μM (X———X) added in ethanol) or ethanol (10 μl (●———●)). At times indicated in the figure, duplicate tubes were harvested by centrifuging at room temperature, decanting the supernatant, and washing the cell pellets twice in cold 0.85% NaCl, pH 7.2. Pellets were then dissolved in 200 μl NCS and counted in a scintillation counter.

Bioassay of A23187

Because of the transient nature of the lymphocyte response to A23187, a bioassay was developed to ascertain whether either instability or metabolism of ionophore under our culture conditions could account for the release of accumulated calcium from the cells. The susceptibility of human red blood cells to hemolysis induced by hypotonic buffer, a process which has been shown to be very sensitive to low doses of A23187 [10], was used to assay for the presence of A23187 both in the lymphocytes and in the incubation medium of ionophore-treated cells.

It was found that at least 95% of A23187 added to lymphocyte cultures remained in the supernatant even after 18 h of incubation at 37°C. A very small (<5%), but consistent percentage of the added ionophore was found associated with the lymphocytes (data not shown).

To confirm the bioassay of A23187 in the culture medium, advantage was taken of the fluorescent properties of the ionophore [1]. It was found that 90% or more of the initially added ionophore could be accounted for in the supernatant of lymphocyte cultures even after 18 h of incubation (data not shown).

Stimulatory capacity of supernatant from A23187-treated cultures

In order to determine if medium from ionophore-treated cultures was capable of stimulating fresh lymphocytes, the following experiment was designed. Lymphocyte cultures were incubated with A23187 for various periods of time before being centrifuged; the medium from these treated lymphocytes was decanted onto fresh lymphocyte pellets; the cells were suspended in this medium; and ^{45}Ca uptake was then measured in these freshly exposed cells. Table I demonstrates that supernatant from cultures treated with A23187 for

TABLE I

THE UPTAKE OF ^{45}Ca INTO LYMPHOCYTES INDUCED BY THE SUPERNATANT OF LYMPHOCYTE CULTURES PREINCUBATED FOR VARIOUS PERIODS OF TIME WITH A23187

Isolated human lymphocytes ($1 \cdot 10^6$ cells/ml) in modified Eagle medium with 1.0 mM Ca^{2+} plus 10% AB serum were incubated with A23187 for 0.25, 0.5, and 19 h. At the end of the incubation period cells were spun down at room temperature and the supernatant decanted onto a fresh lymphocyte pellet ($1 \cdot 10^6$ cells). ^{45}Ca (5 μCi) was immediately added and incubation continued for 30 min at 37°C. Cells were then harvested as described in Methods. For the control and standard tubes, ethanol (10 μl) or A23187 (3 or 4 μM) in ethanol was added to 1 ml of modified Eagle medium containing 10% serum. Immediately thereafter, this medium was decanted onto fresh lymphocyte pellets, ^{45}Ca added, and incubation at 37°C for 30 min carried out as above. All data are given as the averages of duplicate determinations.

Culture condition	^{45}Ca counts/30 min
15 min supernatant	2068 ± 102
30 min supernatant	2258 ± 171
19 h supernatant	1851 ± 11
A23187 3 μM standard	995 ± 233
A23187 4 μM standard	1988 ± 73
Control	161 ± 56

0.25 to 19 h was as capable of inducing calcium uptake into fresh lymphocytes as an ionophore solution not previously incubated with cells.

A23187 effects on calcium uptake in the absence of serum

Since A23187 is a lipophilic compound [2], it was probable that most of the A23187 shown to be present in lymphocyte culture supernatants was bound to serum lipoproteins. Therefore lymphocytes incubated in the absence of serum should require much less ionophore to maximally take up calcium. Fig. 2 shows a dose vs. response curve of A23187-induced calcium uptake into lymphocytes incubated in the presence and absence of 10% serum. This experiment had to be carried out at 25°C because human lymphocytes are not viable in the absence of

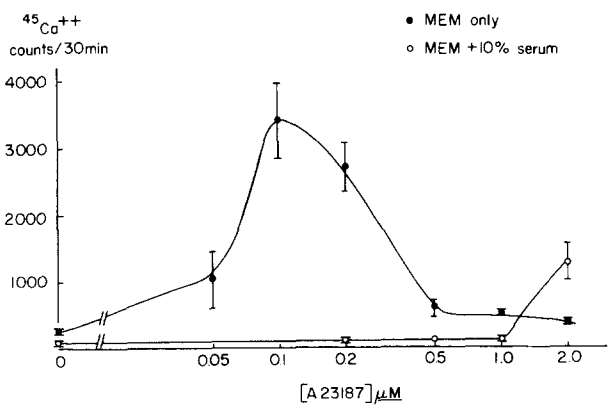

Fig. 2. Dose vs. response curves of A23187-induced ^{45}Ca uptake into lymphocytes incubated in the presence (○——○) or absence (●——●) of serum. Freshly prepared lymphocytes ($1 \cdot 10^6$ cells/ml) were incubated either in modified Eagle medium (MEM) with 1.0 mM Ca^{2+} or in modified Eagle medium with 1.0 mM Ca^{2+} plus 10% serum at 25°C. ^{45}Ca (5 μCi) was added to each tube, followed immediately by either ethanol (10 μl) or the listed concentrations of A23187 (10 μl in ethanol). Incubation was continued for 30 min at 25°C and harvesting then carried out as described in Fig. 1.

TABLE II

THE EFFECT OF FCCP ON A23187-INDUCED ^{45}Ca UPTAKE IN THE PRESENCE AND ABSENCE OF SERUM

Human lymphocytes were suspended at $1 \cdot 10^6$ cells/ml in 1-ml cultures in modified Eagle medium with 1.0 mM Ca^{2+} in the presence or absence of 10% serum. Cultures containing serum were incubated at 37°C, and those without serum, at 25°C. FCCP (20, or 0.5 μM) in ethanol or ethanol (10 μl) was added to replicate cultures at time zero. 15 min later appropriate tubes were given A23187 (4 or 0.2 μM) or ethanol (5 μl); all tubes were immediately thereafter given ^{45}Ca (5 μCi). Incubation was continued for an additional 30 min and then harvesting and counting carried out as described in Methods. All data are given as the averages of duplicate determinations.

Culture condition	^{45}Ca counts/30 min
+ serum	
Control	81 ± 1
A23187 (4 μM)	1010 ± 230
FCCP (20 μM)	118 ± 8
A23187 + FCCP	160 ± 35
— serum	
Control	302 ± 164
A23187 (0.2 μM)	1904 ± 16
FCCP (0.5 μM)	454
A23187 + FCCP	527 ± 83

serum at 37°C, as measured by Trypan Blue exclusion [11]. In the absence of serum, a maximal effect on calcium uptake was obtained at 0.1—0.2 μM A23187, while even 2 μM A23187 was a submaximal dose in the presence of serum in this experiment.

Sensitivity of calcium uptake to FCCP

Mitochondria are capable of accumulating large amounts of calcium via a mechanism sensitive to inhibition by respiratory uncouplers [12,13]. To determine if calcium taken up by lymphocytes treated with A23187 was accumulated by mitochondria, lymphocytes were preincubated with the uncoupler *p*-trifluoromethoxycarboxylcyanide phenylhydrazone (FCCP) and then stimulated with A23187. Table II shows that FCCP (20 μM for cultures in the presence of serum and 0.5 μM for cultures in the absence of serum) caused an approximate 90% inhibition of the A23187-induced calcium uptake. The much larger concentration of FCCP required to block A23187-induced calcium uptake in the presence of serum is accounted for by the ability of serum lipoproteins to bind lipophilic agents, such as FCCP, and hence reduce their free concentrations. To establish that FCCP was acting as an uncoupler, the rate of cellular respiration in the presence and absence of FCCP was measured. Oxygen consumption in control cells was 0.076 and in FCCP-treated cells 0.241 nmol O_2/min per $2 \cdot 10^6$ lymphocytes. In spite of this stimulation of respiration, FCCP at 20 μM in the presence of serum had no significant effect on the ATP levels of the lymphocyte within the time span of this experiment (Table III). The data in Table III also illustrate the point that A23187 lowered lymphocyte ATP levels by approx. 30% after a 15 or 30 min incubation; FCCP had no effect on this decrease.

TABLE III

THE ATP CONTENT OF LYMPHOCYTES TREATED WITH FCCP WITH AND WITHOUT A23187

At time zero ethanol (10 μl) or FCCP to a final concentration of 20 μM in ethanol was administered to human lymphocytes ($1 \cdot 10^6$ cells/ml) in 1-ml cultures in modified Eagle medium with 1.0 mM Ca^{2+} plus 10% serum at 37°C. At 15 min one set of control and FCCP-treated cultures were harvested by centrifugation at room temperature for 2 min, the medium was decanted, the inside of the tubes wiped and the cells extracted with 1 ml of 1% $HClO_4$. After centrifugation to remove the precipitate, the extract was frozen in a CO_2/acetone bath and stored at −20°C for several hours until assay. Also at 15 min, A23187 (4 μm) in ethanol or ethanol (5 μl) was added to both control cultures and cultures treated with FCCP at time zero. Incubation was carried out for an additional 15 or 30 min and harvesting carried out as described above. ATP was assayed in these extracts with a minor modification of the luciferase enzyme method of Stanley and Williams [9], as described in Methods.

Total incubation time (min)	pmol ATP/10^6 lymphocytes			
	Control	FCCP ($2 \cdot 10^{-5}$ M)	A23187 (4 μM)	A23187 + FCCP
15	345 ± 35	345 ± 5	—	—
30	345 ± 35	390 ± 30	255 ± 5	285 ± 15
45	355 ± 5	375 ± 5	230 ± 5	205 ± 5

Sensitivity of calcium uptake to external calcium concentration

Lymphocyte suspensions containing 0.2, 0.7, or 1.2 mM external calcium were treated with 2 μM A23187 and ^{45}Ca uptake determined as a function of time. Fig. 3 shows that the extent of calcium uptake in control cells was not affected by external calcium in this range, but the A23187-induced calcium

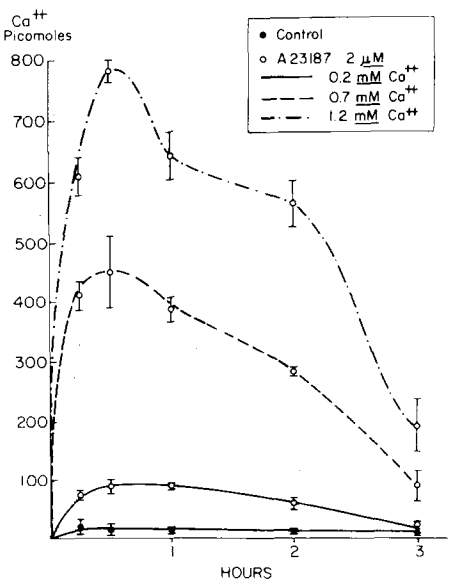

Fig. 3. The uptake of ^{45}Ca into lymphocytes induced by 2 μM A23187 at medium calcium concentrations of 0.2 (○———○), 0.7 (○- - - - -○) and 1.2 (○· — · — ·○) mM. Lymphocytes ($1 \cdot 10^6$ cells/ml) were incubated in modified Eagle medium plus 10% serum with a final calcium concentration of either 0.2, 0.7 or 1.2 mM. ^{45}Ca (5 μCi) was added to each tube followed immediately by A23187 (2 μM) or ethanol (10 μl). Incubation was carried out at 37°C for the indicated times and harvesting carried out as described in Fig. 1.

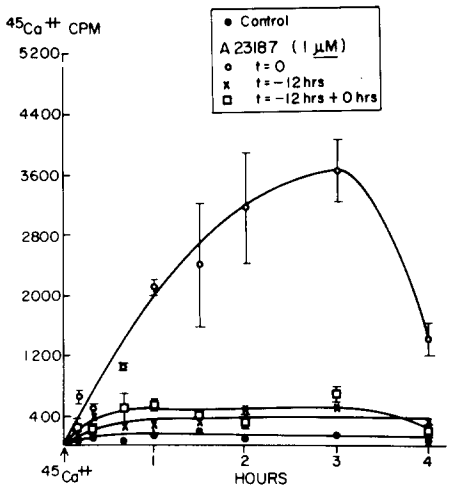

Fig. 4. The effect of a preincubation with A23187, in the presence of calcium, on the time course of the ^{45}Ca uptake into lymphocytes induced by a second A23187 addition. Lymphocytes ($1 \cdot 10^6$ cells/ml) were incubated at 37°C for 12 h in modified Eagle medium with 1.0 mM Ca^{2+} plus 10% serum. Two sets received A23187 (1 μM). At the end of 12 h, all tubes were given tracer ^{45}Ca (5 μCi). The control set had no other additions (•———•). A second set was given A23187 (1 μM) immediately after ^{45}Ca (○———○). A third set, which had been given A23187 (1 μM) 12 h before, had no other additions (X———X). A fourth set, which had been given A23187 (1 μM) 12 h before, was given a second dose of A23187 (1 μM) immediately after ^{45}Ca (□———□). Incubation at 37°C was carried out for the indicated times and harvesting done as described in Fig. 1.

uptake was very dependent on external calcium. The extent of calcium uptake at the plateau increased linearly with increasing external calcium, but all curves showed a decline in the amount of calcium accumulated after 1 h of incubation.

Effect of preincubation with A23187

The following experiment examined in a slightly different way the transient nature of the lymphocyte response to ionophore. Four sets of isolated lymphocytes were incubated overnight (12 h) at 37°C in the presence of 1.1 mM Ca^{2+}; two sets were treated with 1 μM A23187. After 12 h, i.e. at time zero, tracer ^{45}Ca was added to all cultures. Also at time zero, a second dose of 1.0 μM A23187 was added to one set of cultures which had been treated with ionophore 12 h before; and 1.0 μM A23187 was added to the third set which had not been previously exposed to ionophore. The results of these manipulations on calcium uptake are shown in Fig. 4. Lymphocytes which were incubated with ionophore for 12 h prior to the addition of tracer calcium showed a calcium uptake only slightly above control. Similarly, lymphocytes which were given two doses of ionophore, one 12 h prior to ^{45}Ca and a second dose with ^{45}Ca at time zero, also showed uptake only slightly above control levels. A normal biphasic uptake curve was found for cells incubated overnight, and then given only one treatment with A23187 at time zero. Thus not only was the effect of ionophore on calcium uptake transient, but prior treatment with A23187 actually altered the ability of cells to accumulate calcium in response to a second dose of ionophore.

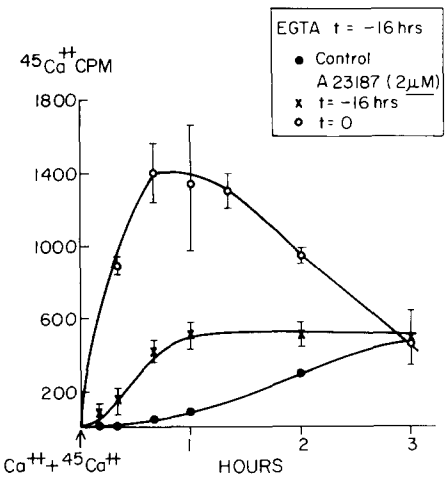

Fig. 5. The effect of a preincubation with A23187, in the absence of calcium, on the time course of the ^{45}Ca uptake into lymphocytes. Isolated lymphocytes ($1 \cdot 10^6$ cells/ml) were incubated for 16 h at 37°C in modified Eagle medium with 1.0 mM Ca^{2+} plus 10% serum, and 1.05 mM EGTA. One set of tubes was given A23187 (2 μM) (X———X). At the end of 16 h, all tubes were given $CaCl_2$ (10 μl of 0.1 M solution) and ^{45}Ca (5 μCi). One set was also given A23187 (2 μM) (○———○). Incubation at 37°C was carried out for the indicated times and harvesting done as described in Fig. 1.

Effect of preincubation with A23187 and EGTA

The following experiment was designed to determine if the time-dependent change in the response of the lymphocyte to A23187 required the presence of calcium in the medium. Lymphocytes were incubated overnight with 1.05 mM EGTA in the presence or absence of A23187 in modified Eagle medium plus 10% serum (Fig. 5). At time zero, calcium plus ^{45}Ca was added to all cultures to give a final free calcium concentration of approx. 1.1 mM. A23187 was added to one set which had not previously been given ionophore, and calcium uptake was determined with time. Control cells accumulated calcium slowly, and lymphocytes given A23187 as well as calcium at time zero showed the normal biphasic uptake curve. Cells preincubated with ionophore overnight showed a much lower uptake of calcium than those which had been given ionophore at time zero.

Discussion

The present data illustrate that the ionophore A23187 in mitogenic doses, causes a very rapid increase in the uptake of calcium into human peripheral lymphocytes, as measured both by total cell calcium determination and by isotopic means using ^{45}Ca. However, the large increase in calcium uptake is not maintained; over a period of many hours the calcium content of ionophore-treated lymphocytes declines to values approaching control.

Because the respiratory uncoupler FCCP is capable of preventing greater than 90% of the A23187-induced calcium accumulation, it is reasonable to conclude that most of the accumulated calcium is sequestered in the mitochondria. We have shown that FCCP at $2 \cdot 10^{-5}$ M (in cultures with serum) causes a 3-fold increase in the oxygen consumption of the lymphocytes, but does not alter the

cellular ATP content (Table III). The large glycolytic capacity of the lymphocyte [14,15] is most likely responsible for the maintenance of the normal ATP levels even when the few mitochondria of the cell are uncoupled.

Three possible explanations were considered here for the biphasic nature of the calcium uptake in ionophore-treated cells. First, with time the ionophore undergoes either chemical inactivation or metabolic conversion to an inactive metabolite. Second, as the ionophore-treated cells accumulate more and more calcium, the capacity of their mitochondria to accumulate calcium is exceeded and further loading results in irreversible damage to the mitochondria. This possibility was considered because isolated mitochondria, after taking up massive amounts of calcium, become uncoupled [13]. Third, with time there is a redistribution of ionophore within the cell from its initial site of action at the plasma membrane to intracellular, including mitochondrial, membranes.

To examine the first possibility we have used, as a sensitive bioassay for A23187, the known effect of ionophore upon the response of the human red cell to osmotic shock [10]. We are able to show that there is no apparent chemical inactivation or metabolic conversion of ionophore to an inactive metabolite.

The second possible explanation for the biphasic nature of the ionophore-induced calcium uptake in the lymphocyte is that massive accumulation of calcium by the mitochondria of these cells causes irreversible damage to the organelles resulting in their uncoupling. However, there are several facts that argue against this explanation. First, it follows from this hypothesis that whether one increases the uptake of calcium into the cell by increasing external calcium at a fixed ionophore concentration (Fig. 3) or by increasing ionophore concentration at a fixed external calcium concentration (Fig. 1), the mitochondria should take up a fixed, critical amount of calcium before becoming uncoupled. However, they do not. Another argument against the second hypothesis is illustrated by the data in Fig. 5. These data show that a 12 h preincubation of cells with ionophore in the absence of external calcium causes a marked decrease in the uptake of subsequently added calcium. Thus, even in the absence of external calcium, the response of the cells to ionophore changes as a function of time after ionophore addition.

All of our data, as well as earlier studies on the interaction of A23187 with isolated mitochondria [7], are consistent with the third hypothesis; i.e. the biphasic nature of the calcium uptake curve is due to a time-dependent redistribution of ionophore within the cells. The early effects of the ionophore are exerted primarily at the level of the plasma membrane, causing an increase in calcium entry into the cell; however, with time the ionophore also exerts an influence on the internal (mitochondrial) membranes, resulting in a net loss of accumulated calcium from these organelles and thereby a loss of calcium from the cells. The loss of calcium from the cell probably is an ATP-dependent process, and it occurs because even in ionophore-treated cells, ATP levels are maintained at 70% of control (Table III).

Whether external calcium is present (Fig. 4) or absent (Fig. 5), preincubation of cells with ionophore alters the subsequent response of the cells to either ionophore readdition or calcium addition. Furthermore, the higher the ionophore concentration, the more rapid is the peak uptake of calcium, and the

more rapid the decline in uptake (Fig. 1), implying that the higher the ionophore concentration, the sooner an effective intracellular (mitochondrial) ionophore content is reached. This reasoning would also explain the biphasic nature of the ionophore dose vs. response curve shown in Fig. 2. As the amount of ionophore added to these serum-free cultures was increased from 0.05 to 0.1 μM, there was a marked (8-fold) increase in calcium accumulation in 30 min, but when cells were incubated in 0.5 μM ionophore, the calcium uptake was considerably less than those incubated with 0.1 μM ionophore, being only 2-fold higher than that seen in control cells. Our interpretation of these data is that with higher added ionophore concentrations, a critical internal content of ionophore was reached almost immediately upon addition of A23187 to the cells. This critical amount of ionophore caused an increase in the rate of calcium efflux from mitochondria, thus preventing significant net calcium accumulation by these organelles, and hence by these cells.

This interpretation of the biphasic nature of the calcium uptake curve is also based on the results of Reed and Lardy [2] and Rottenberg and Scarpa [3], who have found that A23187 causes a leak of calcium from the mitochondria. At low A23187 concentrations the mitochondria are capable of reaccumulating this calcium at the expense of electron transport, but when the ionophore concentration exceeds some critical value, the mitochondria are no longer able to reaccumulate calcium as rapidly as it is being lost.

The present results indicate that although A23187 is a useful tool with which to explore cell function and cellular responses to changes in calcium metabolism, its effects upon cellular calcium metabolism are complex and time dependent. This makes it difficult to draw simple conclusions about the role of calcium in the cellular response under study.

Acknowledgements

This work was supported by grants (AM09650 and 7PD1AM19813-01) from the National Institutes of Health. We greatly appreciate the advice of Dr. Peter Nowell during the conduct of this research.

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