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Total and Exchangeable Calcium in Lymphocytes: Effects of PHA and A23187

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Calcium has been suggested as an internal second messenger when lymphocytes are stimulated by mitogens to enter the cell cycle. We have assessed the effect of 2 lymphocyte stimulants, the plant lectin phytohemagglutinin (PHA) and the calcium ionophore A23187, on human lymphocyte nucleic acid synthesis, total cell calcium content, and ^{45}Ca labeling. We have used an ultrasensitive method for the measurement of total cell calcium in the same samples used for radiolabeling. Mitogenic concentrations of A23187 ($\sim$.25 μmole/liter) caused an increase in both total cell calcium and ^{45}Ca labeling. These increases were almost completely blocked by inhibitors of mitochondrial respiration, suggesting that the calcium increment after ionophore treatment was located in the mitochondria. In contrast, total cell calcium was not altered at optimal mitogenic PHA concentrations (0.1 μg/ml and above). However, at the minimum PHA concentrations that caused stimulation (0.025 to 0.1 μg/ml), the dose response of ^{45}Ca uptake was very similar to that of DNA sysnthesis. Importantly, we could not stimulate DNA synthesis with PHA without increasing lymphocyte ^{45}Ca labeling. Thus, an increase in total cell calcium is not essential for mitogenesis; however, an increase in ^{45}Ca exchange is closely associated with the mitogenic effects of A23187 and PHA.

Key words: lymphocyte, calcium, phytohemagglutinin, A23187

Calcium ions may play a role in the initiation of lymphocyte mitosis. Several laboratories have reported that there is an early increase in ^{45}Ca uptake when lymphocytes are stimulated by mitogens [1–5] and that the calcium ionophore A23187 is mitogenic for lymphocytes [6–9]. However, there is a wide variation in the reported magnitude, duration, and concentration dependence of mitogen-induced ^{45}Ca uptake by lymphocytes, and one laboratory found no increase in ^{45}Ca uptake at mitogenic concentrations of A23187 or concanavalin A [10]. One difficulty in studying lymphocyte calcium metabolism has been the inability to measure accurately total cell calcium in small numbers of cells. Such measurements are necessary to determine if increased accumulation of ^{45}Ca by lymphocytes represents an increase in total or exchangeable calcium.

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In this paper, we have examined the effects of mitogenic concentrations of A23187 and phytohemagglutinin (PHA) on human lymphocyte calcium content and exchange. We have used an ultrasensitive graphite-furnace atomic absorption spectrophotometer in conjunction with a standard radiolabeling technique to measure both total lymphocyte calcium and cell associated ^{45}Ca in the same cell samples [11].

METHODS

Lymphocyte Preparation

Lymphocytes were isolated from the mononuclear cell-rich residues prepared during the plateletpheresis of normal donors [12]. The mononuclear cells in these residues were suspended with iron filings in tissue culture medium 199 with Earle's salts (TC-199) plus 1% (V/V) heat-inactivated fetal bovine serum (FBS) for 30 min at 37°C. The lymphocytes were separated by centrifugation over a metrizoate-ficoll gradient as described by Boyum [13]. Iron-adherent monocytes, granulocytes, and erythrocytes sedimented through the gradient. Lymphocytes were harvested from the gradient interface, washed twice, and resuspended in TC-199 with 20% V/V FBS plus penicillin (100 U/ml) and streptomycin (100 μg/ml). The cell concentration was adjusted to 5×10^6 cells/ml and incubated in horizontally placed tissue culture flasks under air and 7% CO_2 at 37°C. After 18 h, during which residual monocytes adhered to the plastic flasks, the lymphocytes were resuspended in TC-199 with 10% (V/V) FBS (Na, 145 mM; K, 5.5 mM; Ca, 1.8 mM). The lymphocyte concentration was adjusted to $4–5 \times 10^6$ cells/ml, and the suspension was preincubated for 30 min at 37°C prior to experimental manipulation. The final cell suspensions contained greater than 95% lymphocytes, as determined by morphology of stained smears and by cell size distribution. Cell viability determined by trypan blue dye exclusion was greater than 95%.

^3H-Thymidine and ^3H-Uridine Incorporation into DNA and RNA

The mitogenicity of different concentrations of PHA and A23187 was assayed by measuring the incorporation of ^3H-uridine and ^3H-thymidine into the acid-precipitable fraction of lymphocytes. Lymphocytes were suspended at 1×10^6 cells/ml in TC-199 plus 10% (V/V) FBS, and 1 ml aliquots of the suspension were placed into culture tubes with the appropriate amounts of either PHA or A23187. Uridine incorporation was assayed by adding ^3H-uridine 1 μCi/ml, (specific activity = 9 Ci/mmole) at the beginning of the incubation and incubating the cells under air and 5% CO_2 for 18 h before harvesting. Thymidine incorporation was assayed by adding ^3H-thymidine 2 μCi/ml, (specific activity = .52 Ci/mmole) after the lymphocytes were exposed to mitogen for 48 h. After an additional 24 h (72 h total), the cells were harvested and processed as previously described [14]. The β-radioactivity of the extracted RNA and DNA was determined by liquid scintillation spectrophotometry.

Sample Preparation and Calcium Analysis

The procedures for sample preparation and analysis of total and radioactive calcium were described previously [11]. In brief, 1.0 ml of a cell suspension containing $4–5 \times 10^6$ lymphocytes was sedimented over 0.5 ml of corn oil: butyl phthalate, 3:10 (V/V) at 8000g for 1 min in a 1.5 ml polypropylene tube. Twenty-five μl of the supernatant were removed and placed with 0.5 ml deionized water in 10 ml of Bray's solution for determin-

ation of ^{45}Ca β-radioactivity by liquid scintillation spectrophotometry. The remaining supernatant was removed with a Pasteur pipette, and the tube was inverted to drain the oil. Measurement of ^{45}Ca β-radioactivity in samples from the oil indicated that there was no significant partitioning of ^{45}Ca or A23187-bound ^{45}Ca in the oil phase. The end of the tube containing the cell pellet was severed and placed into a 3-ml polypropylene vial. One ml of deionized water was added, and the cells were dispersed and disrupted by sonication. One-half milliliter of the sonication suspension was placed into 10 ml of Bray's solution for determination of ^{45}Ca β-radioactivity. Differences in quenching between pellet and supernatant were measured by the addition of ^{45}Ca as an internal standard.

The remaining 0.5 ml of the pellet suspension was analyzed for total calcium by graphite-furnace atomic absorption spectrophotometry, using an IL 555 flameless atomizer and IL 351 atomic absorption spectrophotometer. A peak-height mode of analysis was used at 422.7 nm. The recovery of the cells by the oil-gradient separation technique was measured under each experimental condition by a radioactive chromium-labeling technique [15] and was found to be 100% ± 5% (SE) for all experiments. Blank samples were prepared in the same manner as cell samples using buffer with no cells. The background calcium measured in blank samples accounted for 10% ± 5% (SE) of the total measured calcium in each cell sample. The amount of calcium in the cell pellet due to trapped extracellular buffer was measured by using ^{14}C-sucrose as an extracellular marker. For each experimental condition, a duplicate set of samples was prepared as described above, except that ^{45}Ca was omitted from the experimental incubation and ^{14}C-sucrose (specific activity = 602 mCi/mmole) was added 1 min prior to sampling to achieve a final concentration of 1 μCi/ml. The volume of the trapped buffer was calculated from the radioactivity of the supernatant and the pellet. The trapped buffer accounted for 15% ± 4% (SE) of the total calcium in the sample when 5×10^6 untreated cells were separated.

Calculation of Cell-Associated Calcium

The calcium content of the lymphocytes was calculated as previously described [11] using the following equation:

$$Ca = [T - (B + S)] \div (N \times V),$$

where Ca = lymphocyte calcium content (mmoles/liter of cells); T = calcium in cell pellet (mmoles/sample); B = calcium in cell-free blank samples (mmoles/sample); S = calcium in extracellular trapped buffer (mmoles/sample); N = number of cells in pellet (number of cells/sample); V = median volume of cells in suspension (liters/cell).

RESULTS

Thymidine and Uridine Incorporation in PHA- and A23187-Treated Lymphocytes

The effect of varying concentrations of PHA on DNA and RNA synthesis in 6 lymphocyte populations is shown in Figure 1. PHA maximally stimulated ^{3}H-thymidine incorporation into DNA (approximately 15 times the control) at a concentration of 0.25 μg/ml. There was a gradual decrease in thymidine incorporation as the PHA concentration was increased to 8 μg/ml. The effect of PHA on ^{3}H-uridine incorporation into RNA was similar. Maximal uridine incorporation (approximately 4 times the control) was observed at a PHA concentration of 0.50 μg/ml.

The effects of A23187 on thymidine and uridine incorporation in 6 lymphocyte populations are shown in Figure 2. The ionophore maximally stimulated ^{3}H-thymidine incorporation into DNA (approximately 4 times the control) at a concentration of 0.40 μmoles/liter. The magnitude of ^{3}H-thymidine incorporation decreased to the control level as A23187 was increased to 1 μmole/liter. The maximal stimulation of ^{3}H-uridine incorporation (approximately 3 times the control) occurred at an A23187 concentration of 0.5 μmoles/liter. Uridine incorporation remained at least twice the control level as the ionophore was increased to 1 μmole/liter.

The morphology of lymphocytes exposed to PHA or A23187 after 72 h of culture was examined on Wright-stained cytocentrifuge smears. Cells were identified as stimulated lymphoblasts if they displayed a combination of characteristics including increased size, enlarged nuclei, prominent nucleoli, and basophilic cytoplasm. The percentages of lymphocytes that had undergone blast transformation by these criteria after 72 h were 9.5, 80, 77, and 69 at PHA concentrations of 0, 0.1, 0.5, and 1.0 μg/ml, respectively; the percentages were 36, 52, and 53 at A23187 concentrations of 0.25, 0.4, and 1.0 μmole/liter, respectively. In cultures with 0.25 μmoles/liter of A23187, cell survival was 50%, but with 1.0 μmole/liter, it was only 5% at 72 h. Although 53% of the lymphocytes were lymphoblasts after treatment with 1.0 μmole/liter of A23187, this represented a very small number of proliferating lymphocytes. This explains why no increase in ^{3}H-thymidine incorporation was observed at 1.0 μmole/liter of A23187, although greater than 50% of the surviving cells were blasts.

The Effect of A23187 on Total and Exchangeable Lymphocyte Calcium

The total and exchangeable calcium content in 6 lymphocyte populations was measured after 1 and 30 min exposure to 0.1, 0.25, and 1.0 μmole/liter of A23187 (Table I). Treatment with A23187 did not alter cell viability or survival at 30 min. At 1 min, no significant change in total cell calcium was measured at any of the ionophore concentrations tested. After 30 min, no change in total calcium was measured at 0.1 μmole/liter of A23187. Total calcium content increased significantly ($P < .01$) at 0.25 and 1.0 μmole/liter of A23187. Calcium-45 labeling was significantly increased ($P < .01$) at the 3 ionophore concentrations at both 1 and 30 min (Table I).

Figure 3 shows the time course of total and radioactively-labeled calcium in un-

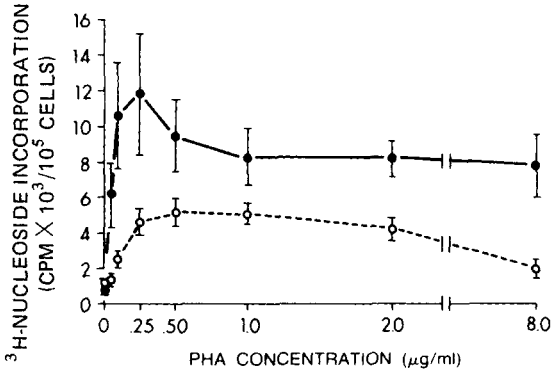

Fig. 1. The effect of PHA on DNA and RNA synthesis in human lymphocytes. ^{3}H-thymidine incorporation (closed circles) and ^{3}H-uridine incorporation (open circles) were measured in 6 cell populations as a function of PHA concentrations from 0 to 8 μg/ml. The data represent the mean ± SE.

treated lymphocytes and lymphocytes exposed to 0.25 μmoles/liter of A23187 during 8 h incubation. This concentration of A23187 was chosen because it is mitogenic and because cell viability remained greater than 95% for 8 h. A23187 caused a time-dependent increase in total cell calcium that was approximately 50% complete by 30 min and complete by 2 h. The cell calcium remained elevated in A23187-treated cells at the time of the last measurement, 8 h.

Cell associated ^{45}Ca increased 3.5 times, from a control value of .21 ± .09 mmoles/liter of cells to 0.75 ± .15 ($P < .01$), after 1 min exposure to A23187. This increase in ^{45}Ca labeling occurred before a significant increase in total cell calcium was measured This reflects an ionophore-induced increase in exchange of cell and medium calcium before net influx occurs. Later increases in cell ^{45}Ca parallel the increase in total cell calcium, reflecting net influx of calcium from the medium.

The Effect of Mitochondrial Inhibitors on the A23187-Induced Changes in Cell Calcium Content

The total and radioactivity-labeled cell calcium in the presence and absence of A23187 and mitochondrial inhibitors was measured in 4 lymphocyte populations (Fig. 4). The total lymphocyte calcium content of untreated cells was 1.41 ± .05 mmoles/liter of cells. Thirty minute exposure to the mitochondrial inhibitors did not alter this value significantly. A23187 at 0.25 μmoles/liter caused a 3.4-fold increase in total cell calcium to 4.79 ± .63 mmoles/liter of cells ($P < .01$), and this increase was almost completely blocked by the mitochondrial inhibitors. The cell calcium content in the presence of 0.25 μmoles/liter of A23187 plus inhibitor was 2.0 ± .16 mmoles/liter of cells with 100 μmoles/liter of dinitrophenol, 1.47 ± .05 mmoles/liter of cells with 1.0 mmoles/liter of sodium azide, and 1.37 ± .12 mmoles/liter of cells with 1.0 mmoles/liter of sodium cyanide. A23187 at 1.0 μmoles/liter caused a 6.4-fold increase in total calcium to 8.97 ± .86 mmoles/liter of cells ($P < .01$). This increase was less effectively blocked by the mitochondrial inhibitors. The cell calcium content in the presence of 1.0 μmole/liter of

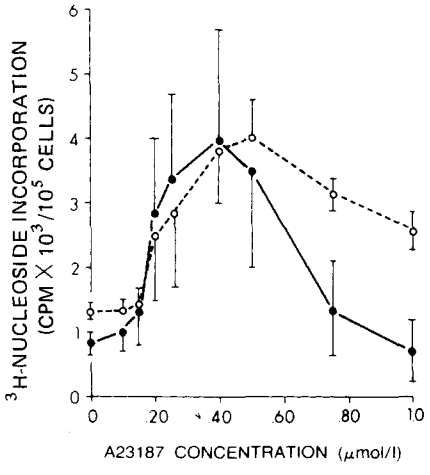

Fig. 2. The effect of A23187 on DNA and RNA synthesis in human lymphocytes. ^{3}H-thymdine incorporation (closed circles) and ^{3}H-uridine incorporation (open circles) were measured as a function of A23187 concentration in the same 6 cell populations as in Figure 1. The data represent the mean ± SE.

TABLE I. Total and Exchangeable Calcium in A23187-Treated Lymphocytes*

Time	A23187 μmole/liter	Total Ca	^{45}Ca-labeling
		mmole/liter of cells	
1 min	0	2.33 ± 0.40	.31 ± 0.06
	0.1	2.09 ± 0.40	.42 ± 0.07
	0.25	2.36 ± 0.40	.47 ± 0.07
	1.0	2.49 ± 0.48	.91 ± 0.21
30 min	0	2.28 ± 0.39	.35 ± 0.08
	0.1	2.22 ± 0.26	.67 ± 0.11
	0.25	4.28 ± 0.72	2.81 ± 0.47
	1.0	7.06 ± 1.10	6.38 ± 1.29

*The data represent the mean ± 1 SE of duplicate measurements in 6 lymphocyte populations.

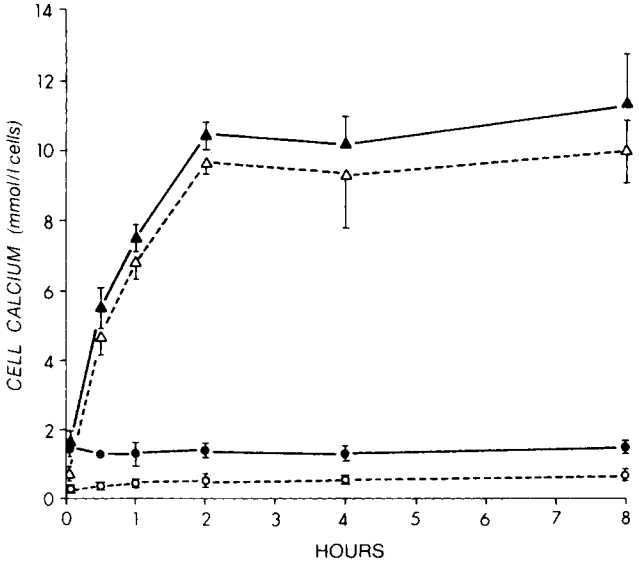

Fig. 3. Total cell calcium and calcium-45 labeling as a function of time in A23187-treated lymphocytes. The mean ± SE of total calcium content (closed triangles) and the mean ± SE of cell ^{45}Ca (open triangles) of 3 cell populations exposed simultaneously to ^{45}Ca and 0.25 μmoles/liter A23187 is shown between 1 min and 8 h. The total cell calcium (closed circles) and cell ^{45}Ca (open circles) in untreated cells is also shown.

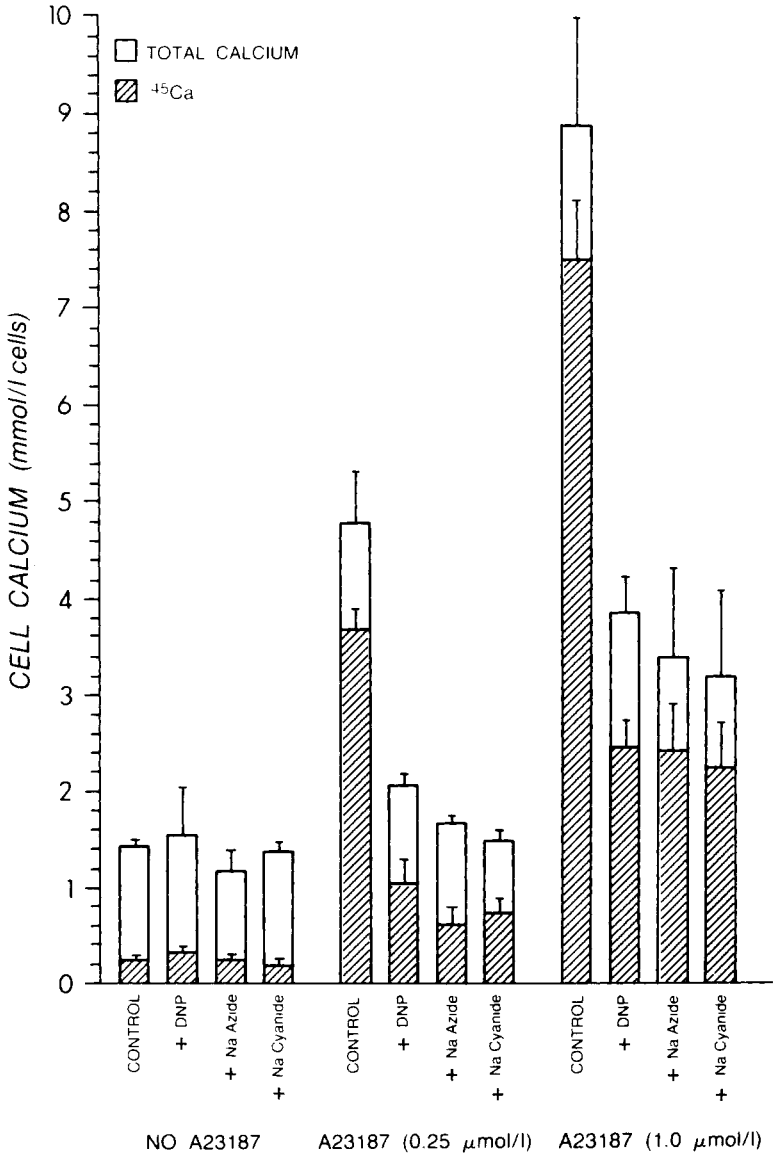

Fig. 4. The effect of mitochondrial inhibitors on A23187-induced uptake of calcium. The mean ± SE of total calcium content (total height of columns) and the ^{45}Ca-labeled fraction (shaded columns) were measured in 4 lymphocyte populations exposed to 0, 0.25, or 1.0 μmoles/liter A23187 plus no inhibitor, 100 μmoles/liter DNP, 1 mmole/liter Na azide or 1 mmole/liter NaCN. Cells were first incubated in the presence of inhibitor for 15 min, then incubated in the presence of inhibitor plus A23187 for an additional 15 min before measurement.

A23187 plus inhibitor was 3.56 ± .53, 3.16 ± .78, and 3.00 ± 1.06 mmoles/liter of cells with dinitrophenol, sodium azide, and sodium cyanide, respectively; these values were significantly higher ($P < .01$) than in untreated cells. Cell associated ^{45}Ca rose proportionately with total cell calcium in ionophore-treated cells without mitochondrial inhibitors. In the presence of mitochondrial inhibitors, the ionophore caused a greater increase in cell ^{45}Ca than the increase in total cell calcium. For example, in the presence of sodium cyanide, 0.25 μmoles/liter of A23187 caused an increase in the cell ^{45}Ca of 0.52 mmoles/liter of cells over control ($P < .01$), while total cell calcium rose by only .08 mmoles/liter of cells, not significantly different from the control ($P > .05$). This reflects an ionophore-induced increase in exchange of medium calcium with cell calcium without net accumulation.

The Effect of PHA on Total and Exchangeable Lymphocyte Calcium

The results of the analysis of total and radioactive calcium in samples from 6 lymphocyte populations are shown in Table II. The lymphocyte calcium content was 2.33 ± .40 (SE) mmoles/liter of cells in untreated cells and did not change significantly after 1 or 30 min exposure to PHA at 0.5, 1, or 8 μg/ml. In untreated cells, the amount of cell calcium that was radioactively labeled after 1 min was 0.31 ± .06 mmole/liter of cells (13% of the total cell calcium). After 30 min, 0.35 ± .08 mmole/liter of cells (15% of the total cell calcium) was labeled. PHA caused an increase in the exchangeable calcium that was related to the concentration of lectin. After 1 and 30 min, the amount of cell calcium labeled with ^{45}Ca increased significantly ($P < .05$) at all 3 PHA concentrations (Table II).

The Relationship of ^{45}Ca Uptake and the Mitogenic Response to PHA

To determine if ^{45}Ca uptake was an antecedent or a result of lectin-induced blastogenesis, we examined both processes at PHA concentrations at or below the optimum for ^{3}H-thymidine incorporation into DNA.

Figure 5A shows the dose response curve for ^{3}H-thymidine incorporation in 6 lymphocyte populations at PHA concentrations from 0 to 0.2 μg/ml. Maximal PHA stimulation of ^{3}H-thymidine incorporation into DNA (approximately 15 times the control) occurred at a concentration of 0.1 μg/ml.

Calcium-45 labeling in the same 6 lymphocyte populations exposed to low concentrations of PHA is shown in Figure 5B. In untreated cells, 0.25 mmoles of Ca/liter of cells was radioactively labeled after 30 min. The amount of cell calcium labeled with ^{45}Ca increased with increasing PHA concentrations to 0.51 mmoles/liter of cells at 0.1 μg/ml PHA. The lowest concentration of PHA that stimulated DNA synthesis also increased ^{45}Ca labeling.

DISCUSSION

We have examined the effects of mitogenic concentrations of the calcium ionophore A23187 as well as PHA on total cell calcium and cell ^{45}Ca to further define the role of calcium as an ionic signal in surface-stimulated mitogenesis. We observed an increase in total lymphocyte calcium and ^{45}Ca labeling after treatment with mitogenic or inhibitory supramitogenic concentrations of A23187. These findings are consistent, in general, with previous reports of the early effects of A23187 on total lymphocyte calcium [16] and ^{45}Ca uptake [6, 17].

TABLE II. Total and Exchangeable Calcium in PHA-Treated Lymphocytes*

Time	PHA μg/ml	Total Ca	^{45}Ca-labeling
		mmole/liter of cells	
1 min	0	2.33 ± 0.40	.31 ± 0.06
	0.5	2.34 ± 0.36	.47 ± 0.11
	1.0	2.22 ± 0.31	.46 ± 0.07
	8.0	2.20 ± 0.36	.75 ± 0.11
30 min	0	2.28 ± 0.39	.35 ± 0.08
	0.5	2.24 ± 0.36	.57 ± 0.10
	1.0	2.09 ± 0.23	.58 ± 0.08
	8.0	2.01 ± 0.20	.80 ± 0.09

*The data represent the mean ± 1 SE of duplicate measurements of 6 lymphocyte populations.

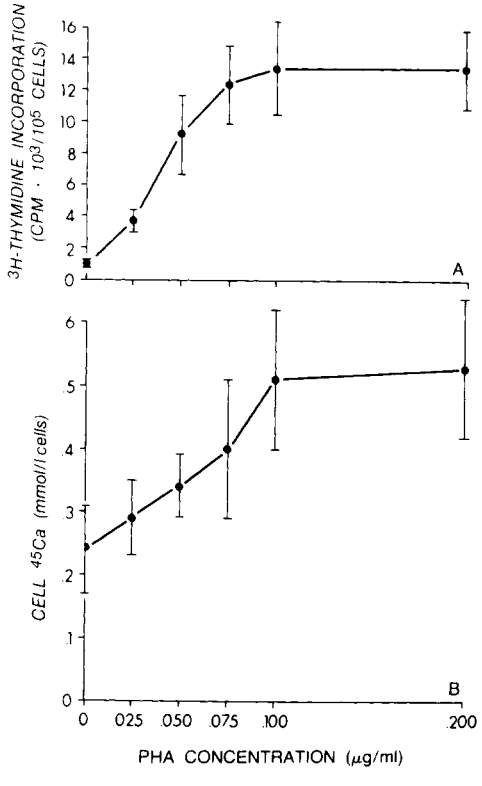

Fig. 5. A: ^{3}H-thymidine incorporation as a function of PHA concentrations from 0 to 0.2 μg/ml. The data represent the mean ± SE in 6 lymphoctye cell populations. B: Cell ^{45}Ca as a function of low PHA concentrations. The mean ± SE of cell ^{45}Ca after 30 min of PHA treatment is shown for the same cell populations studied in 5A.

The increase in cell calcium induced by mitogenic concentrations of A23187 was blocked almost completely by mitochondrial inhibitors [17, 18]. This suggests that the bulk of the calcium entering lymphocytes after ionophore treatment was sequestered in the mitochondria. When mitochondrial sequestration was blocked, the cell calcium remained low while calcium labeling increased. Under these conditions, the incoming calcium may be extruded via a plasma membrane calcium transport ATPase, preventing a rise in total cell calcium. In this regard, we have described a calcium-activated Mg-dependent ATPase and active calcium transport in lymphocyte plasma membrane vesicles [19]. At very high ionophore concentrations (1 μmole/liter) the magnitude of the calcium influx may have exceeded the capacity for plasma membrane extrusion, so that the total lymphocyte calcium increased even in the presence of mitochondrial inhibitors. Taken together, these data suggest that the lymphocyte can regulate the calcium concentration in the cytosol by both mitochondrial sequestration and active calcium extrusion.

PHA did not affect total calcium but caused a small, though consistent, increase in ^{45}Ca labeling of lymphocytes that was concentration dependent. The magnitude of the increase in cell ^{45}Ca after PHA treatment, even at optimal mitogenic concentrations, is small compared with previously reported measurements [5, 20]. Higher levels of cell ^{45}Ca were observed if excessive concentrations of PHA were used, if the influence of the serum concentration on lymphocyte calcium content was not considered [11], or if a correction was not made for extracellular calcium trapped in cell pellets during the separation of lymphocytes from their incubation medium.

The unaltered total calcium after PHA treatment is consistent with one previously reported measurement of total cell calcium in PHA-stimulated pig lymphocytes [16]. Since total cell calcium was unchanged, we interpret the PHA-dependent increase in ^{45}Ca as an increase in the exchangeable pool of lymphocyte calcium. This interpretation assumes that we would be able to measure a small increase in total cell calcium if the increase in ^{45}Ca uptake represented a net accumulation of calcium. In this regard, lymphocytes treated with 8 μg/ml PHA showed an increase in ^{45}Ca uptake that was greater than twice the coefficient of variation for the measurements of total cell calcium in the study, and if a true increase in total cell calcium had occurred, it should have been detected. Thus, PHA does not cause an early increase in lymphocyte calcium content, but rather alters the size of the exchangeable pool of cell calcium. This increase in calcium exchange is closely associated with the mitogenic effects of both PHA and A23187 and thus may be critical for the sequential events leading to lymphocyte mitosis.

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