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CELL CALCIUM IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND THE EFFECT OF MITOGEN

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The apparent cell concentration of calcium in human peripheral blood lymphocytes is 143.3±17.7 μM, as measured by two different techniques using ^{45}Ca. The steady-state level of accumulation, and possibly the rate of uptake, are increased in the presence of succinyl-concanavalin A. Initiation of the mitogen-induced alteration of cell calcium occurs within 1–2 min and the change is complete within 5–10 min. Determinations of cell calcium in cells suspended in low Na media indicate that (1) there is no difference in cell calcium between cells incubated in 142 mM extracellular Na and cells incubated in 63 mM extracellular Na, and (2) the mitogen-induced increase in cell calcium is unaffected by a decrease in extracellular sodium concentration (to 63 mM).

Introduction

We have recently reported [1] that mitogen-stimulated DNA synthesis in human peripheral blood lymphocytes is dependent on extracellular Na. Several possibilities may account for the observed Na effect, some of which are: (1) The external [Na] may affect Na-dependent transport systems in the plasma membrane, thus limiting 'substrate' availability for protein and/or DNA synthesis. (2) Decreased extracellular Na may cause an increase in intracellular Ca due to a reduced rate of Na-Ca exchange. (3) Mitogen-stimulation may induce an initial membrane depolarization that is necessary for the proliferative response; this event would be inhibited by decreased external sodium. (4) It may be that a Na-H exchange is operative and is required for the mitogenic response. As part of our ongoing research on the effects of extracellular Na concentration [1] we have now investigated the second possibility, namely that the Na-dependent proliferation might be mediated via alterations in intracellular calcium due to a Na-Ca exchange. Such exchanges have been shown to exist in the plasma membranes of nerve [2,3], muscle [4–6], secretory tissues [7–9], epithelia [10,11], kidney slices [12] and liver slices [13]. It seemed relevant to investigate the possibility of a sodium-induced change in cell calcium since calcium has been shown to be a critical factor in cell proliferation [14,15]. In particular, when human peripheral blood lymphocytes are treated with a variety of plant lectins, they are transformed from quiescent cells into proliferating ones [16,17] and calcium uptake, as measured by ^{45}Ca, increases [18–21]. Furthermore, a dependence of mitogenesis on extracellular [Ca] has been reported [22,23]. The exact nature of this dependence is controversial.

Our findings demonstrate that lymphocyte cell calcium is increased 30–70% by mitogen and is not regulated by extracellular Na. Furthermore, our

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

studies indicate that two early events in mitogenesis, namely an Na-dependent signal for DNA synthesis and the mitogen-induced increase in cell calcium, are not coupled events, i.e., the enhanced cell calcium response to mitogen addition still occurs in a low Na medium that inhibits proliferation.

Methods and Materials

Lymphocyte preparation

Heparinized human venous blood was collected from healthy donors. The lymphocytes were separated by gradient centrifugation using a modified Ficoll-Hypaque technique as previously described [1]. Cell number was determined using a Coulter Counter and lymphocyte viability was determined by Trypan blue exclusion. Cultures were prepared by adding $5 \cdot 10^5$ cells to a 1 ml solution containing Eagle's minimum essential medium plus 10% pooled human AB serum, 1% added L-glutamine and 1% penicillin-streptomycin. These were incubated at 37°C in humidified air containing 5% CO_2. DNA synthesis in the absence and presence of phytohemagglutinin (25–50 μg/ml) and of succinyl-concanavalin A (25–50 μg/ml) was assayed by use of [^{3}H]thymidine as a marker. [^{3}H]Thymidine (0.25 μCi) was added 8 h before the cultures were collected and washed (Model M12 Cell Harvester from Brandel), at which point the incorporation of [^{3}H]thymidine was measured by counting in a Beckman-7000 Liquid Scintillation Counter.

Calcium uptake measurements

Radioisotopic determinations yield a measure of total exchangeable calcium. If there are non-exchangeable calcium pools in lymphocytes, then the total exchangeable calcium will be somewhat smaller than total cell calcium, as determined by atomic absorption or electron microprobe. If non-exchangeable pools do not exist, then the exchangeable cell calcium will be equal to total cell calcium.

(1) Microcentrifugation method. Cells suspended in the appropriate HCO_3^--free minimum essential medium, which contained 20 mM Hepes and 1% penicillin-streptomycin, were incubated at room temperatures, with stirring, with 1.8 mM Ca. Radioactive Ca (2 μCi, 2.8 μM) was added and at the appropriate times (0–45 min), samples were carefully layered on a multi-layer column of liquid in a 0.4 ml polypropylene centrifugation tube and centrifuged in a Beckman Microfuge. The layers consisted of 20 μl of 1.05 g/ml density silicone oil (bottom), 200 μl Ficoll-Hypaque-Hank's wash (density 1.04 g/ml, middle) and 20 μl of 1.03 g/ml density silicone oil (top). The Ficoll-Hypaque-Hank's wash was made by taking Hank's balanced salt solution and adding, dropwise, small amounts of Ficoll-Hypaque (1.077 g/ml) until the measured density (Hydrometer, 1.000–1.220 spec. gr, VWR Scientific Inc.) was 1.04 g/ml. The cell suspension was layered on top of the 1.03 g/ml density oil and the centrifuged samples were worked up as described previously [24]. This technique of separating cells effected a $77.8 \pm 15.8\%$ ($n = 6$) wash-out of the trapped extracellular volume in the pellet, as measured by ^{14}C-labeled poly(ethylene glycol).

(2) Filtration method. Cells suspended in the above-mentioned medium (either 1.8 mM or 0.5 mM calcium) were incubated at 37°C in a shaker-bath. Mitogen and ^{45}Ca (2 μCi; 2.8 μM) were added at the appropriate times according to the specific experimental design. Samples were assayed by addition of 0.1 ml aliquots to 4 ml of ice-cold dilution medium, vortexing and rapid (5 s) filtration on a Brandel Cell Harvester (Model M12). Eight to twelve samples were harvested simultaneously and washed twice with ice-cold wash medium. The entire process takes approx. 40 s. The filters (Brandel No. FR-12) were dried and then counted directly in ACS II scintillation fluid (Amersham).

Hank's balanced salt solution, Ca^{2+} and Mg^{2+} free, was obtained from Grand Island Biological Co. Eagle's minimum essential medium with Earle's salts was made up from its individual components. Normal, unsubstituted Earle's medium contained 116 mM NaCl, 5.2 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, 6 mM glucose and 26 mM $NaHCO_3$. Low Na media contained 158 mM mannitol, 37 mM NaCl, 5.2 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1 mM H_2PO_4, 6 mM glucose, and 26 mM $NaHCO_3$. Dilution medium was a Hank's balanced salt solution which contained 5 mM $CaCl_2$ and 0.8 mM $MgSO_4$. The

wash medium contained 116 mM NaCl, 5.2 mM KCl, 0.8 mM $MgSO_4$, 6 mM glucose, 1 mM NaH_2PO_4, 20 mM Hepes, 5 mM $CaCl_2$ and Phenol red (0.001%). Ficoll was from Pharmacia, and Hypaque (sodium salt, 50% solution) from Winthrop Laboratories. Phytohemagglutinin-M was obtained from Difco Laboratories and succinyl-concanavalin A from Polysciences. [^{3}H]Thymidine and $^{45}CaCl_2$ were obtained from New England Nuclear.

Cell water measurements.

Cell water was measured by assaying tritiated water and the extracellular marker, ^{14}C-labeled poly(ethylene glycol) (M_r 4000) in the supernatant solutions and pellets of samples centrifuged through a single, silicone oil layer (1.03 g/ml). The poly(ethylene glycol) (2.5 μCi) and water (5 μCi/mM) were always added to the cell suspension in such a way as to preserve a ratio of 2:1 between ^{3}H and ^{14}C counts in the pellet fraction. Since the lymphocyte pellet was so small (2.5 μl), this optimized the measurement of cellular and trapped extracellular water.

Results

When ^{45}Ca was added to a suspension of lymphocytes ((20–50) · 10^6 cells/cm^3) in minimum essential medium-20 mM Hepes (HCO_3^--free, 0.5 mM Ca) the equilibration of tracer radioactivity occurred as shown in Fig. 1. The half-time, $t_{1/2}$, for this equilibration is 6.3 ± 1.2 min ($n = 7$) and the calculated apparent cell concentration of calcium is 143.3 ± 17.7 μM ($n = 9$), a large portion of which is presumably bound. This value was calculated from the measured specific activity of Ca in the cell suspension, the cell water content [24,25] and the measured number of cells. Cell water was 0.223 ± 0.018 μl/10^6 cells ($n = 4$), in agreement with previously reported values [24,25], and 0.212 ± 0.029 ($n = 3$) for lymphocytes suspended in normal Na and low Na media, respectively. In both cases, stimulation with succinyl-concanavalin A produced little or no cell shrinkage (< 15%). The same values of $t_{1/2}$ and of the calculated apparent cell [Ca] were obtained from experiments carried out over a range of cell concentrations from 10 · 10^6 to 50 · 10^6 cells/cm^3. In addition to these results, which were obtained with a filtration technique (Brandel Cell Harvester), similar results ($t_{1/2}$ = 5 min; [Ca] = 156 μM) were obtained with microcentrifugation techniques. When lymphocytes were pre-incubated with succinyl-concanavalin A for 0.5 h at 37°C, the total steady-state levels of accumulation, and possibly the rate of uptake, of ^{45}Ca were increased in comparison to unstimulated cells (Fig. 1). The half-time is 3.9 ± 1.0 min ($n = 3$) and total calcium is increased by a factor of 1.51 ± 0.23 ($n = 24$). This is qualitatively consistent with other reports in the literature [18–21], but differs from them quantitatively.

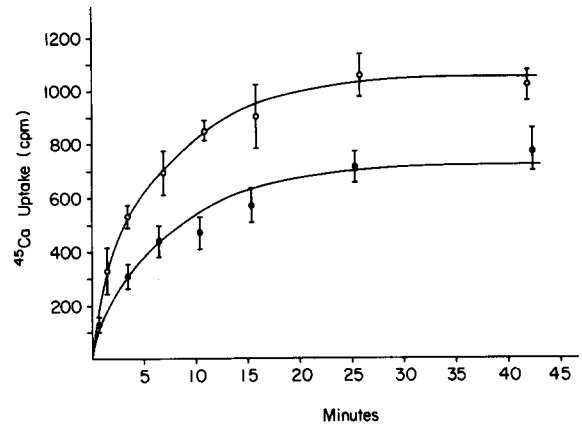

Fig. 1. Time course of ^{45}Ca uptake in succinyl-concanavalin A-treated human lymphocytes. Cells were suspended at 47.7 · 10^6 cells/cm^3 in minimum essential medium (pH 7.3) containing 0.5 mM Ca, 20 mM Hepes and no HCO_3^-. Cells were incubated at 37°C in a shaker-bath. Succinyl-concanavalin A (2.0 mg/ml) was added (○), and an equivalent volume of saline was added to control (●) cells. After 0.5 h, ^{45}Ca was added to the suspensions, and quadruplicate samples taken at the times indicated. Values are given as mean ± S.D.

In order to optimize the signal-to-noise ratio these experiments were carried out at (30–50) · 10^6 cells/cm^3 and at 0.5 mM extracellular calcium. This introduces two considerations: (1) the dependence of cell calcium on extracellular calcium concentration and (2) the appropriate mitogen concentration to use with cells that are suspended at concentrations which are above those used in normal culture. In consideration of both of these points, cell calcium was measured at extracellular concentrations of 1.8 and 0.5 mM in stimulated and unstimulated cells, and viability was evaluated for lymphocytes incubated under conditions iden-

TABLE I

Lymphocytes were suspended at $30 \cdot 10^6$ cells/cm^3 in normal minimum essential medium-20 mM Hepes (142 mM Na, HCO_3^--free) and in low Na minimum essential medium-20 mM Hepes (63 mM Na, 158 mM mannitol. Cells were incubated at 37°C in a shaker bath. Suspensions of stimulated cells contained 3.0 mg/ml succinyl-concanavalin A. Values for viability are given as mean ± S.D. for quadruplicate samples. Cells incubated in normal and low Na media were cultured at the indicated times in the presence of succinyl-concanavalin A (50 μg/ml). Peak ^{3}[H]thymidine incorporation was assayed on Day 3 of cultivation. Values are given as mean ± S.D. for triplicate samples.

	Incubation time (min)	Viability (%)	Incubation time (min)	Peak ^{3}[H]-thymidine incorporation (cpm)
Control	55	96.8 ± 0.8	0	55064 ± 4160
	65	98.7 ± 1.1	10	54323 ± 2413
			20	53649 ± 994
			30	56501 ± 3320
			40	51925 ± 2064
Low Na, control	85	97.6 ± 0.9	0	56361 ± 7609
	95	99.0 ± 1.0	10	48716 ± 3441
			20	51528 ± 1152
			30	56311 ± 4336
			40	51571 ± 1917
Stimulated	74	96.9 ± 1.1		
	82	97.6 ± 2.2		
Low Na, stimulated	115	95.3 ± 2.1		
	122	97.9 ± 0.5		

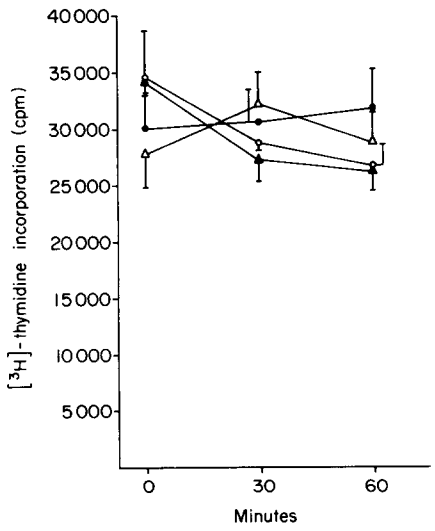

Fig. 2. [^{3}H]Thymidine incorporation in succinyl-concanavalin A-treated human lymphocytes. Cells were suspended at $31 \cdot 10^6$ cells/cm^3 and incubated at 37°C in a shaker-bath. Conditions were identical to those used in the ^{45}Ca uptake studies described in Fig. 1. Succinyl-concanavalin A was present in the incubation media as follows: ●, control cells; ○, 500 μg/ml; ▲, 1250 μg/ml; △, 2000 μg/ml. At the appropriate times, samples were diluted to a final volume of 1 ml with normal culture media (see Methods) such that the final cell concentration was $0.5 \cdot 10^6$ cells/cm^3 and the final succinyl-concanavalin A concentration was 50 μg/ml. [^{3}H]Thymidine incorporation was monitored on Day 3 of the culture period. Values are given as mean ± S.D. for triplicate samples.

tical to those of our calcium uptake experiments. The status of these cells was assessed in two ways: (1) by determining Trypan blue exclusion and (2) by determining the amount of stimulated DNA synthesis in cultures derived from the experimental suspensions. These results are shown in Table I and Fig. 2. There is no decrease in viability over the duration of these experiments when cells are incubated at 37°C in minimum essential medium containing 0.5 mM extracellular calcium, and with succinyl-concanavalin A (2–3 mg/ml). Likewise, there is no impairment of stimulated growth when these cells are placed in culture. Furthermore, the cells were metabolically intact during the experiment, since intracellular [K], as measured by atomic absorption (for methods see Refs. 1,24,25), was 170.9 ± 17.2 mM ($n = 3$) and 177.1 ± 12.4 mM ($n = 3$) for lymphocytes suspended in normal Na-media (145.8 mM) and low Na media (57.8 mM),

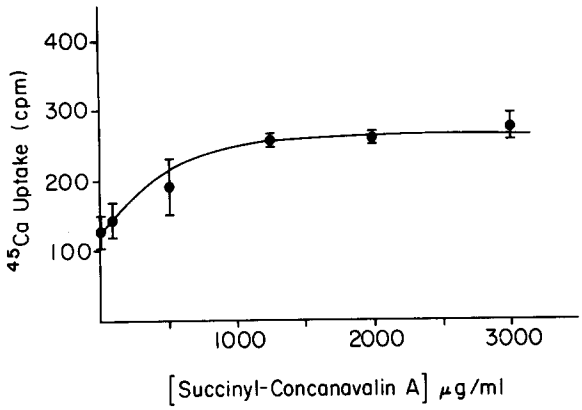

Fig. 3. Dose-response curve of the succinyl-concanavalin A-induced ^{45}Ca uptake in human lymphocytes. Cells were suspended at $30 \cdot 10^6$ cells/cm^3 and incubated at 37°C in a shaker-bath. Succinyl-concanavalin A was added at the appropriate concentrations. After 15 min, ^{45}Ca was added to the suspensions and quadruplicate samples taken after an additional 15, 20 and 25 min. For each mitogen concentration, values are given as a mean ± S.D. for the equilibrated level of calcium.

respectively. The measured cell calcium (filtration techniques) in lymphocytes suspended in minimum essential medium containing 1.8 mM calcium was 234.0 ± 23.5 μM ($n = 7$) and cell calcium is increased by a factor of 1.53 ± 0.18 ($n = 5$) upon stimulation (first 40 min). Although the absolute amount of cell calcium is dependent on the extracellular calcium concentration (at 0.5 and 1.8

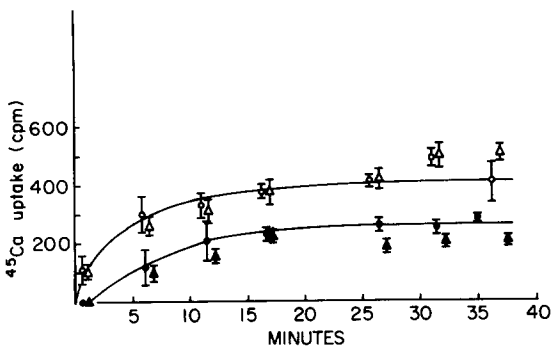

Fig. 4. ^{45}Ca uptake in serum-treated lymphocytes. Cells were suspended at $19.2 \cdot 10^6$ cells/cm^3 as described in Fig. 1. Circles represent serum-free suspensions and triangles represent cells incubated with 10% mixed human serum. Succinyl-concanavalin A (○, △) concentration was 2.0 mg/ml. Closed symbols (●, ▲) represent control cells. Values are given as mean ± S.D. for quadruplicate samples.

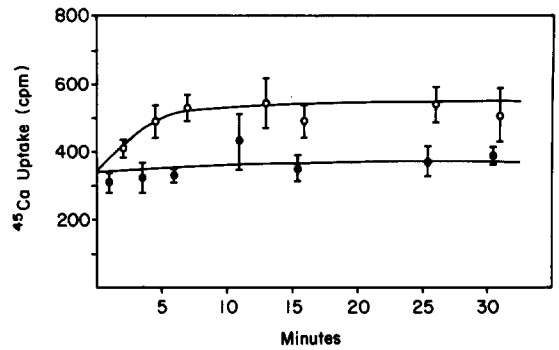

Fig. 5. Mitogen-induced changes in cell calcium. Cells were incubated at $30 \cdot 10^6$ cells/cm^3 at 37°C in minimum essential medium-20 mM Hepes (HCO_3^--free) containing 0.5 mM calcium. ^{45}Ca was added to the suspension and 0.5 h later, succinyl-concanavalin A (3.0 mg/ml, ○) was added to one cell suspension and an equivalent volume of saline was added to control cells (●). Quadruplicate samples were taken at the times indicated. Values are given as mean ± S.D.

mM) the mitogen-induced enhancement of the ^{45}Ca uptake is not.

The effect of mitogen stimulation on calcium uptake was also studied as a function of mitogen concentration. According to the results shown in Fig. 3, for a lymphocyte suspension of $30 \cdot 10^6$ cells/cm^3, that is, approx. 60-times cultured concentrations, the enhanced response of stimulated cells saturates at approx. 0.75 mg/ml of succinyl-concanavalin A. Additional studies were carried out to investigate the effects of serum on the rate and amount of calcium uptake. In these experiments 10% mixed human AB serum was added to the incubation suspensions. As shown in Fig. 4, serum does not alter the amount of cell calcium or the kinetics of ^{45}Ca uptake in either mitogen-stimulated or unstimulated cells.

In order to characterize the kinetics of the observed mitogen-induced alteration in cell calcium, ^{45}Ca was allowed to equilibrate during a 30 min pre-incubation period, then succinyl-concanavalin A was added to the suspension. Samples were assayed over the subsequent 30 min. The initiation of mitogen-induced changes in cell calcium is rapid (Fig. 5), i.e., within the first 1–2 minutes, and the change is complete within 5–10 min.

As noted in the introduction, one of our goals was to determine cell calcium in lymphocytes sus-

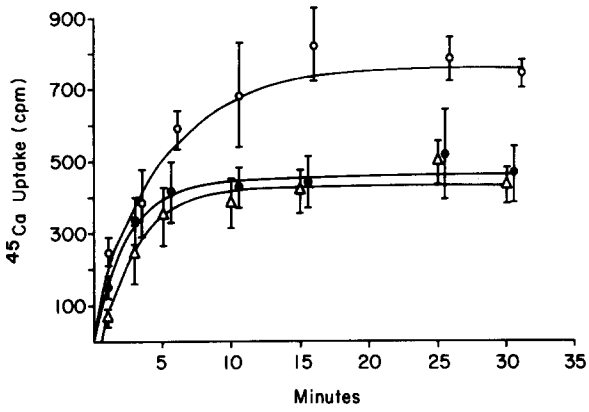

Fig. 6. Time course of ^{45}Ca uptake in lymphocytes suspended in normal minimum essential medium (142 mM Na) and low Na minimum essential medium-20 mM Hepes (63 mM NaCl, 158 mM mannitol). Cells were incubated at 37°C in normal minimum essential medium (△, $40.6 \cdot 10^6$ cells/cm^3) or low Na (●, $26.1 \cdot 10^6$ cells/cm^3) in shaker-bath. Succinyl-concanavalin A (2.0 mg/ml) was added to low Na suspension of lymphocytes (○, $26.1 \cdot 10^6$ cells/cm^3) and an equivalent volume of saline was added to unstimulated normal minimum essential medium cells (△) and unstimulated, low Na minimum essential medium cells (●). After 0.5 h, ^{45}Ca was added and quadruplicate samples were taken at the times indicated. Values are normalized to $40.6 \cdot 10^6$ cells/cm^3 and given as mean ± S.D.

pended in media of low (63 mM) extracellular [Na]. In a previous paper [1] we found a similar Na-dependent mitogen-induced DNA synthesis in cells which had been suspended in each of the following media: (1) hypotonic media containing decreased extracellular Na; (2) media containing decreased Na and equiosmolar replacement with mannitol; and (3) media containing decreased Na and equimolar replacement with choline. Therefore, in the currently reported study, we used a low Na medium in which NaCl had been replaced with equiosmolar mannitol. In this case, as shown in Fig. 6, cell calcium was similar to that of lymphocytes incubated in normal, high Na media. Furthermore, the increase in cell calcium that occurs upon mitogen stimulation of lymphocytes in normal, high Na media occurs to a similar extent in lymphocytes suspended in low Na media.

Discussion

Calcium ions are involved in the control of cell proliferation [14,15]. As such, calcium has been a likely candidate as a trigger of mitogenic activation in lymphocytes. First, because mitogenic lectins produce a rapid, initial calcium influx [18–21]; second, because calcium is required for DNA synthesis some 18–72 h after the mitogenic stimulus [22,23]; and third, because the mitogenic activity of A23187 has been shown to be calcium-dependent. However, there is a convincing body of data which points to alternative theories of the role of calcium. Removing lectins [26–29] or decreasing extracellular calcium after 6 to 36 h after the original stimulation (and initial rise in intracellular calcium) [30–32] inhibited DNA synthesis. Furthermore, there is no correlation between calcium influx induced by different concentrations of A23187, concanavalin A and phytohemagglutinin and the amount of DNA synthesis. There are reports of the uncoupled behavior of the mitogen-induced Ca-influx and the mitogen-induced proliferation: (1) A23187, in the presence of certain concentrations of Mg^{2+} and Mn^{2+}, did not cause calcium influx, yet still stimulated proliferation [33,34]; and (2) a reputed calcium-blocking drug, D600, was shown to inhibit proliferation induced by either phytohemagglutinin or A23187, but had no effect on either phytohemagglutinin- or A23187-induced ^{45}Ca uptake [35].

The reports with respect to ^{45}Ca uptake itself in mitogen-stimulated cells are conflicting. Some workers report an increase in ^{45}Ca uptake during the initial exposure to mitogens [18–21,35] while others [36] did not observe such a change. Their results are not reported as cellular concentrations, but rather as moles/10^6 cell or as relative changes in calcium. These values vary considerably, apparently as a consequence of the method used. Whitney and Sutherland [37], for example, using a technique that involved washing (twice) and resuspending a centrifuged pellet, obtained values of cell calcium that vary from 4 to 25 nmol/10^6 cells (i.e., approx. 14 to 88 mM intracellular calcium!). These results are not consistent with the results of other workers (see discussion below). Jensen and Rasmussen [41] also assayed ^{45}Ca in unstimulated lymphocytes by a similar centrifugation technique. However, they report values of cell calcium of 0.880–0.580 nmol/10^6 cells (i.e., approx. 3 mM intracellular calcium). These authors do not comment on the large discrepancy (5- to 30-fold) between their results and those of Whitney and

Sutherland. The procedures used by both sets of authors for centrifugation, washing and resuspension of cells are not fast enough to prevent a transplasma membrane readjustment of cell calcium. (We observe half-times on the order of 3–6 min.) Furthermore, such procedures are questionable since metabolic alterations will certainly occur in the pelleted cells and subsequent resuspensions. Our centrifugation procedures obviate this effect by using a multi-layer wash system in which transit times through the wash solutions are on the order of seconds and resuspension of the cellular pellet is not entailed. As discussed below, we have been able to obtain similar values of cell calcium by two different methods, both rapid centrifugation techniques and filtration techniques, each rapid enough to accurately measure processes with half-times of 3–6 min.

Confusion exists regarding not only the amount of cell calcium but also the rate of ^{45}Ca uptake and the mitogen-induced enhancement of cell calcium. Allwood et al. [18] used a filtration technique, and found uptake of ^{45}Ca within the first 2 min (their first sampling time) that remained unchanged over the next 2 h. Either the half-time for ^{45}Ca equilibration is on the order of seconds, or their measured ^{45}Ca does not represent actual cellular accumulation. They did not report the actual amount of Ca associated with the cellular fraction. These authors find that phytohemagglutinin increases ^{45}Ca uptake by a factor of 1.7 over a 1-h period. Jensen and Rasmussen [41] reported that A23187 increased cell calcium by 3–4.5-fold within the first hour of incubation and that after 8–12 hours cell calcium decreased to a level that was 1.5–2.0-fold higher than that of control cells. Whitney and Sutherland [37] report up to a 3-fold increase in cell calcium when cells are incubated with phytohemagglutinin; they find a half-time of 15.5 min for ^{45}Ca uptake by stimulated cells. We find that the half-times for ^{45}Ca equilibration are 6.3 min and 3.9 min for unstimulated and succinyl-concanavalin-A-treated lymphocytes, respectively. These disagreements regarding the values of cell calcium in stimulated lymphocytes may, in part, be due to the use of different mitogens, and disagreements in the kinetics may be due to variations in the methods of measurement.

Our studies sought quantitative clarification of these issues and have been directed toward (1) determining the total cell concentration of calcium, (2) characterizing the mitogen-induced alteration in cell calcium and (3) assessing the effect of low extracellular [Na] on cell calcium levels of both stimulated and unstimulated lymphocytes. The latter goal was aimed at testing the hypothesis that Na-dependent mitogenesis is mediated by alterations in cytosolic calcium concentration.

Our values for total exchangeable calcium, 0.143–0.234 mM, are consistent with values of total cell calcium reported for other biological systems: in frog toe muscle cytoplasm the total cell-associated calcium is 1 mmol/kg dry wt. (0.3 mM); it is 4 mmol/kg dry wt. (1 mM) in cytoplasm of toadfish swimbladder as determined by electron microprobe analysis (38) and is in the range of 0.3–0.4 mM (of which only 0.09–0.2 μM is the free ion) in the cytosol of nonactivated axons [39,40].

Our studies of unstimulated cells indicate that total exchangeable calcium is not affected by a decrease in extracellular [Na] from 142 to 63 mM. Furthermore, the same increase in cell calcium is observed when lymphocytes are stimulated in low Na media or in high Na media. Yet low extracellular [Na] inhibits subsequent stimulated DNA synthesis in mitogen-treated lymphocytes [1]. Thus, we conclude that the Na-dependent signal for stimulated proliferation is not dependent on the mitogen-induced calcium change and that calcium does not participate in the initial signalling events of proliferation, but may play a key role in later events of mitosis.

Acknowledgments

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