The Role of Calcium in Lymphocyte Proliferation
(An Interpretive Review)

By Andrew H. Lichtman, George B. Segel, and Marshall A. Lichtman

A small quantity of extracellular calcium is required for the stimulation of lymphocytes by mitogens such as plant lectins. Lectin binding to the lymphocyte surface and early postbinding events that eventually lead to DNA synthesis are calcium dependent. Mitogenic lectins such as PHA and Con-A rapidly increase the size of an exchangeable pool of cell calcium and cause a smaller rise in intracellular ionized calcium. The increase in ionized calcium is so small (100–200 nM), however, that no increase in total cell calcium is measurable. When lymphocytes are stimulated by a lectin, the rate of calcium entry into the cell increases, but the plasma membrane calcium extrusion pump can prevent the total cell calcium from increasing measurably. The calcium ionophore A23187 is a lymphocyte mitogen and causes an increase in the exchangeable, ionized, and total cell calcium. The former two effects may be causal in mitogenesis; the latter effect is cytotoxic. With A23187 treatment, the rate of calcium influx exceeds the maximum rate of the plasma membrane extrusion pump and cell calcium increases in proportion to the concentration of A23187. The mitochondria, by virtue of their high membrane potential, provide a sink for the buffering of cytoplasmic calcium after A23187 treatment. Thus, the plasma membrane or mitochondria regulate the distribution of lymphocyte calcium when the cell is stimulated by mitogenic lectins or ionophores. The evidence strongly suggests that an alteration in calcium pools or an increase in cytoplasmic ionized calcium plays a role in the initiation of the biochemical reactions that lead to mitogen-induced lymphocyte proliferation in vitro and, perhaps, to the immune response.

Knowledge of the mechanisms of mitotic stimulation of lymphocytes is basic to an understanding of the normal immune response and immunopathology. Experimental evidence indicates that calcium serves as a messenger in the initiation of cell responses to stimuli. Calcium may act directly on cellular enzymes and in conjunction with other cellular metabolites, such as cyclic nucleotides, to regulate cell functions. Alteration in the ionized calcium concentration in the cytosol has been implicated in the initiation of secretion, contraction, and cell proliferation. In this review, we will discuss the evidence that calcium is involved in lymphocyte mitogenesis. Other physiologic changes associated with lymphocyte transformation and the role of cyclic nucleotides in lymphocyte mitosis have been reviewed extensively elsewhere.

Lymphocyte mitogenesis usually is gauged by measuring $^{3}$H-thymidine incorporation into DNA 48–72 hr after exposure of cells to mitogens. While antigens recruit only small numbers of cells into the cell cycle, polyclonal mitogens stimulate most of the cells in a lymphocyte population. Polyclonal mitogenic stimulation of lymphocytes is similar to antigenic stimulation in that lectins interact with specific receptors (sugar) on the surface of lymphocytes, show B- and T-lymphocyte specificity, and induce a cascade of biochemical events leading to mitosis. The use of polyclonal mitogens permits measurement of many metabolic events in a lymphocyte population that would not be possible using antigens because the latter stimulate a small fraction of cells. Such techniques have allowed the study of calcium transport and content during lymphocyte mitogenesis (Fig. 1).

Extracellular Calcium Requirements for Lectin-Induced Lymphocyte Mitogenesis

Calcium and Lectin Binding

The calcium requirement for lectin-induced mitogenesis may reflect a calcium requirement for lectin...
Calcium Requirement After Lectin Binding

The studies of the calcium requirements for mitogenesis often employ the use of divalent cation chelators, such as disodium ethylene diamine tetraacetic acid (EDTA) or EGTA, to reduce the ionized calcium concentration in the external medium. The use of chelators is complicated by the fact that they bind at least 1-2 divalent cations other than calcium. For example, the addition of zinc or copper to the culture medium may restore mitogenesis by displacement of calcium from EGTA rather than by direct effect of the ions added. Many studies are difficult to evaluate because the effects of the addition of divalent cations other than calcium have not been examined, and measurements of the ionized calcium in the culture medium have not been made.

In spite of these problems, studies have indicated that extracellular calcium is required for lectin-stimulated lymphocytes to proceed through the cell cycle to blastogenesis and mitogenesis. Two studies found an early requirement for calcium during the first 2 hr of lectin stimulation of 3H-thymidine into lymphocyte DNA. For example, calmodulin–calcium can accelerate the calcium transport ATPase and active calcium extrusion from the cell. Calmodulin–calcium influences also the rates of adenylyl and guanylylcyclases and phosphodiesterases, determining the concentration of two critical regulatory nucleotides, cyclic AMP and GMP. Phosphokinases are stimulated by the calmodulin–calcium complex, and these enzymes phosphorylate and activate critical phosphoproteins, such as the proliferation-related phosphoprotein, which presumably modulates DNA replication.
thymidine showed that calcium in the micromolar range is required at least during the first 20 hr of mitogen-stimulated proliferation of lymphocytes.

When considering extracellular calcium requirements, it is important to note that a large portion of culture medium calcium is not ionized even in serum-free systems. An ionized calcium concentration of 30 \( \mu M \) was necessary for Con-A stimulation of hexose transport in thymocytes, and 50 \( \mu M \) was necessary for stimulation of RNA synthesis in blood lymphocytes. Studies using an electrode to measure the ionized calcium in the extracellular medium indicate that DNA synthesis can be detected initially at 20 \( \mu M \) ionized calcium, and maximum DNA synthesis is observed at 200 \( \mu M \) ionized calcium. Since the ionized calcium in the cytoplasm is in the nanomolar range (see next section), these data suggest that a large gradient of ionized calcium from outside to inside the cell is required to initiate and sustain lymphocyte mitosis.

**EFFECT OF LECTINS ON LYMPHOCYTE CALCIUM UPTAKE AND CONTENT**

Evidence pointing to an increment in cytoplasmic calcium as a signal in lymphocyte proliferation includes the enhancement of radioactive calcium (\(^{45}\)Ca) labeling of mitogen-stimulated lymphocytes. Exposure of lymphocytes to PHA or Con-A results in a significant increase in lymphocyte-associated \(^{45}\)Ca radioactivity. Initial reports indicated that mitogenic lectins caused a prolonged enhancement of \(^{45}\)Ca uptake. Other reports suggested a rapid, but unsustained, increase in radiolabel uptake. An increase in cell-associated \(^{45}\)Ca could represent a net increase in total cell calcium or an increase in the exchangeability of some pool of calcium, without a net gain by the cell. The exchangeable pool includes the ionized calcium in the cytoplasm and calcium bound to various intracellular ligands (Fig. 1). Studies using sensitive techniques to measure both radiolabeled and total cell calcium indicate that the \(^{45}\)Ca uptake is an exchange process in which cell calcium does not increase measurably (Table 1). Moreover, studies of the dose–response curves of \(^{3}H\)-thymidine incorporation and \(^{45}\)Ca exchange at the lowest stimulatory doses of PHA show that the two processes are closely associated. Thus, DNA synthesis cannot be stimulated by PHA without increasing \(^{45}\)Ca exchange. These findings indicate that PHA and presumably other mitogens increase exchange of medium calcium with some cellular calcium pool. The influx of calcium could result from an increase in the plasma membrane permeability to cations that rapidly follows lectin treatment. A recent report suggests that calcium influx after lectin treatment may be regulated by early membrane phospholipid methylation. However, calcium uptake preceded methylation of phospholipids in these studies. The persistence of methylation in calcium-free medium was difficult to interpret since chelators were not added to scavenge trace amounts of medium calcium, and calcium release from internal pools was not measured. The early increase in cellular \(^{45}\)Ca also could represent binding to the external surface of the cell as well as entry into internal pools, a distinction that is difficult to make experimentally. Lymphocyte cell calcium includes an ectocellular pool that can be displaced when millimolar lanthanum is present in the external medium. Use of this technique could help to exclude surface binding as the explanation for the association of \(^{45}\)Ca with the lymphocyte.

Mitogen-stimulated \(^{45}\)Ca uptake into human lymphocytes is influenced by the lipoprotein composition of the external medium. Low density lipoproteins inhibit both \(^{45}\)Ca accumulation and mitogenesis in a concentration-dependent fashion. Inferences could not be drawn, however, regarding the role of lipoproteins in the regulation of calcium influx into stimulated lymphocytes in vivo. Nevertheless, the data are compatible with an inciting role for calcium uptake in the proliferative process.

There is a large body of work concerning the role of calcium and cyclic nucleotides in mitotic stimulation of thymic lymphoblasts. Experimental manipulations that would tend to change steady-state transmembrane distribution of calcium, such as raising extracellular calcium or adding parathormone, serve to stimulate mitotic activity in these thymic lymphoblasts. Although this work supports the calcium-regulated proliferation hypothesis, the cells that have been studied are a “rapidly responding” subpopulation in a presynthetic phase of the cell cycle and are different from the resting premitotic blood lymphocyte.

An innovative technique to measure ionized calcium in the cell employs an acetomethoxyester that fluoresces in proportion to the ionized calcium concentration. A study using this technique has shown that there is a twofold rise in the ionized calcium from 100 to 200 nM

**Table 1. Total and Exchangeable Calcium in PHA-Treated Lymphocytes**

<table>
<thead>
<tr>
<th>Time (nmole/Liter)</th>
<th>Total Cell Calcium</th>
<th>(^{45})Ca-Labeling</th>
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<tbody>
<tr>
<td>1 min</td>
<td>2.3 ± 0.40</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>3 min</td>
<td>2.3 ± 0.36</td>
<td>0.47 ± 0.11</td>
</tr>
<tr>
<td>30 min</td>
<td>2.3 ± 0.39</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td>3.6</td>
<td>2.2 ± 0.36</td>
<td>0.57 ± 0.10</td>
</tr>
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Data are presented for human blood lymphocytes treated with an optimally mitogenic concentration of PHA.
in the cytoplasm of lectin-stimulated lymphocytes\textsuperscript{33} (Fig. 2). This is a much smaller change than the lectin-induced increase in $^{45}$Ca labeling ($\sim 150 \mu M$) and is consistent with the interpretation that most of the increased $^{45}$Ca radiolabeling represents calcium–calcium exchange.\textsuperscript{26}

The rate of pyruvate oxidation by isolated lymphocyte mitochondria is accelerated by an increase in the extramitochondrial free calcium from 1 to 50 nM,\textsuperscript{34} indicating that key physiologic processes can respond to alterations in ionized calcium in the nanomolar range.

**CALCIUM IONOPHORE A23187 AS A LYMPHOCYTE MITOGEN**

Further evidence supporting a role for calcium in lymphocyte mitosis derives from the response of lymphocytes to the calcium ionophore A23187. This carboxylic acid antibiotic derived from *Streptomyces chartreusensis* increases the diveralent cation permeability of biologic membranes. Two molecules of the ionophore bind with one calcium ion, and the complex moves freely across the cell and subcellular membranes.\textsuperscript{35,36} The ionophore is not selective for calcium; it binds magnesium with equal affinity and other divalent cations such as manganese with higher affinity. Despite this lack of specificity, studies have shown that the biologic effects of the ionophore depend on the presence of calcium.\textsuperscript{1}

A23187 eliminates the permeability barrier to calcium that exists at the plasma membrane (Fig. 1). The rate of calcium entry into the cell can exceed the outward pumping of calcium and can result in a rise in intracellular calcium content. A23187-induced activation of cellular functions includes mainly secretory processes such as mast cell histamine release,\textsuperscript{37,38} pancreatic exocrine secretion,\textsuperscript{39} pancreatic insulin release,\textsuperscript{40} fly salivary gland secretion,\textsuperscript{41} and platelet secretion and aggregation.\textsuperscript{42,43} These effects of A23187 are consistent with the hypothesis that a signal for the initiation of intracellular physiologic processes is mediated by an influx of calcium.

Presumably, the ionophore also can gain access to intracellular membranes, such as the inner mitochondrial membrane or endoplasmic reticulum, which sequester calcium, and thus can lead to calcium reentry into the cytoplasmic pool. For example, A23187 has a mitogenic effect on sea urchin eggs, amphibian eggs, and hamster oocytes even in the absence of external calcium, presumably by causing release of calcium from organelles into cytoplasm.\textsuperscript{44,45} A23187 causes a biphasic alteration of $^{45}$Ca labeling in bovine sperm, which results from distinct temporal associations of the ionophore, first with the plasma membrane, then with intracellular membranes.\textsuperscript{46} A similar interpretation was proposed for the biphasic nature of A23187 effects on human lymphocyte $^{45}$Ca labeling.\textsuperscript{47}

A23187 and $^3$H-Thymidine Incorporation Into DNA

A23187 stimulates $^3$H-thymidine incorporation and blast transformation in pig mesenteric lymph node lymphocytes,\textsuperscript{46,49} rabbit spleen and lymph node lymphocytes,\textsuperscript{50} and human blood lymphocytes.\textsuperscript{26,51–54} These data have been taken as evidence for the mitogenic role of calcium influx into lymphocytes and are consistent with the association of lectin-induced calcium influx and lectin-induced mitogenesis. A comparison of PHA and A23187 stimulation of human lymphocyte DNA synthesis in the same culture conditions is shown in Fig. 3. The ionophore is not as effective as mitogenic lectins in stimulating DNA synthesis.\textsuperscript{48} This may be a result of the toxic effects described below.

The mitogenic effects of A23187 depend on the concentration of calcium in the suspending medium\textsuperscript{48,49,52,54} and on the serum concentration of the culture medium, presumably because of partitioning of the ionophore into the lipid fraction of the serum.\textsuperscript{48,49} Concentrations of A23187 above a narrow mitogenic peak concentration are severely cytotoxic. The ionophore causes proportional increases in cell $^{40}$Ca and lysis of rat thymocytes\textsuperscript{29} and of human lymphocytes.\textsuperscript{46} Cytotoxicity is a function of the massive changes in cell calcium content and distribution that occur at higher concentrations of A23187. The intracellular calcium buffering mechanisms, such as respiration-dependent mitochondrial calcium uptake and sequestration, can be overloaded after exposure to A23187.\textsuperscript{26} The iono-
CALCIUM AND LYMPHOCYTE PROLIFERATION

Fig. 3. Comparison of PHA and A23187 stimulation of human lymphocyte DNA synthesis. DNA synthesis is assayed by measuring the incorporation of \(^{3}H\)-thymidine into the acid-precipitable fraction of human lymphocytes. Lymphocytes are cultured at a density of \(10^6\) cells/ml in tissue culture medium 199 containing 10% (v/v) fetal bovine serum. Thymidine incorporation is measured during the last 24 hr of a 72-hr total incubation. The data represent the mean + SE of measurements in 6 different lymphocyte populations. The peak DNA synthesis in A23187-treated lymphocytes is 30% of that in PHA-treated cells, but cell survival and viability are impaired after 72 hr of culture in A23187. PHA and A23187 are mitogens that may result in similar DNA synthesis if the toxic effects of excessive calcium influx after A23187 treatment are considered and the \(^{3}H\)-thymidine incorporation is corrected for cell loss in the culture.

The Effect of A23187 on Uptake, Exodus, and Content of Calcium

Optimal mitogenic concentrations of A23187 increase the total and radiolabeled cell calcium 4–5-fold in human lymphocytes. Intracellular buffering of this added calcium is necessary for cell survival. Mitochondrial accumulation of calcium is an important mechanism for maintaining relatively low and nontoxic levels of cytoplasmic calcium. Calcium uptake across the inner mitochondrial membrane down an electrical gradient is maintained by oxygen-dependent electron transport (Fig. 1), i.e., respiration. Mitochondria isolated from human blood lymphocytes exhibit calcium uptake that is dependent on respiratory substrates such as acetate or pyruvate. The increase in cell calcium induced by mitogenic concentrations of A23187 is blocked by inhibitors of respiration (e.g., cyanide, azide, dinitrophenol) (Fig. 5); thus, the bulk of the calcium entering lymphocytes is sequestered in the mitochondria. Mitochondrial sequestration of calcium also has been shown in studies of rabbit and rat thymocytes. In these studies, the ATP-dependent cellular uptake of calcium was blocked by mitochondrial inhibitors such as rotenone, oligomycin, and atractyloside. In rat thymocytes prelabeled with \(^{45}\)Ca, mitochondrial inhibitors increase calcium exodus. The exodus of calcium from mitochondria in excitable tissues is mediated by an exchange process during which sodium enters the mitochondria (Fig. 1). The role of this exchange process in lymphocytes has not been established, and alternative processes such as calcium-proton exchange may be present.

When lymphocytes are exposed to the calcium ionophore A23187, the magnitude of calcium influx exceeds the capacity of the plasma membrane calcium extrusion pump and total lymphocyte calcium is increased. However, at mitogenic concentrations of A23187, lymphocyte calcium initially remains at a normal level, at a time when \(^{45}\)Ca uptake is increased. This increase is very similar in magnitude to that seen after lectin stimulation. Within minutes, however, total cell calcium increases markedly. These data lend support to the concept that increases in exchangeable and ionized calcium are very early events in lymphocyte mitogenesis. An apparent inconsistency with this inference is that A23187 at a concentration of 10 nM has been shown to cause a marked increase in lymphocyte ionized calcium (~1000 nM) in a serum-free system within minutes of exposure. Yet it requires about 200 nM A23187 to produce near maximal DNA synthesis in a serum-containing system. These differences may reflect differences in partitioning of A23187 between serum and the lymphocyte plasma membrane and differences in the external ionized concentration of calcium in these different circumstances. Also, the higher concentration of A23187 produces a sustained rather than transient increase in cell calcium.
Fig. 4. Transmission electron micrographs of human blood lymphocytes treated with the calcium ionophore A23187. (A and B) Untreated cells. In untreated cells mitochondrial ultrastructure is intact. (C and D) Cells exposed to 0.25 μM A23187 for 60 min. At a mitogenic concentration of A23187, electron-dense material obscures the mitochondrial matrix space, and the cristae are distorted. (E and F) Cells exposed to 1.0 μM A23187 for 60 min. At a toxic concentration of A23187, mitochondria are distorted and swollen with obliteration of their normal ultrastructure.
Table 2. Calcium Transport and Calcium ATPase in Isolated Human Lymphocyte Plasma Membrane Vesicles

<table>
<thead>
<tr>
<th></th>
<th>ATP-Dependent Ca Transport</th>
<th>Ca-ATPase</th>
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<tbody>
<tr>
<td>$K_\text{Ca}$ (μM)</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>$K_\text{ATP}$ (μM)</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>Mitochondrial inhibitors</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Lectins</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Enhances</td>
<td>Enhances</td>
</tr>
</tbody>
</table>

Fig. 5. The effect of mitochondrial inhibition 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) in 4 lymphocyte populations exposed to 0, 0.25, 1.0 μmole/liter A23187 in the presence or absence of 1 mmole/liter NaN$_5$ is shown. The cells were first incubated in the presence of cyanide for 15 min, then in the presence of cyanide plus A23187 for an additional 15 min before measurement of total and exchangeable calcium. The distribution ratio of internal to external calcium far exceeds one (external calcium about 1.8 mM) as a result of an enormous accumulation of calcium by a relatively small number of mitochondria. At a mitogenic concentration (0.25 μmole/liter) of A23187, cyanide markedly reduces the mitochondrial uptake of calcium. As a result, total calcium content is the same as in untreated lymphocytes, but the exchangeable fraction (%Ca) is increased. At a toxic concentration (1.0 μmole/liter) of A23187, total calcium is reduced by cyanide treatment but remains markedly elevated, indicating that the capacity of the plasma membrane calcium extrusion system (Ca-ATPase) has been exceeded.

ROLE OF PLASMA MEMBRANE IN CELLULAR CALCIUM REGULATION

All mammalian cells have an active calcium extrusion mechanism that maintains a large chemical gradient across the plasma membrane. An ATP-dependent plasma membrane calcium pump has been described in erythrocytes. The presence of a lymphocyte plasma membrane calcium extrusion pump has been shown indirectly in rat thymocytes. Calcium that was accumulated during the process of cell preparation was extruded when the cells were oxygenated and incubated in media with substrates for aerobic metabolism.

Highly purified human lymphocyte plasma membrane vesicles have been shown to have both a Ca-activated ATPase activity and an ATP-dependent Ca-transport activity. The ATPase and the pump have similar affinities for calcium and ATP (Table 2) and cannot function if other nucleotides are substituted for ATP. These parameters indicate the presence of a lymphocyte plasma membrane calcium pump that is qualitatively similar to the erythrocyte pump.

Calmodulin is a ubiquitous cytoplasmic calcium-binding protein with a molecular weight of 17,000 daltons. It has been termed a calcium regulator protein for processes including secretion, contraction, transport, and is probably present in all mammalian cells. It complexes to calcium and enhances most calcium-dependent cellular enzyme activities (Fig. 1). It has been purified from the cytoplasm of human lymphocytes and shown to stimulate the Ca-ATPase and Ca-transport of lymphocyte plasma membrane vesicles. Recent studies have indicated that the calmodulin content of cycling Chinese hamster ovary cells varies with the phases of the cell cycle. Further, naphthalenesulfonamides, which are antagonists of calmodulin also inhibit cell proliferation. These data suggest that variations in calmodulin, as well as alterations in ionized calcium, may play a role in modulating the sequence of biochemical steps that lead to cell proliferation.

Ionized calcium and the calcium-calmodulin complex play key roles in the initiation and progression of lymphocyte proliferation (Fig. 1). When lymphocytes are treated with mitogens, the cytoplasmic ionized calcium concentration increases and interacts with calmodulin to initiate essential enzymatic processes. The incremental change in cytoplasmic calcium is due to a change in the plasma membrane permeability to calcium and the large gradient of calcium concentration from the outside to the inside of the cell. The cytoplasmic ionized calcium is restored toward normal by sequestration of calcium in organelles such as mitochondria and by rapid outward pumping via the calcium transport ATPase. This membrane ATPase is
accelerated by increases in cytoplasmic ionized calcium concentration and is not influenced directly by mitogens such as PHA or Con-A, which bind to the external membrane surface (Table 2).

DISTINCTIONS IN THE ROLE OF CALCIUM IN T- AND B-LYMPHOCYTE PROLIFERATION AND FUNCTION

Most studies in humans have used blood lymphocytes as a source of experimental material. These lymphocytes are primarily T cells, and T-cell mitogens such as PHA and Con-A have been used to initiate mitogenesis. Mouse B lymphocytes also require external calcium for antigen or mitogen-induced antibody responses, and an increase in 45Ca uptake has been observed when mouse B lymphocytes are exposed to B-cell mitogens. Furthermore, an increase in the ionized calcium of mouse B cells was observed after treatment with anti-immunoglobulin. Studies of mouse spleen cells have shown that there is an early requirement for calcium in mouse T, but not in B, lymphocytes. A distinction in the role of calcium for T- and B-lymphocyte proliferation was indicated also by studies that showed that A23187 stimulates DNA synthesis in mouse T, but not in B, splenocytes. However, the calcium requirements for normal human B-lymphocyte proliferation and function have not been defined.

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