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CALCIUM IS REQUIRED FOR THE MITOGENIC ACTIVATION OF LYMPHOCYTES BY PERIODIC ACID OXIDATION

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This investigation of Ca^{2+} requirements for the mitogenic activation of lymphocytes by periodic acid has shown that oxidation by periodate causes an immediate and transient increase of Ca^{2+} influx and efflux in oxidized cells. Oxidized lymphocytes maintained in the medium containing 0.2 mM Ca^{2+} failed to proliferate or to produce IL-2, whereas a 1.4 mM Ca^{2+} concentration was shown to be sufficient to sustain cellular proliferation and IL-2 secretion. These results indicate that mitogenic activation of lymphocytes by periodic acid oxidation is Ca^{2+}- dependent.

Periodic acid is an inorganic mitogen (1) and like lectins induces blastogenesis of lymphocytes (1-3) as well as the production of lymphokines by the oxidized cells (4,5). Transformation of lymphocytes by lectins results in increased transmembrane Ca^{2+} fluxes (6), whereas the sustained proliferation of transformed cells requires extracellular Ca^{2+} (6,7). During blastogenesis Ca^{2+} operates as a second messenger (6) and its role in the proliferation of lymphocytes is well known (7,8).

In contrast to the clearly recognized Ca^{2+} function in activation of lymphocytes by lectins (8,9), the role of Ca^{2+} in periodate-induced blastogenesis has received only scant attention, and owing to the contradictory data remains unresolved. For instance, it was reported by Parker (10) that the oxidation of human lymphocytes by periodate results in an early increase of Ca^{2+} uptake, whereas Larner et al (11) have claimed that Ca^{2+} uptake by rat splenocytes is inhibited by

Abbreviations: interleukin-2, IL-2; phosphate buffered saline, PBS; heat-inactivated fetal calf serum, HI-FCS; inositol 1,4,5-trisphosphate, Ins(1,4,5)P_3; protein kinase C, PKC; physiological saline solution, PSS; Concanavalin A, Con A.

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periodate. In view of the pivotal role of Ca^{2+} in cellular responses to a variety of extracellular oxidants (12) and controversial status of Ca^{2+} in periodate-induced blastogenesis, Ca^{2+} requirements and transmembrane Ca^{2+} fluxes were quantified in human and rat lymphocytes oxidized by periodate. We report that activation of lymphocytes by periodic acid results in a transient increase of transmembrane Ca^{2+} fluxes, and that extracellular Ca^{2+} is required for the proliferation of oxidized cells and their secretion of interleukin - 2 (IL-2).

Materials and Methods

Preparation of lymphocyte suspensions: Spleens from Sprague-Dawley rats (Charles River, Mass.) were teased with a rubber policeman, the dispersed pulp was washed with PBS, and filtered though 84 μm nylon mesh (Small Parts, Inc., Miami, FL). Lymphocytes were isolated from peripheral blood donated by healthy volunteers and from rat spleen cell suspensions by Histopaque (Sigma Chemical Co., St. Louis, MO)density centrifugation. The remaining erythocytes were lysed for 10 min in 100 mM NH_4Cl (Fisher Scientific, Pittsburgh, PA) at 4°C. Dispersed cells were enumerated by a ZBI Coulter Counter and their viability determined by trypan blue exclusion. The media used for culture were CMRL 1415 and RPMI 1640 each supplemented with 10 % v/v HI-FCS (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mg/ml $NaHCO_3$.

H_5IO_6 oxidation of lymphocytes: For optimal blastogenic response, lymphocytes (5 x 10^6 cells) suspended in 50 μl of Ca^{2+} and Mg^{2+}-free PBS, were oxidized for 20 min by 1 mM H_5IO_6, at 0°C (2,3). The reaction was terminated by the addition of 1.0 ml of 37°C CMRL-1415 medium. The time at which the oxidation reaction was terminated was designated to be the 0 h time. Oxidized cells were cultured for 72 h, and for the last 12 h [^{3}H]TdR incorporation was measured by pulsing the cultures with 1 μCi of [^{3}H]TdR in 20μl Ca^{2+} and Mg^{2+}-free PBS. Radioactivity was measured following cell harvesting and the stimulation index determined as follows: Stimulation Index (S.I.) = (cpm [^{3}H]TdR incorporated by oxidized lymphocytes) / (cpm [^{3}H]TdR incorporated by control lymphocytes cultured in 1.4 mM Ca^{2+}-containing medium.)

Assay of IL-2 activity: The IL-2-dependent CT6 lymphocyte cell line was used to assay for the presence of IL-2 activity (4,5). Briefly, CT6 cells (10^6/ml) were maintained for 24 h in RPMI-1640 medium, prior to the addition of medium conditioned by the oxidized cells. The CT6 cells were then washed in PBS and resuspended at 1 x 10^5cells/ml in RPMI-1640 medium and 100 μl of this suspension were pipetted into each well of the microculture plate, followed immediately by serial 100 μl dilutions of the supernatants. After 36 h of incubation, the CT6 cells were pulsed with 1 μCi [^{3}H]TdR and cultured for an additional 16 h. They were then harvested and their radioactivity was determined. The supernatants from rat spleen cells stimulated by 2.5μg/ml Con A served as the IL-2 standard. This gave approximately the half maximal stimulation of 10^4 CT6 cells at a 1/200 dilution. Purified human IL-2 served as an additional IL-2 reference standard (Genzyme Corp., Boston, MA).

DNA flow cytometric analysis: Cells washed in PBS were resuspended in Dulbecco's Ca^{2+} and Mg^{2+}-containing PBS, their nuclei were isolated with 0.6 % v/v Nonidet P-40, stained with 4',6-diamidino-2-phenylindole (13), and then analyzed with a modified ICP-22 flow cytometer (Ortho Instrumentation, Westwood, Mass.). In these determinations, trout red blood cells (5.2 pg DNA/nucleus) served as the DNA standard.

Effects of periodate oxidation on cellular calcium fluxes: Calcium fluxes in intact and in periodate-oxidized cells were determined in accordance with previously described methodology (14) as follows:

Ca^{2+} Influx: Calcium influx into lymphocytes, preincubated for 2 min in physiological salt solution at pH 7.4 (PSS; 140 mM NaCl, 5mM KCl, 1.5 mM $CaCl_2$, 0.75 mM $MgCl_2$, 10 mM D-glucose, 5mM Na Hepes), was measured after oxidation by 1 mM H_5IO_6 (4,5). Oxidized lymphocytes (5 x 10^6/ml) were then loaded for 2 min by 20 μ Ci $^{45}Ca^{2+}$/ml in PSS and the washout rate of $^{45}Ca^{2+}$ was determined thereafter. The cells were washed in La^{3+} - containing PSS solution (140 mM NaCl, 5 mM KCl, 2 mM $LaCl_3$, 0.75 mM $MgCl_2$, 10 mM D-glucose, 10 mM Na Hepes, pH 7.4), harvested at 2 min intervals and their radioactivity determined. The retrograde extrapolation of this intracellular calcium content to the zero time point on the efflux curve represents the influx of $^{45}Ca^{2+}$ during a 2 min post-oxidation period.

Ca^{2+} Efflux: Efflux of free calcium from periodate-oxidized cells was determined after loading the lymphocytes (5 x 10^6/ml) with 10 μCi $^{45}Ca^{2+}$/ml in culture medium. Washed cells were then oxidized by 1 mM H_5IO_6 in PBS as previously described (4,5). The remaining cellular $^{45}Ca^{2+}$ content was determined by liquid scintillation counting.

Results

Effect of H_5IO_6 on Blastogenesis: To confirm the blastogenic transformation of oxidized lymphocytes and to rule out any spurious effect of 18 h pre-incubation of cells in medium containing reduced calcium levels, the rates of nucleic acid synthesis and IL-2 production by oxidized cells were assayed as described elsewhere (4). The stimulation of DNA synthesis in cultured lymphocytes was assayed by [^{3}H]TdR incorporation, while the release of IL-2 into the culture medium by oxidized cells was determined by measuring the ability of the conditioned medium to support proliferation of the IL-2-dependent CT6 lymphocytes. Maximum RNA and DNA synthesis and the maximum number of cells in the S-phase of the cell cycle were observed at 72 hours. These data are summarized in Table 1.

Dependency of mitogenic activation by H_5IO_6 on the presence of extracellular Ca^{2+}: The effect of cellular calcium on certain functional indicators of periodate-oxidized lymphocytes is summarized in Table 1. The reduction of the Ca^{2+} levels in the cell culture medium to 0.2 mM did not significantly affect cell viability, but has decreased the rates of DNA synthesis and IL-2 production by intact as well as by oxidized lymphocytes(Table 1). After 24 h of culture in 0.2 mM Ca^{2+}-containing medium, IL-2 production by oxidized cells remained at control levels whereas a more than ten-fold stimulation of IL-2 secretion by the oxidized cells occurred in the presence of 1.4 mM Ca^{2+}. A decrease in viability of cells cultured in the presence of 0.2 mM Ca^{2+}, as compared to 1.4 mM Ca^{2+}, was not detected at 24 h, but it became quite pronounced at 72 h. At that time the stimulation indices of oxidized and intact cells, cultured in the presence of 0.2 mM Ca^{2+} were below 1.0 whereas the oxidized cells, maintained in 1.4 mM Ca^{2+}-containing medium, were stimulated 9.3 fold. Oxidized lymphocytes cultured for 72 h in 1.4 mM Ca^{2+} - containing medium showed a five-fold increase in the percentage of cells in S+G_2 + M phases of the cell cycle, while oxidized cells cultured in the presence of Ca^{2+}-deficient medium showed less than a two-fold increase.

Table 1

Calcium dependency of mitogenesis and IL-2 production by periodic acid-oxidized lymphocytes

		24 h		72 h		
Oxidation	Ca^{2+}(a)	IL-2	%viability	S.I.(b)	%S+G_2+M	%viability
-	1.4	11± 3	80	1.0±0.1	3.3±0.4	65
+	1.4	115±25(c)	84	9.3±0.1	16.5±0.1	63
-	0.2	<10	83	<1.0	2.9±0.4	53
+	0.2	<10	84	<1.0	5.2±0.1	54

Initially rat spleen lymphocytes (5 x 10^6/ml) were cultured for 12 h in CMRL-1415 medium containing 5 % HI-FCS and 1.4 mM Ca^{2+}. Following 20 min oxidation by 1 mM H_5IO_6 at 0°C in Ca^{2+} and Mg^{2+}-free PBS, oxidized lymphocytes (5 x 10^6/ml) were cultured in CMRL-1415 medium supplemented with 5% v/v HI-FCS and containing either 0.2 mM or 1.4 mM Ca^{2+}. The presence of IL-2 in the conditioned medium was determined 24 h after H_5IO_6 oxidation and assays of cell viabilities by trypan blue exclusion were also made at this time. After 72 h of culture, [^{3}H]TdR incorporation rates, the percentages of lymphocytes in the proliferative phases of the cell cycle and their viabilities were determined.

(a) Extracellular calcium: Residual calcium in Ca^{2+} -deficient medium was determined by Cresolphthalein titration (22) prior to and after the addition of a known quantity of calcium to the medium.

(b) Stimulation Index (S.I.) = (cpm [^{3}H]TdR incorporated by oxidized lymphocytes) / (cpm [^{3}H]TdR incorporated by control lymphocytes cultures in 1.4 mM Ca^{2+}-containing medium.)

[^{3}H]TdR incorporation by intact (control) cells - 2230 ± 253 (cpm ± S.D.)

(c) As compared to the number of CT6 cells in the S-phase of the cell cycle cultured in conditioned medium from intact lymphocytes, the increase in IL-2 production by the oxidized cells corresponded to a thee-fold increase in the number of CT6 cells in the S-phase of the cell cycle following 24 h of culture in the medium conditioned by oxidized lymphocytes.

Effect of periodate oxidation on the cellular calcium fluxes. As evidenced by decreased cellular radioactivity, periodate oxidation of lymphocytes has generated an increased rate of transmembrane efflux of calcium (Figure 1). Although immediately after periodate oxidation the calcium content of oxidized cells remained consistently below the control levels, oxidized cells nonetheless incorporated more $^{45}Ca^{2+}$ than intact lymphocytes (Figure 2). Thereafter, calcium efflux from the oxidized and intact lymphocytes proceeded, in convergence, with both cell cultures registering an identical radioactive calcium content approximately 30 min later (Figure 2). Additional quantitative data pertaining to periodate-generated increased rates of Ca^{2+} efflux and Ca^{2+} influx are given in Figure 1 and Figure 2, respectively.

Discussion

The recognized importance of Ca^{2+} in transformation of lymphocytes by lectins (9) as well as the contradictory data regarding Ca^{2+} status during periodate-induced blastogenesis served as a rationale for the present work. Since the previous studies of Ca^{2+} uptake were performed by

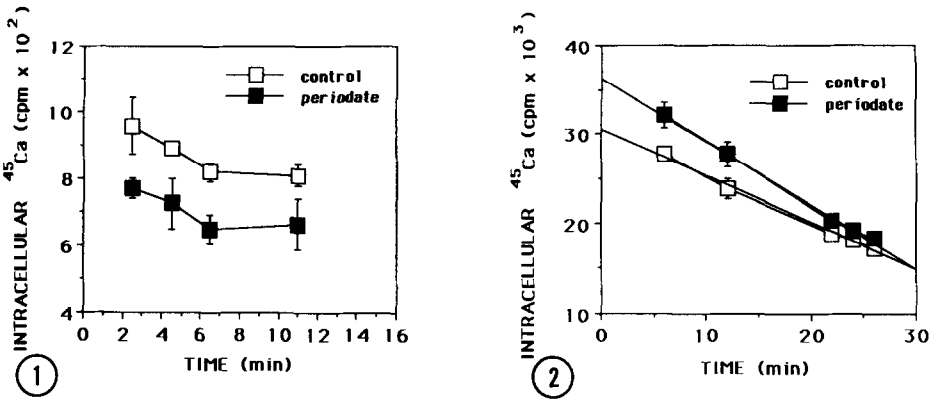

Figure 1: *Rate of calcium efflux from intact and periodate-oxidized lymphocytes.*
Lymphocytes (5 x10^6 cells/ml) were pulsed for 4 h with 10 μCi of $^{45}Ca^{2+}$/ml of medium, washed and resuspended in fresh PBS. Calcium-labeled cells were then oxidized for 2 min by 1 mM H_5IO_6 at 0°C, washed in PBS and their radioactivity determined at 2.5, 4.5, 6.5, and 11 min intervals by liquid scintillation counting Except for the omission of periodate oxidation, control cells were processed identically. The quantitative values were evaluated statistically by employing Student's T-test. Each respective control and experimental value were found to differ significantly ($p<0.0005$). The numerical value for each time represents the mean of six determinations plus/minus the standard deviation.

Figure 2: *Rate of calcium influx into intact and periodate-oxidized lymphocytes.*
Intact and periodate-oxidized lymphocytes were loaded for 2 min by 20 μCi $^{45}Ca^{2+}$/ml. The calcium-loaded cells were rinsed at 2 min intervals in an ice-cold 2 mM lanthanum solution and then washed again with fresh lanthanum solutions. The content of cellular $^{45}Ca^{2+}$ was calculated and the linear segment of the efflux curve extrapolated back to the zero time, so that the initial calcium uptake could be determined over the 2 min period. Each numerical value is the mean of six determinations plus/minus the standard deviation.

Parker(10) and Larner et al (11) on human and rat lymphocytes, respectively, we likewise have used the same cell sources. Moreover, Larner et al (11) have determined that the inhibition of Ca^{2+} uptake by the oxidized rat splenocytes occurred under the conditions that caused enhanced [^{3}H]TdR incorporation - an indicator of the blastogenic response. Therefore, the rate of [^{3}H]TdR incorporation, and the production of IL-2 were quantified in conjunction with Ca^{2+} assays on periodate-oxidized lymphocytes.

Our results, describing $^{45}Ca^{2+}$ incorporation and Ca^{2+} requirements by periodic acid-oxidized lymphocytes, fully support the antedating findings by Parker (10), and contradict those of Larner et al (11). Therefore, it appears that besides lectins (7,9,15) and calcium ionophore A23187 (16), periodate also causes a significant increase of exchangeable Ca^{2+} in lymphocytes. Unlike in lectin-stimulated lymphocytes, where Ca^{2+} functions was studied extensively (9), the mechanisms controlling Ca^{2+} homeostasis in periodate-activated cells remain obscure. Consequently, a few remarks regarding possible mechanisms of periodate-induced metabolic alterations seem to be warranted.

The available evidence (9) suggests that activation of lymphocytes by lectins proceeds via increased phosphoinositide turnover and the subsequent increase in intracellular free Ca^{2+}

concentration. During sustained cellular responses, such as blastogenesis elicited by lectins, Ca^{2+} activates plasma membrane-linked PKC which leads to the phosphorylation of a number of regulatory proteins. In contrast to lymphocytes activated by lectins (9), periodic acid-induced blastogenesis occurs in the absence of increased rates of phosphoinositide turnover (17), which seemingly rules out $Ins(1,4,5)P_3$ as a trigger of intracellular Ca^{2+} release by the oxidized cells, thus suggesting that other mechanisms of Ca^{2+} regulation are triggered by periodate.

Like other extracellular oxidants, oxidation by periodate can modify intracellular PKC(18,19) and, depending on the oxidation conditions, can either activate or inactivate this enzyme. Moreover, extracellular generators of H_2O_2 and superoxide were shown to induce Ca^{2+}/calmodulin-linked phosphorylation of ribosomal S6 protein in JB6 cells, and it was suggested that this protein may be on the pathway of mitogenic stimulation by extracellular oxidants (20). Similarly, an increase in phosphorylation of as yet unidentified protein kinases was induced by periodate under conditions identical with the present study (21). Our data and recent results by other investigators (12, 18-20) jointly suggest that, like other extracellular oxidants, periodate may exert a direct effect on intracellular pathways generally regulated by second messengers.

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