Coordination and Reversibility of Signals for Proliferative Activation and Interleukin-2 mRNA Production in Resting Human T Lymphocytes by Phorbol Ester and Calcium Ionophore*

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Sequential stimulation and washout procedures were employed to examine the kinetics and reversibility of pharmacologically manipulated second messenger signals mediating phenotypic changes and proliferative activation of resting human T lymphocytes. Phorbol dibutyrate (PDBu) was used to stimulate protein kinase C (Ca²⁺/phospholipid-dependent enzyme) while ionomycin was used to manipulate intracellular Ca²⁺ levels. Stimulation by PDBu alone induced phosphorylation of several endogenous substrates and altered expression of phenotypic markers, downregulating expression of CD4 and CD3 while increasing expression of CD2 and the interleukin 2 (IL-2) receptor. Stimulation with ionomycin alone caused an increase in intracellular Ca²⁺ levels but did not induce proliferation or cause major changes in the expression of phenotypic markers (CD2, CD3, CD4, CD8, IL-2, and transferrin receptors). Analysis of endogenous PDBu stimulated phosphosubstrates indicated that some substrates (pp92, pp82, pp65) underwent dephosphorylation, returning to baseline levels following PDBu removal while others (pp61, pp65) showed only partial dephosphorylation, while one (pp28) remained phosphorylated. Washing ionomycin-stimulated cells resulted in an approximately 75% reduction of intracellular Ca²⁺. Ionomycin exposure did not alter the affinity (Kᵤ = 22.3 ± 7.4 nM) or number of receptors (53,497 ± 8,291 receptors/cell) for [³H]PDBu. These data suggest that signals induced by PDBu or ionomycin are reversible following removal of the stimulating agents with respect to proliferative activation of T lymphocytes. Furthermore, a transcriptional mechanism regulating the production of IL-2 mRNA requires simultaneous activation of protein kinase C and elevation of intracellular Ca²⁺.

Phospholipid metabolism and the generation of intermediates with intracellular second messenger functions represent an important pathway for the transfer of information from ligand-receptor interactions in a wide variety of cell types (1, 2). For T lymphocytes, substantial evidence indicates that initial antigen receptor activation signals are transduced by the action of two second messenger intermediates generated from phospholipid metabolism (reviewed in Refs. 3 and 4). Perturbation of the T cell antigen receptor complex activates phosphatidylinositol-4,5-bisphosphate phosphodiesterase which yields inositol polyphosphates and diacylglycerol. Inositol polyphosphates can act within the cell to elevate cytosolic Ca²⁺ levels, while diacylglycerol binds and activates protein kinase C (Ca²⁺/phospholipid-dependent enzyme). These events initiate a further biochemical cascade resulting in the expression of several genes including those coding for IL-2 and IL-2 receptor (5). Subsequent interaction between the IL-2 receptor and IL-2 released from the T helper cells leads to cell cycle progression and proliferation (6, 7).

The precise mechanism is not yet understood by which the elevation of cytosolic Ca²⁺ and the activation of protein kinase C triggers the genetic transcriptional events critical to T lymphocyte activation. Pharmacologic agents such as phorbol esters or Ca²⁺ ionophores which selectively stimulate protein kinase C or elevate intracellular Ca²⁺, respectively, have been important experimental tools for determining which arm of this bifurcating signal transduction pathway is responsible for various events critical to lymphocyte function (8). Many enzymatic systems, in a wide variety of cell types, are regulated by Ca²⁺. The ionophore, ionomycin, is highly specific for Ca²⁺. Following intercalation into membranes, ionomycin allows Ca²⁺ to flow down its concentration gradient (9). The addition of ionomycin to cells in physiological solutions results in elevation of cytosolic Ca²⁺ levels thus mimicking the effects of inositol polyphosphates generated from phospholipid metabolism.

Phorbol 12,13-dibutyrate (PDBu) is a potent activator of protein kinase C but, unlike many other phorbol esters, it is less lipophilic. This characteristic, in conjunction with a rapid rate of receptor dissociation, allows PDBu to be removed from intact cells (8, 10–12). We have previously demonstrated that human macrophage spreading induced by stimulation of protein kinase C by PDBu is rapidly reversible when PDBu is washed from the cells (12). Thus, stimulation, followed by removal of PDBu allows for the kinetic manipulation of protein kinase C activity. Similarly, washing cells following stimulation by ionomycin may be a useful method for kinetically manipulating levels of cytosolic Ca²⁺.

Stimulation of T lymphocytes with protein kinase C-activating phorbol esters has been shown to induce endogenous substrate phosphorylation (13–16), as well as regulate expression of several cell surface components including CD3 (17), antigen receptor (18), CD4 (19–20), and the IL-2 receptor (21, 22).

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The abbreviations used are: IL-2, interleukin 2; PDBu, phorbol 12,13-dibutyrate; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 4-morpholineethanesulfonic acid.
T Cell Activation Signals

22. Protein kinase C stimulation by phorbol ester alone has also been reported to be capable of inducing transcription of several growth-related genes including elements coding for the IL-2 receptor (23–25) and the proto-oncogenes c-myc and c-fos (26, 27). Neither protein kinase C stimulation nor cytosolic Ca++ elevation alone, however, is sufficient to stimulate proliferation of macrophage-depleted resting T lymphocytes (28). Complete mitogenic activation appears to require both activation of protein kinase C and cytosolic calcium elevation (16). One critical event requiring both signals is the production of IL-2 (29).

In this report we analyze the kinetics and the relationship between PDBu and ionomycin-generated signals with respect to mitogenic activation of resting human peripheral blood lymphocytes. We further evaluate the cells for transcription of the IL-2 gene following sequential pharmacologic manipulation of protein kinase C and cytosolic Ca++ levels. The results indicate that the signals from either PDBu or ionomycin are rapidly reversible following removal of the stimulating agents and that both signals must occur simultaneously for activation of resting T lymphocytes and production of IL-2 mRNA. Receptor binding analysis employing [3H]PDBu indicates that the number of Ca++ channels did not alter the PDBu receptor number or affinity. Phosphoprotein analysis further suggests that dephosphorylation events may play a regulatory role in the dynamic balance of signals regulating activation of resting T lymphocytes.

EXPERIMENTAL PROCEDURES

Materials—Complete culture medium: supplemented with 5% heat inactivated human AB serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2.0 mM glutamine, and 1.0 μM 2-mercaptoethanol. All tissue culture reagents were obtained from Gibco. Ionomycin was purchased from Behring Diagnostics, and phorbol esters were purchased from L. C. Services Corp. (Woburn, MA). Unless specified, all other chemicals were obtained from Sigma. Plastic tissue culture ware was purchased from Costar (Cambridge, MA).

Cell Isolation—Mononuclear cells were isolated from the venous blood of healthy volunteers by density gradient centrifugation over a two-step gradient modified from described procedures (31). Briefly, a two-step gradient was prepared with layers (top to bottom) of 43 and 47.5% Percoll in columns and nonadherent cells eluted. Resting T lymphocytes were recovered from the bottom of the 47.5% layer represent the experimental population of cells utilized in these studies. The cells were washed twice in complete phosphate-buffered saline (PBS) and PBS+A (i.e. phosphate-buffered saline + 1.0 mM NaN3) at 4 °C, then incubated with optimum dilutions of fluorescein isothiocyanate labeled monoclonal antibodies (specific activity 30.8 Ci/mmol). (Du Pont-New England Nuclear) was used in the preincubation procedure in order to determine the efficacy of the PDBu removal procedure. Following stimulation and wash-out, aliquots of cells exposed to [3H]PDBu, with and without ionomycin, were collected and the [3H]PDBu retained by the cells measured via liquid scintillation spectrometry.

Proiferation Measurement—Proliferation was measured by the incorporation of [3H]thymidine into cells. Briefly, cells were cultured in 0.2 ml of complete medium in 96-well microtiter plates at 106 cells/ml. The plates were resuspended in 2.0 ml of Dulbecco’s modified Eagle medium and counted. Emission was monitored at 555 nm. Fluorescence intensity ratios were converted to calcium concentrations using a revised version of the Spex CM PROG.

[H]PDBu Binding Analysis—Cells were suspended in RPMI-1640 with 1% autologous serum and 10 μg for 20 min in ice-cold phosphate-buffered saline and fixed in phosphate-buffered saline with 0.1% formalin. The cells were analyzed on an Ortho 2150 Cytofluorograph.

Cell Stimulation, Washout, and Culture—Cells at 1.5 X 106 in 1.5 ml of complete medium were stimulated with experimental agents for various times. The cells were washed 3 times by centrifugation using 1.0 ml of complete medium adjusted to 290 mosmol. Lymphocytes in complete medium were layered on top of the gradients which were then centrifuged. The small dense lymphocytes within 45 min and remained constant for up to 2 h. Further experiments, employing various concentrations of [3H]PDBu, were incubated in 0.2 ml of complete medium containing experimental agents. In parallel experiments, 10.0 nM [3H]PDBu (specific activity 30.8 Ci/mmol) and 1.0 μCi [3H]thymidine (specific activity = 2.0 Ci/mmol) (Du Pont-New England Nuclear) were used in the preincubation experiment in order to determine the efficacy of the PDBu removal procedure. Following stimulation in 0.2 ml of complete medium containing experimental agents, the cells were washed twice with phosphate-buffered saline and fixed in phosphate-buffered saline with 0.1% formalin. The cells were analyzed on an Ortho 2150 Cytofluorograph.

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Intracellular Ca++ Analysis—Lymphocytes in RPMI-1640 were loaded with the fluorescent calcium chelator, fura-2, by means of a 60-min incubation in RPMI-1640 + 1.0% autologous serum at 37 °C with 1.0 μM of the membrane permeant acetoxy methyl derivative of the dye (32–33). Cells were then washed twice, resuspended in RPMI-1640, stored on ice, and used within 1 h. Immediately prior to use, each sample was sedimented in a microcentrifuge, and 20 X 106 cells were resuspended in 2.0 ml of Dulbecco’s modified Eagle’s medium ([Ca++]) = 1.8 mM; at 37 °C. Calcium measurements were conducted in a Spex model CM-2 cation measurement system, with dual excitation at 340 and 380 nm. Emission was monitored at 555 nm. Fluorescence intensity ratios were converted to calcium concentrations using a revised version of the Spex CM PROG.

Phosphorothioate Analysis—Cells were isolated and washed twice in phosphate-buffered saline to remove any nucleic acid contamination. Cells were then incubated with [3H]thymidine in the presence of 1 μCi/ml, and harvested at 24 h. RNA was then isolated and analyzed by polyacrylamide gel electrophoresis. The relative amounts of 5S and 28S RNA were determined by scanning the gel with a densitometer.
glycerol, 6 mM EGTA, 3 mM EDTA, 10 mM NaF, 0.1% disodium pyrophosphate, 13 mM 2-mercaptoethanol and 0.1% bromphenol blue, pH 6.7. The samples were then boiled for one minute.

Cell lysates were fractionated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (35). Each gel employed a 5% polyacrylamide-stacking gel over a separating gel containing a linear gradient of 6-13% total acrylamide. The gels were stained with Coomassie Brilliant Blue, destained, dried, and exposed to Kodak Xar-5 film with an enhancing screen for 12–24 h. Molecular weights of selected proteins were calculated based upon the linear relationship of the logarithm of the Mr of standards versus the logarithm of the percent total acrylamide. The molecular weight standards utilized were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). For quantitation of phosphorylation in selected bands, the autoradiogram was overlayed with the dried gel and appropriate bands marked, excised, and counted in a liquid scintillation counter (36). The relative degree of experimentally induced substrate phosphorylation was calculated based on comparisons with the phosphorylation of the identical substrate from unstimulated cells as well as with other phosphoproteins whose degree of phosphorylation was not altered following cell exposure to PDBu. Thus, % control phosphorylation = 100 X [cpm PDBu-stimulated substrate/cpm PDBu nonresponsive substrate]/[cpm negative control-stimulated substrate/cpm PDBu nonresponsive substrate].

**RESULTS**

**Cell Morphology and Phenotypic Analysis Following Stimulation by PDBu and Ionomycin**—The stimulation of resting T lymphocytes with PDBu (1–100 nM) alone or PDBu + 1.0 μM ionomycin initiated morphologic changes including the development of pseudopods and cell aggregation. The morphologic changes were evident within 60 min of stimulation and persisted throughout the culture period (i.e. up to 3 days). Unstimulated cells and cells exposed to ionomycin alone remained rounded and did not aggregate. To determine whether phenotypic alterations could be induced by PDBu alone or required the elevation of intracellular Ca²⁺ by ionomycin, T cell populations were exposed to experimental stimuli for 24 h, then analyzed for the expression of CD2, CD3, CD4, CD8, IL-2 receptor, and transferrin receptor using a cytofluorograph employing specific monoclonal antibodies (Table I). The expression of CD4 and CD8 was downregulated by PDBu with and without ionomycin. The expression of CD4 was nearly completely eliminated while CD3 expression appeared lower but did not disappear. Although the percent of positive cells expressing CD2 did not change with experimental treatments, there was an increase in the amount of CD2 expressed on cells following PDBu stimulation both with and without ionomycin. Unstimulated cells expressed no receptors for transferrin or IL-2. Stimulation by PDBu alone induced a few cells (17.3%) to express the transferrin receptor and a larger number (37.8%) to express the IL-2 receptor. The combination of PDBu and ionomycin induced substantial expression of both the transferrin (64.4%) and IL-2 (58.0%) receptors. The addition of ionomycin alone had little effect on morphology or cell surface phenotype which remained similar to the unstimulated cells. There were slight increases in the level of CD2 and CD8 expression, a slight decline in amount of CD4/cell, and a very small increase in the percent of cells expressing IL-2 receptor and transferrin receptor.

**Kinetics and Reversibility of Mitogenic Activation by PDBu and/or Ionomycin**—The first series of experiments were designed to determine the kinetics of mitogenic activation by the combination of PDBu and ionomycin as well as to determine whether one signal could prime the cells for activation by the other. Resting T lymphocytes were pharmacologically manipulated by sequential exposure to PDBu and/or ionomycin for various periods (15 min to 4 h) followed by a washing procedure and secondary culture with medium alone, PDBu, and/or ionomycin (Fig. 1). Proliferation was measured by [³H]thymidine incorporation following 72 h in culture. Microscopic observations indicated that polyclonal blastogenic transformation correlated with increased [³H]thymidine incorporation. In parallel experiments, [³H]PDBu was employed to determine the efficacy of the washout procedure. Following preculture stimulation with [³H]PDBu and washout, aliquots of cells were collected and the radioactivity quantitated. In all cases, the amount of [³H]PDBu remaining in washed cells was below detectable limits of the system (i.e. 0.1 fmol/10⁶ cells).

Stimulation of T cells during primary culture with the

**TABLE I**

<p>| Phenotypic profile of T lymphocyte populations following stimulation by PDBu and/or ionomycin |
|---------------------------------|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Stimulus</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>IL-2 receptor</th>
<th>Transferrin receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 0 control</td>
<td>93.5</td>
<td>138</td>
<td>90.5</td>
<td>284</td>
<td>37.8</td>
<td>549</td>
</tr>
<tr>
<td>Medium</td>
<td>94.7</td>
<td>154</td>
<td>88.2</td>
<td>355</td>
<td>41.2</td>
<td>548</td>
</tr>
<tr>
<td>PDBu</td>
<td>93.5</td>
<td>350</td>
<td>89.7</td>
<td>137</td>
<td>6.7</td>
<td>218</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>93.8</td>
<td>233</td>
<td>89.1</td>
<td>310</td>
<td>41.9</td>
<td>452</td>
</tr>
<tr>
<td>PDBu + Ionomycin</td>
<td>95.4</td>
<td>387</td>
<td>92.5</td>
<td>128</td>
<td>5.5</td>
<td>264</td>
</tr>
</tbody>
</table>

Cells were stimulated for 24 h prior to analysis with specific monoclonal antibodies, except for zero time control which were stained immediately following isolation. Cytofluorographic values represent % fluorescence cells as compared to cells stained with a nonspecific control antibody. Mean value represents average fluorescence of positive cells. Concentrations of stimulating agents were 100 nM PDBu and 1.0 μM ionomycin. The data set depicted is representative of four independent experiments.
Fig. 1. Kinetics and reversibility of T lymphocyte proliferative activation of PDBu and/or ionomycin (Io). Peripheral blood T cells were incubated for designated time periods in the experimental agents, then washed as described under "Experimental Procedures." Aliquots of cells (10⁶/well) were then incubated in 96-well plates for 3 days along with various experimental agents. Proliferation was measured by [³H]thymidine incorporation during the terminal 6 h of culture. Concentrations of experimental agents were 1.0 μM ionomycin and 100 nM PDBu. Values are expressed in counts/minute (mean ± S.E., n = 3) from a representative experiment. Similar results were observed in three independent experiments.

<table>
<thead>
<tr>
<th>Primary Culture</th>
<th>Secondary Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>time (min)</td>
<td>stimuli</td>
</tr>
<tr>
<td>15</td>
<td>Io</td>
</tr>
<tr>
<td>240</td>
<td>Io</td>
</tr>
<tr>
<td>15</td>
<td>PDBu</td>
</tr>
<tr>
<td>240</td>
<td>PDBu</td>
</tr>
<tr>
<td>15</td>
<td>PDBu + Io</td>
</tr>
<tr>
<td>30</td>
<td>PDBu + Io</td>
</tr>
<tr>
<td>60</td>
<td>PDBu + Io</td>
</tr>
<tr>
<td>120</td>
<td>PDBu + Io</td>
</tr>
<tr>
<td>240</td>
<td>PDBu + Io</td>
</tr>
<tr>
<td>240</td>
<td>medium</td>
</tr>
<tr>
<td>240</td>
<td>medium</td>
</tr>
<tr>
<td>240</td>
<td>medium</td>
</tr>
</tbody>
</table>

[³H] thymidine incorporation (cpm)

Fig. 2. Alteration of endogenous phosphosubstrates in viable T lymphocytes following manipulation with 10 nM PDBu. Phosphoprotein analysis and cell washing procedures were performed as described under "Experimental Procedures."

Combination of PDBu and ionomycin produced a time-dependent activation of the cells, resulting in a proliferative response when the cells were subsequently washed and cultured in medium alone (Fig. 1). T cells stimulated with either agent alone, then washed, did not proliferate in subsequent culture unless both PDBu and ionomycin were concomitantly present in the post-wash culture. Thus, sequential stimulation was not able to trigger T cell mitogenic activation suggesting that signals mediated by either protein kinase C stimulation or cytosolic Ca²⁺ elevation are unable to prime for one another.

T Lymphocyte Phosphoprotein Analysis Following PDBu Stimulation and Washout—Several lines of evidence indicate that diacylglycerol activation of protein kinase C is a transient event, and our data using PDBu suggests that a mitogenically important signal(s) is reversible following removal of the protein kinase C-stimulating agent. Since protein kinase C activation involves protein phosphorylation, we examined the phosphoprotein pattern of resting T lymphocytes following stimulation and washout of PDBu in order to determine whether the reversibility might also be reflected in dephosphorylation of protein kinase C substrates. The PDBu-induced phosphorylation kinetics of six proteins were analyzed in viable lymphocytes which had been loaded to isotopic equilibrium with [³²P]orthophosphate. These substrates, distinguished by molecular weight on SDS-polyacrylamide gel electrophoresis, included pp28, pp55, pp61, pp65, pp82, and pp92.

As seen in Fig. 2, exposure to PDBu induced a rapid increase in protein phosphorylation of all six substrates which plateaued in 5–10 min and remained constant for up to 60 min as long as PDBu remained in the culture medium. Ionomycin (1.0 μM) did not induce protein phosphorylation nor enhance the effect of PDBu within the resolution limits of our system.
Intracellular $Ca^{2+}$ Analysis—The failure of mitogenic induction following sequential stimulation with ionomycin followed by PDBu also suggests that the pharmacologic increase in cellular $Ca^{2+}$ with ionomycin as well as the activation signals it generates may be a reversible process. To further examine this observation, cytosolic $Ca^{2+}$ levels in resting T cell populations were monitored with the fluorescent $Ca^{2+}$ probe, fura-2 (Fig. 3). Intracellular $Ca^{2+}$ levels in resting T cell populations appeared relatively stable at approximately 35 nM. The addition of 1.0 nM ionomycin to T cells in buffer containing physiologically relevant $Ca^{2+}$ concentrations initiated a rapid (i.e. 120 s) increase in intracellular $Ca^{2+}$ which peaked at 1850 nM then decreased over 120 s before leveling off at 450 nM and remaining relatively stable for up to 10 min, which was the maximum length of the experimental observation. When fura-2-loaded T cells, in which $Ca^{2+}$ had been elevated by 5 min of stimulation with ionomycin, were washed 3 times in warm (i.e. 37 °C) Dulbecco’s modified Eagle’s medium and reanalyzed for intracellular $Ca^{2+}$ content, there had been a reduction of intracellular $Ca^{2+}$ from 450 to 135 nM (Fig. 3, post-wash analysis). The intracellular $Ca^{2+}$ level following ionomycin stimulation and washout remained stable for the duration of observation (up to 10 min) and could be restimulated by the readdition of more ionomycin. The elevation of intracellular $Ca^{2+}$ following the second exposure to ionomycin did not show the initial $Ca^{2+}$ peak and decline, but rather a steady rise to a level (450 nM) comparable to the stable concentration observed following the initial 5-min stimulation by this ionophore.

Effect of Ionomycin on $[^3H]$PDBu Binding—One possible explanation for the mitogenic synergy between PDBu and ionomycin is that the elevation of intracellular $Ca^{2+}$ enhances the availability or affinity of protein kinase C for an activating ligand (i.e. PDBu). This possibility was addressed by determining whether ionomycin could alter the specific binding of $[^3H]$PDBu to intact T lymphocytes. A time course (5–90 min) of specific binding of 10 nM $[^3H]$PDBu at 37 °C showed a gradual increase in binding which plateaued at 20 min and remained stable through 90 min. Scatchard analysis indicated a single class of $[^3H]$PDBu-binding sites both in the presence and absence of 1.0 μM ionomycin. Equilibrium binding analysis of $[^3H]$PDBu at 37 °C in resting human T lymphocytes further indicated a specific ligand binding affinity ($K_d$) of 22.3 ± 7.4 (±S.E., n = 4) with 53,497 ± 8,291 (±S.E., n = 4) binding sites/cell (Table II). Addition of 1.0 μM ionomycin did not significantly alter the $[^3H]$PDBu receptor binding affinity.
affinity ($K_D = 18.3 \pm 8.5$) or receptor number (55,228 ± 13,591).

**Induction of IL-2 mRNA**—We next sought to determine the signal kinetics of protein kinase C activity and cytosolic Ca$^{2+}$ elevation on early genetic events thought to play key roles in proliferative activation of T cells. Following stimulation by PDBu and/or ionomycin for various periods of time, we examined the RNA of lymphocytes for IL-2 mRNA using Northern blot procedures on RNA separated on agarose gels. As seen in Fig. 4, incubation of the cells for up to 4 h in medium alone or in the presence of ionomycin induced no detectable transcription of the IL-2 gene. A small, but detectable, amount of IL-2 mRNA was observed in cells stimulated 30 min with PDBu alone (Fig. 4). This was not a consistent finding, however, and PDBu alone did not induce IL-2 mRNA at other times (1.9-4.0 h) and in other independent experiments. The combination of PDBu and ionomycin induced substantial transcription of the IL-2 gene which was detectable in 30 min, with maximum levels reached by 4 h of exposure to the experimental agents.

**Coordination of PDBu and Ionomycin for Induction of IL-2 mRNA**—The next series of experiments addressed the issue of whether signals induced by one agent, either PDBu or ionomycin, could prime for the other with respect to triggering transcription of IL-2 mRNA. Cells were incubated 2 h in either medium, PDBu, or ionomycin. They were then washed 3 times at 37 °C and cultured an additional 2 h with various experimental agents. As seen in the representative Northern blot analysis depicted in Fig. 5, substantial expression of IL-2 mRNA was detected only when the cells were exposed to both PDBu and ionomycin at the same time.

**Discussion**

Phospholipid hydrolysis and the generation of metabolites capable of stimulating protein kinase C activity and elevating cytosolic Ca$^{2+}$ are important mechanisms critical to T lymphocyte mitogenic activation. Pharmacologic agents such as phorbol esters and Ca$^{2+}$ ionophores have been important tools for selectively stimulating each arm of the bifurcating signal transduction mechanism. The present investigation makes use of a sequential stimulation-washout-stimulation procedure to pharmacologically manipulate protein kinase C activity and cytosolic Ca$^{2+}$ levels in naive, resting human T lymphocytes. These studies indicate that induction of IL-2 mRNA and mitogenesis of T lymphocytes is dependent on the simultaneous stimulation of protein kinase C and elevation of intracellular Ca$^{2+}$. The reversibility of the signals generated by these pharmacologic manipulations were also evaluated by monitoring intracellular Ca$^{2+}$ levels and the phosphorylation/dephosphorylation of endogenous phosphoproteins following experimental manipulations. A major consideration of any study exploring mechanisms of T lymphocyte activation is the cell population employed. The cells utilized in these experiments were resting peripheral blood T cells which were carefully depleted of other cells (e.g. monocytes, B lymphocytes) which may serve an accessory function in the activation process by releasing lymphokines or directly interacting with T lymphocyte membrane components (39). Perturbation of CD2 determinants, as occurs in sheep erythrocyte rosetting, was also avoided since this procedure causes changes in cell activation processes including altered c-myC expression (27).

The activation of protein kinase C is regulated by multiple factors, including Ca$^{2+}$, phospholipid, and an activating ligand whether it be diacylglycerol or arachidonate (40) in a physiologic setting or pharmacologic agents such as PDBu. In some biological systems, particularly those involving secretory events, the physiologic response depends on the synergistic activity of protein kinase C stimulation and intracellular Ca$^{2+}$ mobilization (41). In T lymphocytes, as in a variety of other cell systems, addition of PDBu alone is capable of initiating physiologic responses without requiring an elevation of intracellular Ca$^{2+}$. As demonstrated in the present study and those of others, stimulation of T cells by PDBu alone causes phosphorylation of a variety of endogenous substrates (13-16). It also causes the downregulation of the expression of CD4 (19, 20) and CD3 (17) while not altering the expression of other surface markers such as CD8. The expression of CD2 as well as high affinity receptors for IL-2 are also increased by addition of PDBu alone (21, 22). Elevation of intracellular Ca$^{2+}$ using ionomycin alone caused only slight changes in the expression of these cell surface markers. The expression of IL-2 receptors was greatly augmented by stimulation with the combination of PDBu and ionomycin. The expression of the transferrin receptor, which is a reliable marker of mitogenically activated cells, was induced in only a small subpopulation of cells following stimulation by PDBu alone but was significantly enhanced by stimulation with both PDBu and ionomycin. Thus, PDBu alone activates one class of responses generally attributed to protein kinase C although mechanisms critical to mitogenic triggering, including transcription of the IL-2 gene, require the simultaneous elevation of intracellular Ca$^{2+}$.

One possible mechanism for the synergism between ionomycin and PDBu is that a rise in cytosolic Ca$^{2+}$ promotes translocation and binding of protein kinase C to the cell membrane, thereby increasing the availability of phospholipid cofactor and thus enhancing the binding affinity of protein kinase C for PDBu (42, 43). In human B cells (44) and HL-60 cells (45), the ionophore A23187 has been reported to increase the affinity of protein kinase C for PDBu. Our data, however, indicate that ionomycin does not enhance $[^{3}H]$PDBu binding affinity or receptor number in resting human T lymphocyte populations (Table II). This observation, along with data on substrate phosphorylation, alteration of morphology, and expression of surface markers suggest that PDBu is able to bind and activate protein kinase C at ambient cytosolic Ca$^{2+}$ concentrations. It should be noted that this does not exclude the possible importance of a Ca$^{2+}$-mediated enhancement for protein kinase C binding of physiologic ligands such as diacylglycerol.

Several lines of evidence have implicated Ca$^{2+}$-calmodulin-dependent events as having a critical role in the activation of naive lymphocytes (45). The activation of calmodulin, induced by elevation of cytosolic Ca$^{2+}$, increases the activities of many enzymes within the cytoplasm of the cell, in particular calmodulin-dependent protein kinase, phosphorylases, phosphatases, and enzymes involved in glucose metabolism and energy production. Agents which bind and inhibit calmodulin, such as the potent immunosuppressive agent cyclosporin A, also inhibit early events in T lymphocyte activation (reviewed in Ref. 46) including production of IL-2 mRNA (47). It is thus plausible that the mitogenic synergy between PDBu and ionomycin is due to the coordinated activity of protein kinase C and the Ca$^{2+}$-calmodulin-dependent system.

Another possible mechanism regulating the mitogenic synergy of protein kinase C and Ca$^{2+}$ may involve the proteolytic modification of PK-C by a Ca$^{2+}$-dependent protease (1, 48). In the neutrophil, the Ca$^{2+}$-dependent protease, calpain, has been implicated as regulating the proteolysis of protein kinase C, and by so doing, regulates the response to phorbol ester stimulation (49). It is not yet clear whether a similar system
plays a role in T lymphocyte activation.

The analysis of IL-2 mRNA indicated that the synergistic mechanism triggered by PDBu and ionomycin occurred at a transcriptional/post-transcriptional level rather than a translational or post-translational processing or secretory event. The inability of PDBu or ionomycin to prime for one another with respect to IL-2 gene activation and cell proliferation further suggests that the signals induced by PDBu and ionomycin are rapidly reversed following removal of the stimulating agents. A potential criticism of studies employing phorbol esters is the assumption that they act exclusively by translational or post-translational mechanism triggered by PDBu and ionomycin occurred at a base-line levels while other intracellular compartments retain higher levels of Ca\(^{2+}\). The sequestered Ca\(^{2+}\) may thus not be available to synergize with PDBu-mediated events to trigger mitogenesis. A third possibility is that ionomycin is not completely removed by the washing procedure and Ca\(^{2+}\) continues to flow into the cell. This is unlikely since comparable Ca\(^{2+}\) levels produced by lower concentrations of ionomycin or anti-CD3 antibody are capable of mitogenic synergy with PDBu.

In addition, ionomycin-treated cells which were washed maintained stable intracellular calcium levels as a function of time in a buffer containing 1.8 mM Ca\(^{2+}\). If residual ionomycin were present under these conditions, the cells should continue to accumulate intracellular Ca\(^{2+}\). Future experiments may help clarify the fate of intracellular Ca\(^{2+}\) following pharmacologic manipulation with ionophores or physiologic generation by ionotrope polyphosphates.

The in vivo studies were designed to define the kinetics and coordination requirements of T lymphocyte activation signals generated by pharmacologic manipulation of protein kinase C and cytosolic Ca\(^{2+}\) using phorbol dibutyrate and the calcium ionophore, ionomycin. Stimulation by PDBu alone could induce the phosphorylation of several endogenous substrates and alter the expression of biologically important cell surface markers. Sequential stimulation and washout procedures, however, indicated that the induction of IL-2 mRNA and mitogenesis of peripheral blood-derived T lymphocytes was dependent on the simultaneous stimulation of protein kinase C and cytosolic Ca\(^{2+}\) elevation. The inability of PDBu and ionomycin to prime for one another with respect to IL-2 gene activation and cell proliferation emphasize the kinetic independence of each of these signals in triggering expression of important regulatory proteins. The phosphorylation of a variety of endogenous substrates following stimulation by PDBu showed that cellular mechanisms are present to allow for the decrease in substrate phosphorylation following removal of the PDBu. These observations provide an experimental basis for further evaluation of the regulatory importance of dephosphorylation events in regulating T cell immune responses. It is hoped that a more complete understanding of the mechanisms regulating T lymphocyte growth and function will lead to substantial improvements in the field of immunotherapy.

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REFERENCES


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