Inhibition of DNA Fragmentation in Thymocytes and Isolated Thymocyte Nuclei by Agents That Stimulate Protein Kinase C*

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Glucocorticoid hormones and Ca\(^{2+}\) ionophores stimulate a suicide process in immature thymocytes, known as apoptosis or programmed cell death, that involves extensive DNA fragmentation. We have recently shown that a sustained increase in cytosolic Ca\(^{2+}\) concentration stimulates DNA fragmentation and cell killing in glucocorticoid- or ionophore-treated thymocytes. However, a sustained increase in the cytosolic Ca\(^{2+}\) level also mediates lymphocyte proliferation, suggesting that apoptosis is blocked in proliferating thymocytes. In this study we report that phorbol esters, which selectively stimulate protein kinase C (PKC), blocked DNA fragmentation and cell death in thymocytes exposed to Ca\(^{2+}\) ionophore or glucocorticoid hormone. The T cell mitogen, concanavalin A, which stimulates thymocytes by a mechanism that involves PKC activation, caused concentration-dependent increases in the cytosolic Ca\(^{2+}\) level that did not result in DNA fragmentation, but incubation with concanavalin A and the PKC inhibitor H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) resulted in both DNA fragmentation and cell death. Phorbol ester directly inhibited Ca\(^{2+}\)-dependent DNA fragmentation in isolated thymocyte nuclei. Our results strongly suggest that PKC activation blocks thymocyte apoptosis by preventing Ca\(^{2+}\)-stimulated endonuclease activation.

During normal cell turnover, hormone-induced tissue atrophy, or effector-mediated cytosis in the immune system, a suicide process is activated in target cells, termed apoptosis or programmed cell death (1). The most characteristic biochemical marker for apoptosis is DNA fragmentation (2-4). Although the details of the process remain unclear, the fragmentation appears to be due to a Ca\(^{2+}\)-dependent nuclear endonuclease that cleaves host chromatin into oligonucleosome-length fragments (4, 5). Immature thymocytes are sensitive to induction of apoptosis in response to a number of stimuli including glucocorticoid hormones (5, 4), calcium ionophores (6, 9), the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (7), γ-irradiation (8), or antibodies to the CD3- T cell receptor complex (9), and thymocytes have therefore been useful as a model system for characterizing the biochemical mechanisms underlying the process. It has been suggested that thymocyte apoptosis is involved in the thymic cell selection process that generates the mature peripheral T cell repertoire (10).

We recently showed that DNA fragmentation and death induced by glucocorticoid hormones is dependent on the extranuclear Ca\(^{2+}\) concentration (12). Calcium-dependent DNA fragmentation in thymocytes is dependent on a sustained increase in the cytosolic Ca\(^{2+}\) level (11). In addition, the rate of DNA fragmentation in rat liver nuclei is strictly dependent on the extranuclear Ca\(^{2+}\) concentration (12). Calcium-dependent DNA fragmentation in thymocytes directly results in cell death (11).

Paradoxically, lymphocyte proliferation is also a process that is dependent on a sustained increase in cytosolic Ca\(^{2+}\) level. T cell mitogens such as concanavalin A (ConA) stimulate growth, DNA synthesis, and cell division in lymphocytes by means of sustained increases in cytosolic Ca\(^{2+}\) concentration (13) that are comparable to those stimulating DNA fragmentation in thymocytes (7, 10, 11). The mechanism of mitogen-induced elevation of lymphocyte Ca\(^{2+}\) level involves stimulation of polyphosphoinositide hydrolysis (13), a process that also results in the release of diacylglycerol and the activation of protein kinase C (PKC) (14). In fact, one of the most effective proliferative stimuli for lymphocytes is a combination of calcium ionophore and phorbol ester to mimic the mitogenic stimulus (15). Since phorbol esters selectively stimulate protein kinase C (14), these results indicate that PKC activation is required for Ca\(^{2+}\)-dependent lymphocyte proliferation.

We report here that agents which stimulate PKC block DNA fragmentation in thymocytes without detectably affecting the increase in cytosolic Ca\(^{2+}\) level. We discuss the possible roles DNA fragmentation and PKC activation may play in the differential responses of thymocytes to Ca\(^{2+}\) signals during cell selection in the thymus.

EXPERIMENTAL PROCEDURES

Preparation and Incubation of Cell Suspensions—Suspensions of thymocytes from immature (3 weeks old) 50-65-g male Sprague-Dawley rats were prepared as described previously (7) in RPMI 1640 medium supplemented with 1% (w/v) bovine serum albumin. Thymocytes (50 x 10^6 cells x ml\(^{-1}\) in the RPMI medium described above) were loaded with 10 μM fura-2 tetraacetoxymethyl ester for at least 45 min at 25 °C (16). Cells were then treated with various agents for the time periods indicated. Aliquots of 1 ml were taken, washed free of extra-

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PKC Activation Inhibits DNA Fragmentation

Results and Discussion

It was reported in a recent study (19) that treatment of cortical thymocytes with either phorbol ester or the cation ionophore A23187 resulted in cell death, while incubation of cells with both of these agents did not. Since ionophore treatment has been shown to stimulate endonuclease activity in thymocytes through a sustained increase in cytosolic Ca\(^{2+}\) concentration (6, 9, 11), we tested whether the phorbol esters TPA and PDBu affected the DNA fragmentation and cell killing induced by A23187. The ionophore stimulated concentration-dependent DNA fragmentation in thymocytes (Table I) that appeared to be optimal with 300 nM A23187 (Fig. 1).

**Table I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PDBu +PDBu</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>+ 300 nM A23187</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>+ 500 nM MP</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>+ 10 μg x ml(^{-1}) ConA</td>
<td>90 ± 3</td>
</tr>
</tbody>
</table>

Endonuclease activation was closely associated with loss of cell viability at concentrations of ionophore up to 1 μM (Tables I and II). In contrast, phorbol ester completely blocked both DNA fragmentation (Fig. 1) and cell killing (Table II) in thymocytes treated with 300 nM A23187. At higher concentrations of ionophore the effect was less profound, and DNA fragmentation was not apparently linked to cell death in the presence of the highest concentration of ionophore tested (Table I).

Because glucocorticoid hormones also stimulate thymocyte apoptosis by a mechanism that is dependent on a sustained increase in cytosolic Ca\(^{2+}\) level (10), we tested the effects of phorbol esters on glucocorticoid-treated thymocytes. The glucocorticoid hormone methylprednisolone stimulated concentration-dependent DNA fragmentation and cell death in thymocytes (Table I). With moderate concentrations of glucocorticoid, phorbol ester blocked both glucocorticoid-stimulated endonuclease activation and cell killing (Table I).

Phorbol ester inhibition of glucocorticoid-induced DNA fragmentation (Fig. 1) and cell killing (Table II) were most pronounced in thymocytes incubated with 500 nM methylprednisolone. With higher concentrations of methylprednisolone, DNA fragmentation and cell killing were less sensitive to the effects of phorbol ester (Table I). Phorbol esters did not measurably affect the Ca\(^{2+}\) levels in thymocytes treated with A23187 or methylprednisolone (Table III).

Mitogen-induced T cell proliferation is dependent on sustained increases in the lymphocyte Ca\(^{2+}\) level (13). Mitogens

### Table II

**Effects of phorbol ester or H-7 on Ca\(^{2+}\)-induced thymocyte killing**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PDBu +PDBu</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>+ 300 nM A23187</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>+ 500 nM MP</td>
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Mitogen-induced T cell proliferation is dependent on sustained increases in the lymphocyte Ca\(^{2+}\) level (13). Mitogens
PKC Activation Inhibits DNA Fragmentation

TABLE III

Phorbol esters or H-7 do not affect the cytosolic Ca2+
in thymocytes incubated with or without ionophore
A23187, methylprednisolone, or ConA

<table>
<thead>
<tr>
<th>Cytosolic Ca2+ concentration</th>
<th>Control</th>
<th>A23187</th>
<th>MP</th>
<th>ConA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>80 ± 8</td>
<td>327 ± 21</td>
<td>305 ± 9</td>
<td>302 ± 7</td>
</tr>
<tr>
<td>+ 1 nM TPA</td>
<td>70 ± 12</td>
<td>320 ± 20</td>
<td>286 ± 13</td>
<td>ND</td>
</tr>
<tr>
<td>+ 50 nM PDBu</td>
<td>75 ± 5</td>
<td>330 ± 14</td>
<td>291 ± 15</td>
<td>ND</td>
</tr>
<tr>
<td>+ 50 μM H-7</td>
<td>85 ± 11</td>
<td>ND</td>
<td>ND</td>
<td>324 ± 12</td>
</tr>
</tbody>
</table>

Fig. 2. ConA stimulates a sustained increase in thymocyte cytosolic Ca2+ concentration. Thymocytes (50 x 10^6 cells x ml⁻¹) were loaded with 10 μM fura-2 AM, and Ca2+ changes were measured as described under "Experimental Procedures." Additions: □, control; ▲, 1 μg x ml⁻¹ ConA; △, 10 μg x ml⁻¹ ConA; ♂, 100 μg x ml⁻¹ ConA. Results are of one experiment that was typical of three replicates.

have been shown to induce diacylglycerol accumulation (20) and increases in the cytosolic Ca2+ level (21) in thymocytes. Because we have shown that many agents that induce a sustained rise in the cytosolic Ca2+ level in thymocytes also stimulate DNA fragmentation (7, 10, 11), we tested whether effects of the T cell mitogen ConA on the cytosolic Ca2+ concentration were linked to endonuclease activation and cell death in thymocytes. ConA stimulated sustained increases in the thymocyte Ca2+ level (Fig. 2). However, ConA by itself did not stimulate DNA fragmentation (Fig. 1). Because the diacylglycerol produced in response to ConA stimulation is the physiological agonist for PKC and phorbol esters blocked ionophore- and glucocorticoid-induced DNA fragmentation and cell death in thymocytes, we tested the effects of H-7, a potent PKC antagonist (18), on DNA fragmentation and cell death in thymocytes treated with ConA (Fig. 1). ConA plus H-7 stimulated DNA fragmentation (Fig. 1) and cell death (Table II) in thymocytes, suggesting that the PKC inhibitor blocked a process that normally protected ConA-treated cells from apoptosis. The inhibitor did not significantly affect the intracellular Ca2+ level (Table III), indicating that its effects were not due to potentiation of Ca2+ responses.

Incubation of isolated thymocyte nuclei with millimolar Ca2+ concentrations results in extensive DNA fragmentation (4), and it is known that lymphocyte nuclei contain PKC (22). To determine whether the PKC-dependent inhibition of thymocyte DNA fragmentation was due to direct effects on the nucleus, we measured Ca2+-dependent DNA fragmentation in isolated thymocyte nuclei in the presence or absence of phorbol ester (Fig. 3). Phorbol ester blocked Ca2+-dependent DNA fragmentation in thymocyte nuclei. Thus, direct phorbol ester inhibition of DNA fragmentation in isolated thymocyte nuclei can account for the effects observed in the intact cells. This also supports the conclusion that the inhibition of Ca2+-dependent DNA fragmentation in thymocytes by agents that stimulate PKC is not due to effects on the cytosolic Ca2+ level.

Our results stand in contrast to those published previously (9), where phorbol ester did not appear to inhibit ionophore-stimulated DNA fragmentation in cultured murine thymic lobes. We cannot explain this discrepancy at present. However, the concentration of calcium ionophore used in the previous study was higher than that found to be optimal in the present one, and we found that high concentrations of ionophore reduced the capacity of phorbol ester to inhibit DNA fragmentation (Table I); the differences may therefore be due to the different concentrations of Ca2+ ionophore used. We have found that phorbol esters also prevent DNA fragmentation and cell death in suspensions of human thymocytes, suggesting that PKC inhibition of Ca2+-dependent DNA fragmentation and death in thymocytes is not limited to cells of rat origin.

The thymus is the site in the immune system where deletion of self-reactive lymphocytes occurs before they are allowed to migrate to the periphery. This cell selection process is known as tolerance. Recent work has shown that the subpopulation of cells sensitive to induction of apoptosis (9) is also the direct precursor pool for mature T lymphocytes (23), suggesting that the cell killing process may be involved in tolerance induction. However, certain cells within this subpopulation must be allowed to mature, and the biochemical mechanism that saves

PKC Activation Inhibits DNA Fragmentation

these precursors has not been identified. Mature T cell proliferative responses to Ca²⁺ stimuli may provide a clue to this mechanism. It is well established that stimulation of mature T lymphocytes via the T cell receptor, resulting in a sustained increase in the Ca²⁺ level, does not result in an optimal response without an additional signal generated by lymphokines (24). This requirement can be bypassed with phorbol esters, and it has been shown that interleukin-1 stimulates diacylglycerol production in lymphocytes (25) and that interleukins 2 and 3 induce PKC activation and translocation (26, 27), strongly suggesting that the lymphokine-induced signal involves PKC activation. Lymphokines appear to be rate-limiting for growth in the thymus (28), and we have found interleukin-1 to be as effective as phorbol esters in preventing Ca²⁺-dependent DNA fragmentation and cell killing in suspensions of human thymocyte. It is possible that endonuclease activation occurs in thymocytes as the result of “unbalanced signaling,” where a Ca²⁺ increase occurs with insufficient PKC activation. Lymphokine-mediated PKC activation may therefore be the physiologically relevant biochemical mechanism that spares the appropriate T cell precursors during the thymic cell selection process, although further investigation is required to verify this hypothesis.

REFERENCES


D. J. McConkey, unpublished observation.