Biochemical Pharmacology, Vol. 44, No. 8, pp. 1563–1568, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

MULTIPLE EFFECTS OF STAUROSPORINE, A KINASE INHIBITOR, ON THYMOCYTE FUNCTIONS

COMPARISON WITH THE EFFECT OF TYROSINE KINASE INHIBITORS

YAEL ZILBERMAN* and YEHUDA GUTMAN
Department of Pharmacology, The Hebrew University—Hadassah Medical School, Jerusalem, Israel

(*Received* 3 *March* 1992; *accepted* 6 *July* 1992)

Abstract—The effects of staurosporine, a protein kinase inhibitor, on the signal transduction and proliferation of thymocytes were studied. Signal transduction in response to Concanavalin A (Con A) as well as Concanavalin A (Con A)-induced augmentation of [^{3}H]inositol incorporation into phospholipids were inhibited by staurosporine ($\geq 10^{-8}$ M). Staurosporine inhibited thymocyte proliferation in response to Con A in the presence or absence of the phorbol ester, phorbol myristate acetate (TPA) (10 nM). This inhibition was observed regardless of whether staurosporine was added together with Con A or 3 hr later. High concentrations of staurosporine ($>10^{-8}$ M) inhibited thymocyte proliferation induced by the calcium ionophore A23187 and the phorbol ester TPA, whereas lower concentrations of the inhibitor ($\leq 10^{-8}$ M) enhanced thymidine incorporation in response to these activators. This dual effect of staurosporine was also observed in the presence of the staurosporine-related kinase inhibitor, K252a. In contrast, the tyrosine kinase inhibitor, tyrphostin AG490, inhibited the response to A23187 and TPA at all concentrations of the inhibitor and no augmentation was seen. Interleukin 2 (IL-2)-driven mitogenesis in IL-2-dependent cells was also inhibited by staurosporine. We suggest that the inhibition of thymocyte proliferation by staurosporine results from inhibition of both protein kinase C and tyrosine kinase: the augmentation of the response to A23187 and TPA results from inhibition of protein kinase C. Inhibition of signal transduction as well as inhibition of IL-2-driven mitogenesis result from inhibition of tyrosine kinase.

Activation of thymocytes and T cells by T cell receptor ligands and mitogenic lectins involves activation of phospholipase C, which generates the two intracellular second messengers, inositol triphosphate (IP_3†) and diacyl glycerol (DAG) [1–3]. IP_3 and DAG activate intracellular release of calcium ions and protein kinase C, respectively. The importance of these two second messengers is illustrated by the fact that the combined effect of A23187 and phorbol esters, which mimick the intracellular events resulting from activation of the T cell receptor, lead to mitogenesis [4].

Activation of protein kinase C can have opposing effects on thymocyte responses. On the one hand, phorbol esters augment the responses to Concanavalin A (Con A) or mitogenic antibodies. Phorbol esters also have co-mitogenic effects to that of calcium ionophore. On the other hand, short pretreatment of the cells with phorbol esters inhibits the signal transduction process in many cell systems [5] but not in others [6]. A possible approach to elucidate the role of protein kinase C at the various stages of the mitogenic process may involve pharmacological modulation of the enzyme activity at these points.

Staurosporine, formerly known as a protein kinase C inhibitor, is now known to inhibit a variety of protein kinases *in vitro* [7, 8]. We have used it in conjunction with various activators of thymocytes in order to analyse the role of protein kinases in thymocyte signal transduction and mitogenesis. In addition to protein kinase C, tyrosine kinases have been suggested to be involved in the signal transduction of the T cell receptor [9]. Tyrosine kinases are also associated with the interleukin 2 (IL-2) receptor. In order to resolve the effects of staurosporine on protein kinase C and on tyrosine kinases, we have compared its effects to those of other kinase inhibitors, namely the staurosporine-related kinase inhibitor, K252a, and the tyrosine kinase inhibitor, tyrphostin AG490.

MATERIALS AND METHODS

Materials. Basal medium Eagle diploid (modified) was obtained from Flow Laboratories (Rickmansworth, U.K.). RPMI 1640 medium was obtained from Gibco (Paisley, U.K.). [^{3}H]Inositol and [^{3}H-methyl]thymidine were obtained from Amersham (U.K.); fetal calf serum, glutamine and antibiotics were obtained from Beit Ha'emek (Israel). Earle's basal salt solution, Dowex 1 × 8, Con A, A23187, TPA and PDBu were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Dowex 1 × 8 was converted to the formate form. All the reagents were of analytical grade. Staurosporine and K252a were from Kyowa Hakko Co. (Tokyo Research labs, Japan). Tyrphostin AG490 was a generous gift from Prof. A. Levitzky, Department of Biochemistry,

* Corresponding author.

† Abbreviations: Con A, Concanavalin A; IP_3, inositol trisphosphate; PDBu, phorbol dibutyrate; TPA, phorbol myristate acetate; IL-2, interleukin 2; DAG, diacyl glycerol.

The Hebrew University, Jerusalem. IL-2 was from Cetus Corp. (Emeryville, CA, U.S.A.).

Cell preparation. Cells were prepared from thymuses of 4–6-week-old Balb/C mice. For thymidine incorporation studies, thymocytes were prepared by teasing the thymus into RPMI 1640 medium, supplemented with sodium bicarbonate (24 mM), antibiotics and glutamine (2 mM), and 5% fetal calf serum, followed by washing into the same medium.

Thymidine incorporation. Thymidine incorporation was measured in cells incubated in RPMI medium as described above. The cells were incubated at a cell density of 1–2 × 10^6 cells/mL in 96-well flat bottom plates for 72 hr, during the last 4 hr of which [^{3}H-methyl]thymidine (1 μCi/well) was present. At the end of this incubation, the cells were harvested onto glass fibre filter paper and thymidine incorporation was measured.

IP_3 accumulation experiments. The thymus was teased and washed into basal medium Eagle diploid (modified), supplemented with bicarbonate (24 mM), Tris (20 mM), glutamine and antibiotics at pH 7.3. The cells were incubated in a 5% CO_2, 100% humidity incubator at 37° for 16–18 hr, in the presence of [^{3}H]inositol (20 μCi/mL). After this incubation, the cells were washed into Earle's basal salt solution supplemented with glucose (11 mM, final concentration) and HEPES (10 mM), pH = 7.3, at a final density of 5–10 × 10^6 cells/mL and incubated for 15 min in the presence of 5 mM LiCl. The cells were then stimulated with Con A. The reaction was terminated 20 min later and inositol (poly)phosphates were extracted and separated using Dowex 1 columns (formate form) as described [10]. Results are shown for the fraction eluted between 0.4 and 1.0 M ammonium formate in 0.1 M formic acid, which contains I-1,4,5-P_3, I-1,3,4-P_3 and I-1,3,4,5-P_4.

Incorporation of [^{3}H]inositol into phospholipids. The thymus was teased and washed into basal medium Eagle diploid (modified), supplemented with bicarbonate (24 mM), Tris (20 mM), glutamine and antibiotics at pH 7.3. The cells were incubated in a 5% CO_2, 100% humidity incubator at 37° at a cell density of 5–10 × 10^6 cells/mL. The cells were incubated for 2 hr in the presence of [^{3}H]inositol (20 μCi/mL), and in the presence or absence of activators and inhibitors. The incubation was terminated and phospholipids extracted using a modification of the method of Bligh and Dyer [11]. Briefly, 3.8 volumes of $CHCl_3$:Methanol:HCl (1:2:0.05 v/v) were added to the incubation mixture to obtain one phase. Phases were separated by the addition of $CHCl_3$ and H_2O. The organic phase was washed twice with KCl (1 M):methanol:HCl 1:1:0.05 (v/v), dried and radioactivity counted after addition of scintillation fluid.

IL-2-dependent mitogenesis. Thymocytes were cultured in tissue culture flasks at a cell density of 1–2 × 10^6 cells/mL. The cells were stimulated with Con A (2 μg/mL) and rIL-2 (50 U/mL). Four days later, the cells were divided and restimulated with rIL-2 (50 U/mL). After another 4 days, the cells were washed thoroughly and cultured at a cell density of 1 × 10^6 cells/mL, in 96-well microplates. The cells were stimulated with various concentrations of rIL-2, and [^{3}H]thymidine incorporation was determined 48 hr later.

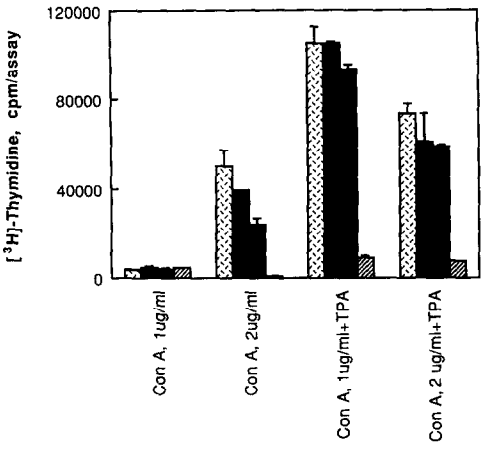

Fig. 1. The effect of staurosporine on [^{3}H]thymidine incorporation in response to Con A, or Con A and TPA. (▨) control. Staurosporine: (■) 10^{-11} M; (▩) 10^{-10} M; (▨) 10^{-9} M.

Each experiment was performed 2–5 times and each measurement done in triplicate. Due to variations in the response to the mitogens between experiments, each figure contains results from one representative experiment. Results are shown as means ± SE.

RESULTS

The effect of staurosporine on the mitogenic effect of Con A in thymocytes is shown in Fig. 1. TPA increased the response to Con A and also lowered its optimal concentration so that in the presence of TPA, 2 μg/mL caused a smaller response than 1 μg/mL of the lectin. This shift in the dose–response curve has been noted before [2]. Staurosporine inhibited the response to Con A at concentrations of 10^{-11} M or more. The response of the cells to treatment with Con A and TPA was also inhibited by staurosporine at the same concentration range. In order to find out which step is sensitive to staurosporine, we also added staurosporine 3 hr after the initiation of proliferation by Con A, or Con A and TPA (results not shown). The same pattern of responses could be observed: staurosporine still inhibited the proliferative responses to both Con A, and Con A and TPA, at the same concentration range of inhibitor. This suggests that one of the steps of the thymocyte response which is sensitive to staurosporine occurs later than the early steps of activation. To study this possibility, two approaches were taken. The first was to study the effects of staurosporine on A23187 and phorbol ester-induced mitogenesis, and the second was to study the effects of staurosporine on IL-2-dependent mitogenesis in cells rendered sensitive to this lymphokine.

Thymocytes can be activated by the combination of the calcium ionophore A23187 and phorbol esters

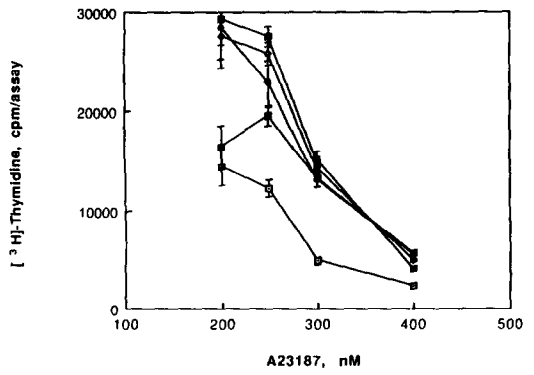

Fig. 2. The effect of staurosporine on the mitogenic responses to A23187 and TPA. Cells were incubated in the presence of TPA, 10 nM, and various concentrations of A23187. Staurosporine concentrations: (□) none; (◆) 10^{-11} M; (■) 10^{-10} M; (◇) 10^{-9} M; (■) 10^{-8} M. The experiment was done in triplicate.

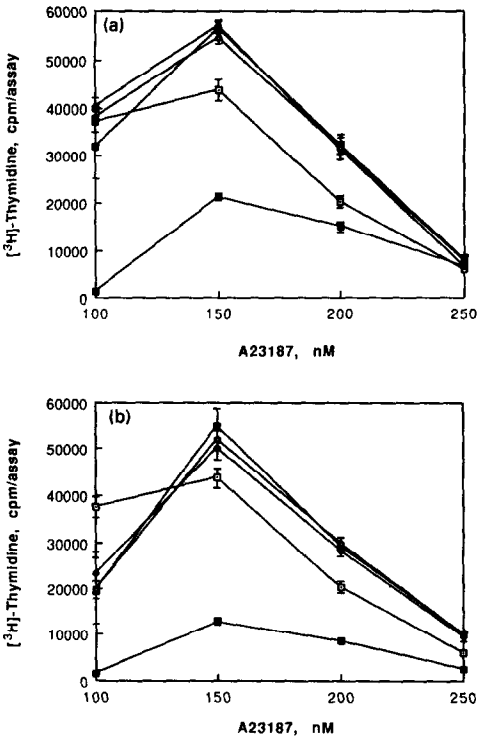

Fig. 3. Comparison of the effects of protein kinase inhibitors on the mitogenic responses of thymocytes to A23187 and PDBu. Thymocytes were incubated in the presence of PDBu, 10 nM, and various concentrations of A23187. (a) Inhibition of the response by staurosporine. Staurosporine concentrations: (□) none; (◆) 10^{-11} M; (■) 10^{-10} M; (◇) 10^{-9} M; (■) 10^{-8} M. (b) Inhibition by K252a. K252a concentrations: (□) none; (◆) 10^{-10} M; (■) 10^{-9} M; (◇) 10^{-8} M; (■) 10^{-7} M. (a) and (b) are parts of the same experiment and each measurement was done in triplicate.

such as TPA or PDBu, which bypass the signal transduction step. The mitogenic response to this mode of activation is biphasic with respect to A23187 at a very narrow concentration range, and is evident at phorbol ester concentrations of 1 nM and above. The optimal A23187 concentration varies somewhat from one experiment to another. We have studied the effects of staurosporine on the mitogenic responses of thymocytes stimulated with A23187 and TPA. In Fig. 2, only the optimal to supra-optimal concentration range of A23187 was tested. Staurosporine at concentrations of 10^{-11}–10^{-10} M augmented the response to A23187 in the presence of TPA by up to 220%. To assess whether this augmentation was manifested at supra-optimal concentrations of A23187 only, we tested the effect of staurosporine over a wider concentration range of A23187, in the presence of PDBu (which acts in a similar manner to TPA). These results are shown in Fig. 3a. We found that staurosporine was inhibitory at concentrations of 10^{-8} M and above. At concentrations of 10^{-9} M and lower, staurosporine had a biphasic effect: it inhibited thymidine incorporation in response to sub-optimal concentrations of A23187 and augmented the incorporation of thymidine at supra-optimal concentrations of A23187. In order to see if the facilitatory effect of staurosporine on A23187 and phorbol esters was shared by other kinase inhibitors, we compared the effects of the staurosporine-related kinase inhibitor, K252a, to those of staurosporine. Indeed, both staurosporine and K252a augmented the response of the cells to supra-optimal concentrations of A23187 and the phorbol ester PDBu (Fig. 3a, b). K252a, however, was slightly less potent than staurosporine, and inhibition of the response could only be seen at 10^{-7} M and above, whereas in the same experiment staurosporine was inhibitory at 10^{-8} M. Thus both staurosporine and K252a rendered the cells less sensitive to supra-optimal concentrations of intracellular calcium. The effect of staurosporine on Con A-induced proliferation differed from its effect on A23187- and phorbol ester-induced mitogenesis in that the responses to Con A, or Con A and phorbol ester were not augmented by staurosporine at any concentration of the inhibitor.

Staurosporine and K252a inhibit both protein kinase C and tyrosine kinases. In order to be able to ascribe the facilitatory effects of these inhibitors on A23187 and phorbol ester-induced thymocytes to inhibition of either protein kinase, we have studied the effects of specific tyrosine kinase inhibitors, the tyrphostins, on the response to A23187 and PDBu. The results of such an experiment are shown in Fig. 4. The tyrosine kinase inhibitor AG490 inhibited the response to A23187 and phorbol ester over the entire concentration range of A23187 and no facilitatory phase was observed. The same pattern of responses was observed with other tyrphostins as well, albeit at different concentration ranges (results not shown). Thus, it is clear that the response to mitogenic lectins on the one hand and to A23187 and phorbol esters, on the other, are dissimilar with respect to their sensitivity to staurosporine.

One of the differences between the mitogenic

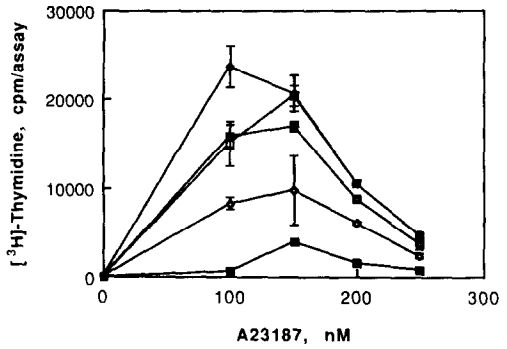

Fig. 4. The effect of tyrphostin AG490 on the mitogenic response of thymocytes stimulated with A23187 and TPA. Cells were stimulated with TPA, 10 nM, and various concentrations of A23187 in the presence of various concentrations of AG490. AG490 concentrations: (⊡) none; (◆) 2.5 μM; (■) 5 μM; (◇) 10 μM; (■) 20 μM.

response induced by Con A and that induced by A23187 and TPA is that the former is mediated by activation of phospholipase C, whereas the latter mode of activation bypasses this step. We therefore studied the effect of staurosporine on the signal transduction of thymocytes in response to Con A. The effect of staurosporine on the accumulation of inositol (poly)phosphates in response to Con A is shown in Fig. 5a. Staurosporine concentrations above 10^{-8} M totally inhibited the accumulation of inositol (poly)phosphates. Some enhancement could sometimes be seen between 10^{-11} and 10^{-8} M. In another set of experiments, we studied the effect of staurosporine on the incorporation of [^{3}H]inositol into phospholipids. Freshly prepared thymocytes incorporate [^{3}H]inositol into phospholipids at a constant rate for more than 2 hr. Con A, as well as other T cell mitogens such as anti-CD3 and anti-Thy 1.2 antibodies, augment this incorporation (unpublished results; and R. Guy, E. Yefenof and Y. Zilberman, submitted for publication). Staurosporine inhibited the Con A-induced augmentation of the incorporation at staurosporine concentrations above 10^{-9} M (Fig. 5b).

A second difference between lectin-induced mitogenesis and A23187- and phorbol ester-induced mitogenesis is the former's dependence upon lymphokine action. The early steps of activation in response to T cell receptor cross-linking or mitogenic lectins are followed by IL-2 secretion and expression of high affinity IL-2 receptors. The subsequent proliferative response is strongly dependent upon interaction of the lymphokine with its receptors. In contrast, the response to A23187 and TPA is at least partially independent of lymphokine action [11, 22]. We have therefore assessed the effect of staurosporine on the response of thymocytes to IL-2. For this purpose, thymocytes were grown in the presence of Con A (2 μg/mL) and IL-2 (50 U/mL) for 1 week to 10 days, washed thoroughly and stimulated with various concentrations of IL-2 (Fig. 6). We found that staurosporine inhibited the response of the cells

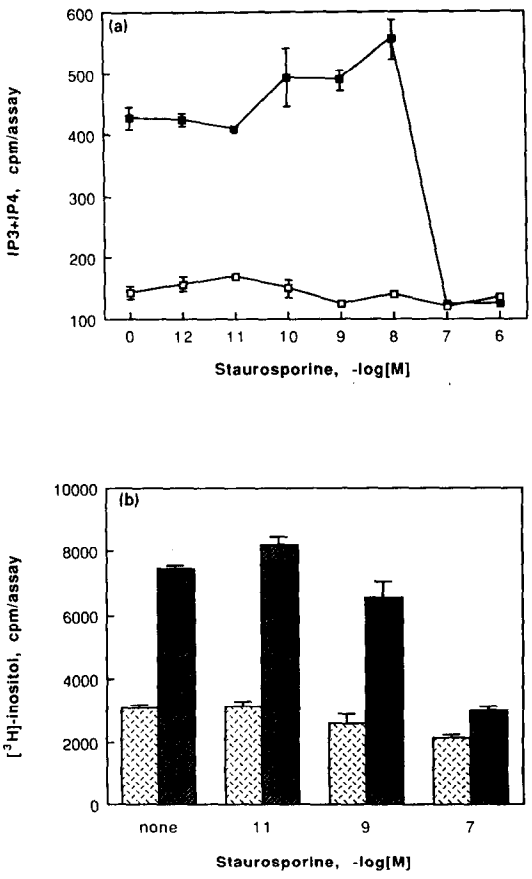

Fig. 5. The effect of staurosporine on phosphoinositide metabolism. (a) accumulation of IP_3 and IP_4 in Con A-stimulated cells. Cells were incubated in the presence (■) or absence (□) of Con A (1 μg/mL) and various concentrations of staurosporine. (b) The effect of staurosporine on the incorporation of inositol into phospholipids. Cells were incubated in the presence (▨) or absence (▨) of Con A, 1 μg/mL, and various concentrations of staurosporine.

to IL-2, at concentrations of 10^{-8}–10^{-7} M. Lower concentrations of staurosporine did not markedly augment the responses of the cells to IL-2 at any concentration. The same degree of inhibition could be seen in cells stimulated with IL-2 concentrations of between 6.25 and 50 U/mL.

DISCUSSION

The mitogenic response of thymocytes consists of the signal transduction step, which results in increased intracellular calcium concentration and activation of protein kinase C, followed by an increase in IL-2 receptor expression and secretion of IL-2. Interaction of IL-2 and its receptor results in mitogenesis. Protein kinase C has been shown to modulate CD4 and CD8, causing their dissociation from the tyrosine kinase p56^{lck} [12], and to down-regulate CD3 [13]. It can thus affect signal transduction both directly and indirectly.

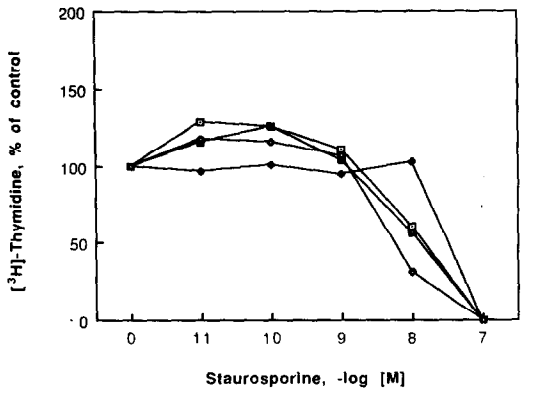

Fig. 6. The effect of staurosporine on IL-2 driven mitogenesis. IL-2 dependent cells were prepared as described in Materials and Methods. The cells were stimulated with various concentrations of IL-2 in the presence of staurosporine. IL-2 concentrations: (□) 6.25 U/mL; (◆) 12.5 U/mL; (■) 25 U/mL; (◇) 50 U/mL. The degrees of stimulation in the presence of IL-2 only were at: 6.5 U/mL, 7950 ± 1824; 12.5 U/mL, 19324 ± 2571; 25 U/mL, 66066 ± 7243; 50 U/mL, 130323 ± 3815 cpm/assay.

Staurosporine is a protein kinase inhibitor, which has higher affinities towards protein kinase C and tyrosine kinases [8]. It has been shown to inhibit the calcium signal in T lymphoblasts in response to mitogenic lectins and the anti-CD3 antibody UCHT1 [14], an effect attributed to inhibition of protein kinase C. The present study demonstrates that staurosporine inhibits both the signal transduction step as well as events further downstream along the mitogenic pathway. Staurosporine inhibited mitogenic responses of thymocytes stimulated by various modes, namely, mitogenic lectins, A23187 and phorbol esters, and IL-2 in IL-2-dependent cells. The effects of staurosporine could not be attributed to inhibition of protein kinase C only. Some of the results presented can be attributed to inhibition of tyrosine kinase.

Inhibition by staurosporine of the mitogenic responses to Con A in the presence or absence of TPA can be explained as resulting from inhibition of either the signal transduction step, later events, or both. Staurosporine inhibits the signal transduction step itself, as evident from the reduced accumulation of inositol phosphates in response to Con A. The augmented incorporation of [^{3}H]inositol into phospholipids, induced by Con A, was also inhibited by staurosporine. These effects cannot be attributed to inhibition of protein kinase C, since in thymocytes activation of protein kinase C has either no effect or a small facilitatory effect on signal transduction (Refs 6, 14, and unpublished results). In this respect, thymocytes differ from other T cells such as LBRM-331A5 [5] or Jurkat [6], in which pretreatment with TPA or indeed DAG generated during the signal transduction step has an inhibitory effect on the signal transduction [5]. Negative modulation of phospholipase C by protein kinase C was suggested to prevail in those cells expressing phospholipase C type β, whereas phospholipase C type γ was thought to be insensitive to modulation by protein kinase C [15]. Whether this underlies the difference between T lymphoblasts and thymocytes, on the one hand, and Jurkat and LBRM-N331A5, on the other, is not known. Inhibition of the Con A-induced accumulation of inositol polyphosphates by staurosporine, however, could result from inhibition of tyrosine kinase. Indeed, by using specific inhibitors it was demonstrated that activation of tyrosine kinase was obligatory for the activation of phospholipase C in T cells (Refs 16, 17, and data not shown).

The experiment described in Fig. 1 demonstrates that staurosporine inhibits the mitogenic response when applied to cells together with Con A, which is compatible with inhibition of the signal transduction; similar results were obtained when the inhibitor was added 3 hr later, when the early events of activation are over. These findings suggest that there are at least two staurosporine-sensitive steps in thymocyte mitogenesis. In order to confirm this hypothesis, we tested the effects of staurosporine on the mitogenic response to A23187 and phorbol ester, which bypass the signal transduction step and elicit the same intracellular responses as Con A, leading to cellular proliferation. We also tested the effects of staurosporine on IL-2-dependent mitogenesis. We found that both A23187 and phorbol ester as well as IL-2-dependent mitogenesis were inhibited by staurosporine, although somewhat higher concentrations of the inhibitor were required for inhibition of these pathways than for Con A- and TPA-induced mitogenesis.

The response to A23187 and TPA is biphasic with respect to A23187 concentrations. This is indicative of two opposing effects of A23187, one of which is stimulatory, while the other, at higher A23187 concentrations, probably reflects toxic effects of high intracellular calcium. Both calcium ions and protein kinase C have been shown to activate programmed cell death (apoptosis), especially in immature thymocytes [18, 19], and it is quite possible that inhibition of mitogenesis by high concentrations of A23187 reflects this process. Staurosporine, at rather high concentrations, inhibited the mitogenic response to A23187 and TPA, while lower concentrations of staurosporine suppressed the toxic effects of higher concentrations of A23187. The latter was manifested as a stimulatory effect of staurosporine. This dual effect was shared by the staurosporine-related inhibitor, K252a, but not by tyrphostin AG490, which had only inhibitory effects. This suggests that inhibition of protein kinase C, rather than tyrosine kinase, underlies the suppression of the toxic effects of supra-optimal concentrations of A23187 and may reflect protection of the cells against apoptosis.

IL-2-dependent mitogenesis is another staurosporine-sensitive step, which may underlie the inhibition of Con A-induced mitogenesis. Tyrosine kinase was suggested to be involved in IL-2 dependent mitogenesis [20], while protein kinase C activation was not obligatory for this response [21]. We therefore suggest that the effect of staurosporine in this process stems from inhibition of IL-2 receptor-coupled tyrosine kinase. Indeed, we found that

tyrphostins inhibit IL-2 dependent mitogenesis (data not shown). The higher dependence of Con A-induced mitogenesis on lymphokine action, as compared to A23187- and phorbol ester-induced mitogenesis, is manifested by the fact that the former is fully inhibited by anti-IL-2 or anti-IL-2 receptor antibodies, whereas the latter is only partially inhibited under the same conditions [22]. This relative independence may allow the protective effect of staurosporine against high concentrations of A23187 in the presence of TPA to be manifested, resulting in an apparent augmentation of mitogenesis.

The results presented here demonstrate that staurosporine has a complex effect on thymocyte mitogenesis. Some of its effects can be attributed to inhibition of tyrosine kinase—notably the inhibition of signal transduction. While this in itself is sufficient to account for the inhibition of mitogenesis in response to lectins, it is not the only step at which staurosporine inhibits mitogenesis. Another staurosporine-sensitive step is the response to lymphokines, which probably stems from inhibition of tyrosine kinase. Inhibition of protein kinase C or of tyrosine kinase may mediate the inhibitory effect of high concentrations of staurosporine. The fact that A23187- and TPA-induced mitogenesis is relatively lymphokine-independent reveals another staurosporine-sensitive step, namely, the protection of the cells against the effects of high intracellular concentrations of calcium.

Acknowledgements—This work was supported by grants from the Leukemia Research Foundation, Illinois, and from the Israel Ministry of Health.

REFERENCES

1. Weiss A, Imboden J, Hardy K, Manger B, Terhorst C and Stobo J, The role of the T3/antigen receptor complex in T cell activation. *Annu Rev Immunol* **4**: 593–619, 1986.
2. Taylor MV, Metcalfe JC, Hesketh TR, Smith GA and Moore JP, Mitogens increase phosphorylation of phosphoinositides in thymocytes. *Nature* **312**: 462–465, 1984.
3. Bonvini E, DeBell KE, Kolber MA, Hoffman T, Hodes RJ and Taplits MS, Hydrolysis of inositol phospholipids induced by stimulation of the T cell antigen receptor complex in antigen-specific, murine helper T cell clones: requirement for exogenous calcium. *J Immunol* **143**: 587–595, 1989.
4. Truneh A, Albert F, Golstein P and Schmitt-Verhulst A, Early steps of activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature* **313**: 318–320, 1985.
5. Mills GB, May C, Hill M, Ebanks R, Raifman C, Mellors A and Gelfand EW, Physiologic activation of protein kinase C limits IL-2 secretion. *J Immunol* **142**: 1995–2003, 1989.
6. Ward SG and Cantrell DA, Heterogeneity of the regulation of phospholipase C by phorbol esters in T-lymphocytes. *J Immunol* **144**: 3523–3528, 1990.
7. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M and Tomita F, Staurosporine, a potent inhibitor of phospholipids/Ca^{2+} dependent protein kinase. *Biochem Biophys Res Commun* **135**: 397–402, 1986.
8. Fallon RJ, Staurosporine inhibits tyrosine kinase in human hepatoma cell membranes. *Biochem Biophys Res Commun* **170**: 1191–1196, 1990.
9. Samelson LE, Patel MD, Weissman AM, Harford JB and Klausner RD, Antigen activation of murine T cells induces tyrosine phosphorylation of a peptide associated with the T cell antigen receptor. *Cell* **46**: 1083–1090, 1986.
10. Berridge MJ, Dawson RMC, Downes CP, Heslop JP and Irvine RF, Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212**: 473–482, 1983.
11. Koretzky GA, Daniele RP, Greene WC and Nowell PC, Evidence for an interleukin-independent pathway for human lymphocyte activation. *Proc Natl Acad Sci USA* **80**: 3444–3447, 1983.
12. Hurley TR, Luo K and Sefton BM, Activators of protein kinase C induce dissociation of CD4, but not CD8, from $p56^{lck}$. *Science* **245**: 407–409, 1989.
13. Cantrell D, Davies AA and Crumpton MJ, Activators of protein kinase C down regulate and phosphorylate the T3/T-cell antigen receptor complex of human T-lymphocytes. *Proc Natl Acad Sci USA* **82**: 8158–8162, 1985.
14. Ward SG, Cantrell D and Westwick J, Inhibition by staurosporine of mitogen-induced calcium mobilisation in human T lymphoblasts. *FEBS Lett* **239**: 363–366, 1988.
15. Rhee SG, Kim H, Suh P-G and Choi WC, Multiple forms of phosphoinositide-specific phospholipase C and different modes of activation. *Biochem Soc Trans* **19**: 337–341, 1991.
16. Mustelin T, Coggeshall KM, Isakov N and Altman A, Tyrosine phosphorylation is required for T-cell antigen receptor-mediated activation of phospholipase C. *Science* **247**: 1584–1587, 1990.
17. June CH, Fletcher MC, Ledbetter JA, Schieven GL, Siegel JN, Phillips AF and Samelson LE, Inhibition of tyrosine phosphorylation prevents T-cell receptor mediated signal transduction. *Proc Natl Acad Sci USA* **87**: 7722–7726, 1990.
18. Kizaki H, Tadakuma T, Odaka C, Muramatsu J and Ishimura Y, Activation of a suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esters. *J Immunol* **143**: 1790–1794, 1989.
19. Tadakuma T, Kizaki H, Okada C, Kubota R, Ishimura Y, Yagita H and Okumura K, CD4+CD8+ thymocytes are susceptible to DNA fragmentation induced by phorbol ester, calcium ionophore and anti CD3 antibody. *Eur J Immunol* **20**: 779–784, 1990.
20. Horak ID, Gress RE, Lucas PJ, Horak EM, Waldmann TA and Bolen JB, T-lymphocyte interleukin 2-dependent tyrosine kinase signal transduction involves the activation of $p56^{lck}$. *Proc Natl Acad Sci USA* **88**: 1996–2000, 1991.
21. Valge VE, Wong JGP, Datlof BMA, Sinskey J and Rao A, Protein kinase C is required for responses to T-cell receptor ligands but not to interleukin 2 in T-cells. *Cell* **55**: 101–112, 1988.
22. Zilberman Y, Ravid A, Liberman UA, Novogrodsky A and Koren R, Stimulatory and inhibitory effects of 1,25-dihydroxyvitamin D_3 on thymocyte mitogenesis induced by phorbol ester and calcium ionophore. *Biochim Biophys Acta* **1134**: 297–302, 1992.