Staurosporine, but not Ro 31-8220, induces interleukin 2 production and synergizes with interleukin 1α in EL4 thymoma cells

Activation of nuclear factor κB as a common signal for staurosporine and interleukin 1α

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Protein kinase C (PKC) has been implicated in interleukin 1 (IL1) signal transduction in a number of cellular systems, either as a key event in IL1 action or as a negative regulator. Here we have examined the effects of two PKC inhibitors, staurosporine and the more selective agent Ro 31-8220, on IL1 responses in the murine thymoma line EL4.NOB-1. A 1 h pulse of staurosporine was found to strongly potentiate the induction of IL2 by IL1α in these cells. In contrast, neither a pulse nor prolonged incubation with Ro 31-8220 affected the response to IL1α. Both agents blocked the response to PMA, however. A 1 h pulse of staurosporine was also found to induce IL2 production on its own, activate the transcription factor nuclear factor κB (NFκB) and increase the expression of a NFκB-linked reporter gene. It synergized with IL1α in all of these responses. Ro 31-8220 was again without effect, although both staurosporine and Ro 31-8220 blocked the activation of NFκB by PMA. Finally, staurosporine caused the translocation of PKC-α and -ϵ, and to a lesser extent PKC-β, but not PKC-θ or -ζ, from the cytosol to the membrane, although a similar effect was observed with Ro 31-8220. The results suggest that PKC is not involved in IL1α signalling in EL4 cells. Furthermore, the potentiating effect of staurosporine on IL1α action does not involve PKC inhibition, and is likely to be at the level of NFκB activation.

INTRODUCTION

The cytokine interleukin 1 (IL1) is a potent pro-inflammatory mediator, activating a large number of cell types with roles in both immunity and inflammation. In spite of much effort, a clear signal transduction pathway for IL1 has yet to emerge (reviewed in [1,2]). A number of post-receptor signals have been described, including G-protein activation, increases in such second messengers as cAMP, diacylglycerol and ceramide, activation of the transcription factor nuclear factor κB (NFκB), and increased protein phosphorylation (reviewed in [1,2]).

Much work has gone into identifying protein kinases activated by IL1. An IL1 type 1 receptor-associated protein kinase has been described [3,4], although direct coupling to the receptor has not been demonstrated. In addition, activation of several members of the mitogen-activated protein kinase (MAP kinase) family, including p42/p44 MAP kinase [5], p38 MAP kinase [6] and p54 MAP kinase [7], has been demonstrated. The potential involvement of protein kinase C (PKC) in IL1 action has also been indicated in a number of studies. A role for PKC was originally suggested because of the ability of IL1 to substitute for PMA in co-stimulation studies in T cells involving antibodies to the T cell receptor or sub-optimal concentrations of mitogenic lectins [8,9]. Studies in the T lymphoma line Jurkat demonstrated increases in the PKC activator diacylglycerol which were due to activation of phosphatidylcholine-specific phospholipase C [10]. Increases in diacylglycerol were also reported in EL4 thymoma cells [11,12], macrophages [13], mesangial cells [14] and mouse pancreatic β cells [15]. In one study [12] the increase in diacylglycerol in EL4 cells was shown to be due to activation of lysophosphatidyl acyltransferase and phosphatidic acid hydrolase. Others have failed to show such changes, however [16]. Direct activation of PKC has also been reported in T helper cells [17], pre-B cells [18], 3T3 fibroblasts [19], astrocytes [20] and pancreatic β cells [15], and in EL4 cells activation of PKC and translocation of PKC-β have been demonstrated [21].

A number of studies have also utilized PKC inhibitors such as the fungal alkaloid staurosporine. This has been shown to inhibit some responses to IL1, such as the induction of matrix metalloproteinases in fibroblasts [22], of vascular cell adhesion molecule-1 in endothelial cells [23] and of tumour necrosis factor (TNF) in astrocytes [20]. However, staurosporine has also been shown either to have no effect on IL1 action [24–26] or alternatively to potentiate IL1 responses such as the induction of IL6 in astrocytes [27], prostaglandin E_2 production in synovial fibroblasts [28] and IL2 and IL4 production in EL4 thymoma cells [29]. In spite of the lack of specificity of staurosporine with regard to PKC inhibition, these studies have concluded that PKC may exert a tonic inhibitory effect on IL1 action, although the target for PKC was not identified. In the present study we have explored further the effects of staurosporine on IL1 signalling in EL4 cells. Using the more selective PKC inhibitor Ro 31-8220, our results suggest that the dramatic potentiating effects of staurosporine on IL1 action are not due to effects on PKC. Furthermore, we have found that a 1 h pulse of staurosporine is capable of inducing IL2 in these cells, and that its potentiating effect on IL1 may be through activation of the transcription factor NFκB, which is a critical response for IL2 induction in these cells. The results question previous conclusions in relation to the inhibitory effect of PKC on IL1 action, and also support work on other cell types demonstrating activatory effects of staurosporine independent of its ability to inhibit PKC.

Abbreviations used: CAT, chloramphenicol acetyltransferase; FCS, foetal calf serum; IL1 (etc.), interleukin 1 (etc.); MAP kinase, mitogen-activated protein kinase; NFκB, nuclear factor κB; PKC, protein kinase C; TNF, tumour necrosis factor.

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EXPERIMENTAL

Materials

The murine thymoma cell line EL4.NOB-1 was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts., U.K.). RPMI 1640 cell culture medium, penicillin/streptomycin solution, L-glutamine and foetal calf serum (FCS) were purchased from Gibco BRL Life Technologies. All other tissue culture materials were obtained from Greiner G.m.b.H. (Frickenhausen, Germany). Recombinant human IL1α was given by Professor J. Saklatvala (Kennedy Institute of Rheumatology, London, U.K.). Monoclonal antibodies against PKC isoenzymes were purchased from the following companies: anti-PKC-α and -β, Seigagaku Corp. (Rockville, MD, U.S.A); anti-PKC-δ, -ϵ and -θ, Transduction Laboratories; anti-PKC-ζ, Santa Cruz Biotechnology Inc. Ro 31-8220 was from Calbiochem (Nottingham, U.K.). Poly(dI-dC) was from Pharmacia Biosystems Ltd. (Milton Keynes, U.K.). T4 polynucleotide kinase and the human recombinant oligonucleotide containing the NFκB consensus sequence (underlined) (5′-AGTTGAG-GGGACTTTCCCAGGC-3′) were purchased from Promega. [γ-^{32}P]ATP (3000 Ci/mmol) and D-THREO [*dichloroacetyl*-1-^{14}C]chloramphenicol (56 mCi/mmol) were purchased from Amersham International (Aylesbury, Bucks., U.K.). The pCAT® Promoter plasmid, in which five NFκB sequence motifs had been cloned into the promoter region of the chloramphenicol acetyltransferase (CAT) gene, was a gift from Dr. Timothy Bird (Immunex Research and Development Corp., Seattle, WA, U.S.A.). TLC-Plastic sheets of silica gel 60 (without fluorescent indicator; 0.2 mm) were purchased from Merck.

Purified anti-(murine IL2), recombinant murine IL2, and biotinylated anti-(murine IL2) were supplied by Pharmingen (San Diego, CA, U.S.A.). Polyacrylamide solutions, Protogel® and Acugel® (29:1, w/v) were supplied by National Diagnostics. All other reagents were of molecular biology or AnalaR grade and were supplied by Sigma, Aldrich or BDH.

Cell culture

Murine EL4.NOB-1 thymoma cells were cultured in RPMI 1640. All medium was supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated FCS. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The passage number of the cells used varied between 10 and 30. The earlier passages produced more IL2 in response to PMA.

IL2 assay

EL4.NOB-1 cells (1×10^6/ml) were seeded in 24-well plates in RPMI 1640 medium containing 10% (v/v) FCS. Following a preincubation for 15 min at 37 °C, cells were treated with various concentrations of staurosporine or Ro 31-5880 for 1 h. Cells were then stimulated with either IL1α (5 ng/ml) or PMA (1 ng/ml) for 24 h at 37 °C. The samples were then harvested by centrifugation at 170 ***g*** for 10 min and the supernatants collected. The supernatants were used directly [or appropriately diluted with PBS/10% (v/v) FCS] in the IL2 ELISA as described previously [30]. In some experiments, cells were washed free of drug prior to addition of IL1α or PMA.

Preparation of subcellular fractions

Subcellular fractions were prepared as described previously [30]. Briefly, EL4.NOB-1 or Jurkat cells [$(1–5) \times 10^6$/ml] were plated into 24-well plates (16 mm diameter) 20 min before stimulation. Cells were activated with IL1α (10 ng/ml) or PMA (100 ng/ml) for different time periods. In some experiments, cells were pretreated with inhibitor for 1 h; the inhibitor was then either left on the cells or washed free before IL1α or PMA treatment. A time course for staurosporine was also performed, with cells either being exposed to the drug for various times up to 4 h, or alternatively being washed free of drug after these times and incubated for a further 4 h in media alone. All treatments were terminated by the addition of 5 vol. of ice-cold PBS, and nuclear and cytosolic extracts were prepared as described [30].

Electrophoretic mobility shift assay

Nuclear extracts were analysed for DNA-binding activity by an electrophoretic mobility shift assay as described [30]. Briefly, nuclear extracts (4 μg of protein, as determined by the method of Bradford [31]) were incubated with 10000 c.p.m. of a 22 bp oligonucleotide containing the NFκB consensus sequence for 30 min at room temperature in the presence of 2 μg of poly(dI-dC). Samples were then electrophoresed in a native 5% (w/v) polyacrylamide gel. Gels were subsequently dried and autoradiographed.

EL4.NOB-1 transfection and assay of CAT activity

Cells were harvested (in exponential phase) and washed twice in 10 ml of PBS. Cells (1.4×10^7) were then incubated for 30 min at room temperature with 10 μg of plasmid in 600 μl of TBS buffer as described [32]. The cells were then washed twice in RPMI 1640 medium and resuspended in 40 ml of RPMI/10% (v/v) FCS medium overnight. Cells were harvested, seeded at 1×10^6/ml and incubated with medium alone, staurosporine or Ro 31-8220 for 1 h. Following this, cells were washed in the case of staurosporine, or left untreated in the case of Ro 31-8220, and stimulated with IL1α (10 ng/ml) or PMA (100 ng/ml) for 22 h. CAT activity was measured in cell lysates using 40 μg of protein, as described [32]. Results are expressed as fold stimulation over control levels of acetylation.

Western blot analysis of PKC isoenzymes

Cytosolic and solubilized particulate fractions were prepared as described previously [33] from cells (1×10^7/ml; 1 ml per sample) treated with staurosporine, Ro 31-8220 or medium control for 1 h. A standard Western blotting procedure for PKC isoenzymes was performed on these fractions as described previously [30]. The PKC antisera dilutions were: anti-PKC-α, 1 μg/ml; anti-PKC-β, 1 μg/ml; anti-PKC-δ, 1 μg/ml; anti-PKC-ϵ, 1:200 (v/v); anti-PKC-θ, 1:250 (v/v); anti-PKC-ζ, 1 μg/ml. The blots were developed using ECL Western blotting detection reagents according to the manufacturer's recommendations (Amersham International).

RESULTS

Staurosporine, but not Ro 31-8220, induces IL2 and potentiates induction of IL2 by IL1α

As shown in Figure 1(A), a 1 h pulse with various concentrations of staurosporine followed by washing inhibited the induction of IL2 by PMA in EL4 cells, with 50 nM staurosporine decreasing IL2 levels from 50 ng/ml to 9 ng/ml. The opposite was observed using IL1α as a stimulus, however. A strong potentiation of IL2 production occurred, with 50 nM staurosporine increasing IL2 production in cells stimulated with 5 ng/ml IL1α by 29-fold. A 1 h pulse with staurosporine on its

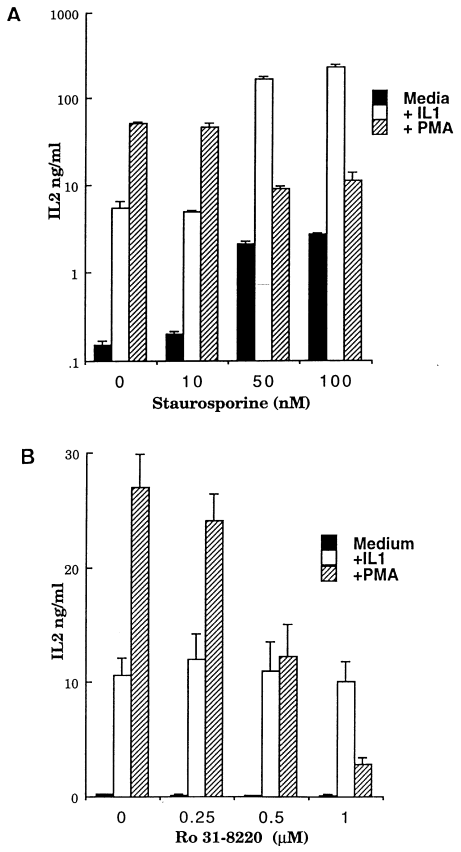

Figure 1 Effects of staurosporine and Ro 31-8220 on IL1- and PMA-stimulated IL2 production in EL4.NOB-1 cells

(**A**) EL4.NOB-1 cells (1×10^6/ml) were pretreated with the indicated concentrations of staurosporine for 1 h at 37 °C. The cells were then washed, resuspended in culture medium and stimulated with either IL1 (5 ng/ml) or PMA (1 ng/ml) for a further 24 h. The same protocol was used for Ro 31-8220 (**B**), except that this compound was not washed from the cells prior to the addition of IL1 or PMA. Supernatants were removed after 24 h and assayed for IL2 by ELISA. Data shown represent means ± S.E.M. for three separate experiments, each carried out in triplicate. The three experiments with staurosporine (**A**) were carried out with earlier cell passages, which produced more IL2 in response to PMA.

own was found to increase IL2 production over 24 h, in a concentration-dependent fashion, such that, at 50 nM staurosporine, IL2 levels reached 2.14 ng/ml from control levels of 0.15 ng/ml. As this concentration was optimal, and as a concentration of 5 ng/ml IL1α was also optimal (results not shown), a strong synergy was therefore occurring. It was necessary to pulse the cells with staurosporine in these experiments, as leaving the cells in contact with the concentrations of the drug used here was cytotoxic, as judged by Trypan Blue exclusion (results not shown). This cytotoxic effect has also been shown by others [34,35].

We next tried the more selective PKC inhibitor Ro 31-8220 (Figure 1B). Like staurosporine, Ro 31-8220 inhibited the induction of IL2 by PMA, with a concentration of 1 μM blocking the response to PMA by 90%. In contrast, however, the inhibitory effect required that Ro 31-8220 be left in contact with the cells, a 1 h pulse having no effect (results not shown). Ro 31-8220 was not toxic under these conditions, as judged by Trypan Blue exclusion (results not shown). Unlike staurosporine, Ro 31-8220, even when in contact with the cells over the whole course of the experiment, did not induce IL2 production at concentrations up to 1 μM, nor was it able to potentiate IL2 induction by IL1α (Figure 1B).

The experiments shown for staurosporine in Figure 1(A) were carried out with cells at an earlier passage than those for Ro 31-8220 shown in Figure 1(B). These cells produced roughly twice as much IL2 in response to PMA, and this was still sensitive to inhibition by staurosporine. The effect of staurosporine on IL1 action was not passage-dependent, as later passages showed a similar potentiation to that shown in Figure 1(A) (results not shown).

Taken together, these results indicate that the effect of staurosporine on IL2 production is unlikely to be due to its inhibitory effect on PKC.

Staurosporine activates NFκB and potentiates the activation of NFκB by IL1α

We next examined the effect of staurosporine on the activation of the transcription factor NFκB, which plays a critical role in the induction of IL2 by IL1 [36]. As shown in Figure 2(A), a 1 h pulse with staurosporine followed by incubation in medium for 4 h was found to activate NFκB (lanes 2 and 3). Furthermore, this treatment did not inhibit the activation of NFκB by IL1. Indeed, a strong potentiation in this response was observed (lanes 5 and 6). A 1 h pulse with staurosporine was found, however, to inhibit the activation of NFκB by PMA (Figure 2B). This experiment required that the cells be treated with PMA for 24 h, as we have shown previously [30,37]. Concentrations of 50 and 100 nM staurosporine substantially reduced the PMA effect (Figure 2B, lanes 5 and 6). A 1 h pulse was again found to activate NFκB, as detected 24 h later (lanes 3 and 4). Staurosporine treatment therefore reduced the NFκB signal in PMA-treated cells to that in cells treated with staurosporine alone. Higher concentrations did not lead to further inhibition (results not shown). These results suggest that sufficient staurosporine must accumulate in the cells after a 1 h pulse, thereby causing inhibition of the PMA response and leading to detectable activation of NFκB at 24 h.

The complexes observed in response to IL1 and PMA in these experiments differed somewhat, in that, although in both cases two bands were visible, the upper band was more prominent in the samples from IL1-treated cells. We had observed this in a previous study [30] and found, by carrying out supershift analysis with specific antibodies to NFκB subunits, that the samples from IL1-treated cells contained more p50/RelA dimers (upper band) than those from PMA-treated cells.

We carried out similar experiments with Ro 31-8220 (Figures 2C and 2D). On its own, this agent did not activate NFκB at any concentration or contact time (as shown for 1 μM over 4 h in Figure 2C, lane 3), and its effect on NFκB activation by IL1 was much less than that of staurosporine (Figure 2C, lanes 4–6). However, Ro 31-8220, at a concentration of 1 μM, was found to inhibit activation by PMA (Figure 2D, lane 6). As observed in the IL2 studies shown in Figure 1(B), it was necessary to leave Ro 31-8220 in contact with the cells to see this effect.

We also carried out a time course for the effect of staurosporine on NFκB (Figure 2E). The effect was detectable at 30 min, with the appearance of a complex of lower electrophoretic mobility, and peaked at 4 h. Later time points showed less induction. To allow for a strict comparison to be made with the experiment described in Figure 2(A), we also carried out an experiment which involved pulsing the cells with staurosporine for various times, washing the cells free of the drug and then incubating them for a further 4 h in medium (Figure 2F). Here, a 1 h pulse was found to be optimal (lane 3), further emphasizing the synergy

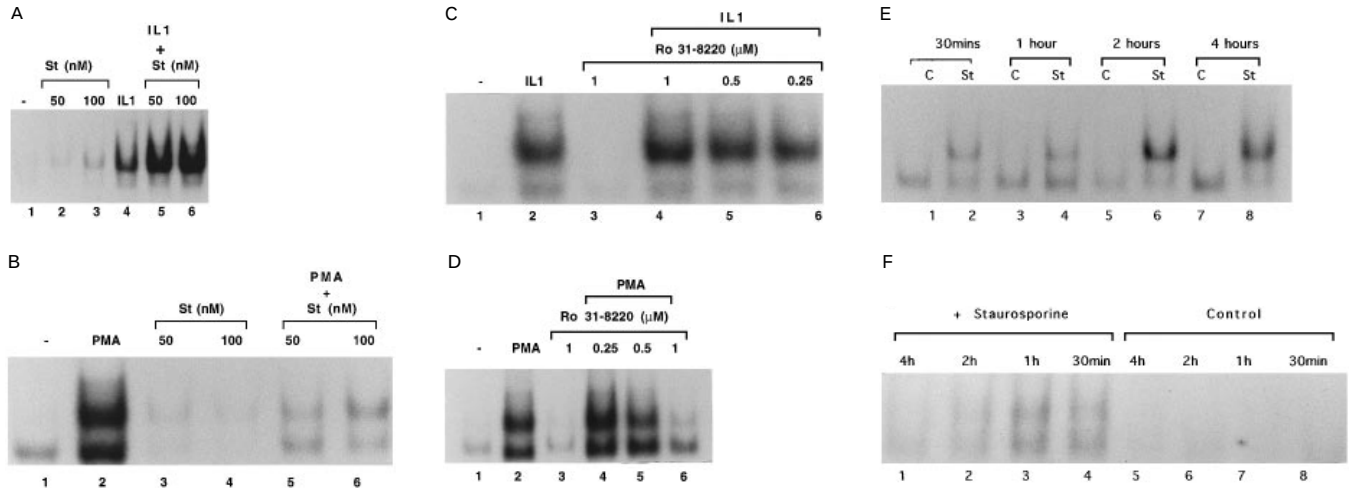

Figure 2 Effects of staurosporine and Ro 31-8220 on IL1- and PMA-induced activation of NFκB in EL4.NOB-1 cells

(**A**)–(**D**) EL4.NOB-1 cells (1×10^6/ml) were treated with the indicated concentrations of staurosporine (St) (**A** and **B**) or Ro 31-8220 (**C** and **D**) for 1 h at 37 °C. Cells were then washed and resuspended in culture medium in the case of staurosporine, or left unwashed in the case of Ro 31-8220, and were then left untreated or were treated with IL1 (10 ng/ml) for 4 h (**A** and **C**) or PMA (100 ng/ml) for 24 h (**B** and **D**), as indicated. These concentrations and contact times were optimal for these stimuli, as shown previously [37]. (**E**) EL4.NOB-1 cells (1×10^6/ml) were left untreated (C) or were incubated with 100 nM staurosporine (St) for the indicated time periods. (**F**) EL4.NOB-1 cells (1×10^6/ml) were left untreated (Control) or were incubated for the indicated times with 100 nM staurosporine, then washed and incubated for a further 4 h in medium alone. Following all of these treatments, nuclear extracts were prepared as described in the Experimental section. NFκB–DNA binding activity was measured using a standard electrophoretic mobility shift assay. Retarded protein–DNA complexes are shown. Each result presented is representative of three separate experiments.

Table 1 Effects of staurosporine and Ro 31-8220 on NFκB-driven CAT expression in EL4.NOB-1 cells

EL4.NOB-1 cells were transiently transfected with a reporter construct comprising five NFκB sites upstream of the gene for CAT, as described in the Experimental section. Cells (1×10^6/ml) were pretreated with 100 nM staurosporine (**a**) or 1 μM Ro 31-8220 (**b**) for 1 h at 37 °C. Cells were then washed and resuspended in culture medium in the case of staurosporine or left unwashed in the case of Ro 31-8220, and were then left untreated or treated with IL1α (10 ng/ml) or PMA (100 ng/ml) for 22 h. In the case of cells treated with IL1α or PMA alone there was a 1 h preincubation with medium followed by the addition of IL1α or PMA for 22 h. CAT activity was assayed in cell lysates as described in the Experimental section, with percentage acetylation being calculated for each sample. Results are expressed as fold increase in CAT activity above that in control samples, and are the means ± S.E.M. for three separate experiments, each experiment being carried out in triplicate.

Additions	Stimulation above control (fold)
(a) Staurosporine	
Staurosporine	6.1 ± 1.0
IL1α	7.0 ± 1.2
IL1α + staurosporine	27.2 ± 3.2
PMA	28.1 ± 3.7
PMA + staurosporine	14.2 ± 2.5
(b) Ro 31-8220	
Ro 31-8220	1.5 ± 1.2
IL1α	11.1 ± 3.8
IL1α + Ro 31-8220	9.8 ± 2.7
PMA	34.0 ± 6.2
PMA + Ro 31-8220	14.6 ± 2.0

between staurosporine and IL1α shown in Figure 1(A). The effect of staurosporine was found to be transient, with a lower response being evident for a 4 h pulse followed by 4 h of incubation in medium. This was similar to the response to IL1α in these cells, which we have shown previously to be transient [37]. Competition analysis with unlabelled wild-type and mutant NFκB probes demonstrated that the complexes were specific for NFκB, in both control and staurosporine-treated cells (results not shown).

Staurosporine increases expression of an NFκB-linked reporter gene and potentiates its induction by IL1α

Because of the observed effect of staurosporine on NFκB activation, as judged by gel shift analysis, we next examined the effect of this inhibitor in a functional NFκB assay, i.e. the induction of a reporter gene, CAT, under the control of five NFκB sequence repeats (Table 1). A 1 h pulse with 100 nM staurosporine followed by washing and incubation with medium for 22 h increased CAT expression in transfected EL4 cells by 6.1-fold. Incubation with IL1α for 22 h had a similar effect, increasing CAT expression 7-fold. Adding IL1α following the pulse of staurosporine led to a 27.2-fold induction, however. As the individual concentrations of both agents were again optimal, a marked synergy was again in evidence for this response. PMA at 100 ng/ml gave a strong induction of CAT which, as expected, was inhibited by 50% by first pulsing the cells for 1 h with staurosporine (Table 1a).

Ro 31-8220 proved incapable of inducing CAT expression, and also had no effect on the induction by IL1α (Table 1b). However, it inhibited the response to PMA by 60%.

Both staurosporine and Ro 31-8220 induce translocation to the membrane of PKC-α and -ε isoforms

Previous workers have demonstrated that staurosporine can induce translocation to the cell membrane of the ε isoform of PKC [38]. As this isoform has been implicated in NFκB activation [39], we attempted to repeat this observation and also examined the PKC-α, -β, -δ, -θ and -ζ isoforms. A 60 min incubation of EL4 cells with 100 nM staurosporine or 1 μM Ro 31-8220 caused

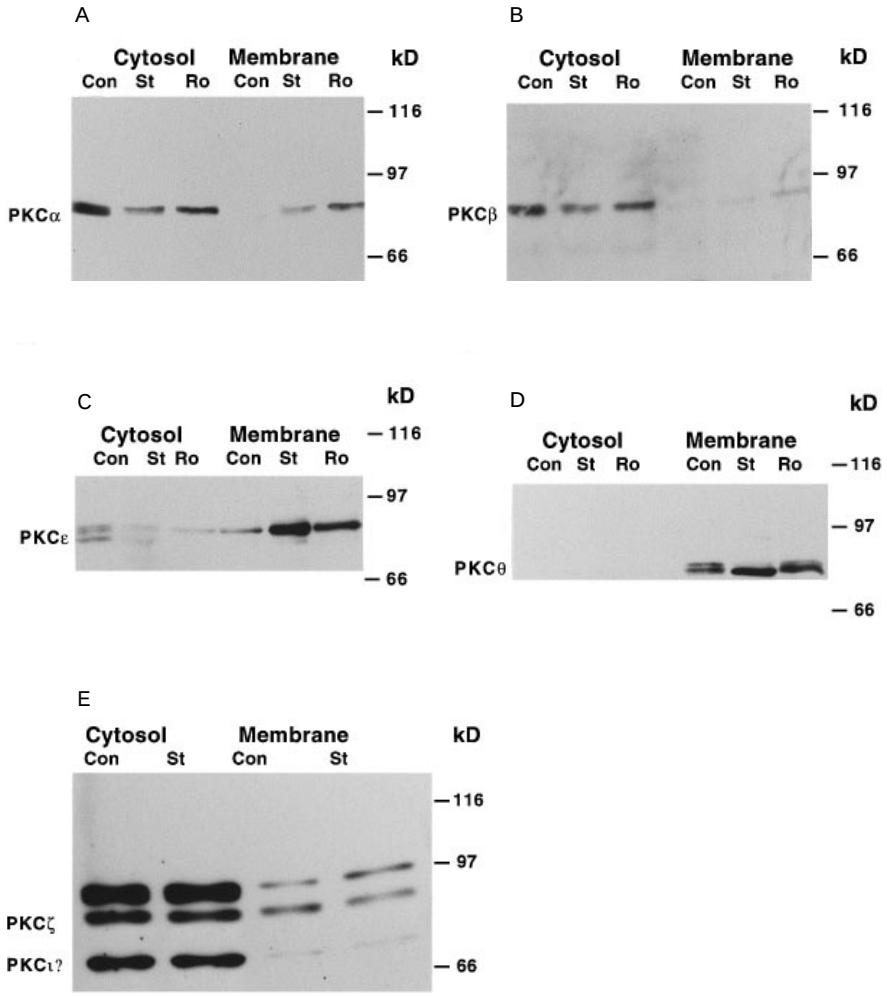

Figure 3 Effects of staurosporine and Ro 31-8220 on the cellular distribution of PKC isoenzymes in EL4.NOB-1 cells

EL4.NOB-1 cells (1×10^7 cells per sample in 1 ml) were left untreated (Con) (**A**–**D**, lanes 1 and 4; **E**, lanes 1 and 3) or were stimulated with 100 nM staurosporine (St) or 1 μM Ro 31-8220 (Ro) as indicated for 60 min. Cells were then lysed and cytosolic and membrane fractions prepared. The fractions were separated by electrophoresis on an SDS/7.5%-polyacrylamide gel, electrotransferred on to nitrocellulose and immunoblotted with anti-PKC-α (**A**), anti-PKC-β (**B**), anti-PKC-ϵ (**C**), anti-PKC-θ (**D**) or anti-PKC-ζ (**E**) as described in the Experimental section. Molecular mass standards (kDa) are shown, and each PKC isoform is indicated. No other bands were seen on the blots. Results shown are representative of three separate experiments.

a marked translocation of PKC-α and -ϵ to the membrane (Figures 3A and 3C). PKC-β showed a marginal decrease in the cytosolic fraction and a slight increase in the membrane fraction, particularly with Ro 31-8220 (Figure 3B). The multiple bands observed in the cytosolic fraction for PKC-ϵ have been noted by Koide et al. [40], who suggested that they represent different phosphorylation states of this isoform. If this is the case, both staurosporine and Ro 31-8220 therefore caused the accumulation of one particular phosphoform in the membrane, as indicated by the increase detected in the upper band (Figure 3C).

No changes in PKC-θ or -ζ were detected (Figures 3D and 3E respectively) following treatment with staurosporine. PKC-θ was membrane-associated in untreated cells (Figure 3D). For PKC-ζ, three immunoreactive bands were observed. Other laboratories have made similar observations with a different antiserum to PKC-ζ [41,42]. The 82 kDa band is probably a cross-reactive species of unknown identity. This was not an atypical PKC, as it translocated to the membrane upon treatment with PMA, unlike the two other bands detected (results not shown). This was also found by Tsutsumi et al. [41]. The 75 kDa band is likely to be PKC-ζ, while the 66 kDa band is PKC-ι, which is cross-reactive with the antiserum used. In any event, none of the complexes were responsive to staurosporine. We also tested for PKC-δ, but found no expression in these cells (results not shown).

The conclusion from these studies is that, since Ro 31-8220 was as effective as staurosporine at inducing translocation of the particular isoenzymes described, the stimulatory effects of staurosporine on NFκB and IL2 were unlikely to be due to this effect.

DISCUSSION

A large number of studies have been published on the potential involvement of PKC in IL1 signal transduction [7–15,17–23]. Protein kinase inhibitors capable of inhibiting PKC have been utilized and shown to inhibit cellular responses to IL1 [20,22,23]. There have also been reports failing to show effects of PKC inhibitors such as staurosporine [24–26], and in three reports this inhibitor potentiated IL1 action [27–29]. Each of these studies discussed the possibility that staurosporine treatment relieved

cells of a tonic inhibition by a staurosporine-sensitive protein kinase, which was suggested to be PKC. The exact target for staurosporine was not determined, however. Our present results confirm the synergistic effect of staurosporine on IL1α action, but indicate that PKC is unlikely to be the target. In addition, the point of synergy with the IL1α pathway may be the transcription factor NFκB.

To determine the importance of PKC in the effect of staurosporine, we tested Ro 31-8220, which was developed as a highly selective inhibitor of PKC [43]. This compound is less potent than staurosporine at inhibiting PKC in cells, but is more selective towards PKC, being at least 10 times less potent an inhibitor of protein kinase A [43]. The concentrations of both compounds used in our study inhibited PMA action very effectively, with Ro 31-8220 being about 10-fold less potent than staurosporine. Neither inhibited NFκB activation by IL1α, as we have shown previously for staurosporine [37]. The potentiating effect on IL1α of staurosporine was not observed with Ro 31-8220. Furthermore, the ability of staurosporine alone to increase IL2 production was not shared by Ro 31-8220, again indicating that PKC is not important for this response. Staurosporine and Ro 31-8220 also differed in another regard. Because prolonged exposure of the cells to staurosporine was toxic, it was necessary to pulse the cells with the drug, wash them and then treat them with medium, IL1 or PMA. This was sufficient to drive IL2 production, synergize with IL1 and inhibit the effects of PMA. Ro 31-8220, on the other hand, was not toxic and had to be left in contact with the cells for it to inhibit the response to PMA. In spite of this prolonged contact, very little effect, either inhibitory or synergistic, on the IL1 response was seen.

The potentiation of IL1 action by staurosporine appeared to be a true synergy, as both agents together caused an effect that was much greater than additive. In spite of the different protocols used for the two drugs, the observation that both failed to significantly block IL1α action in the cells while clearly inhibiting PMA strongly indicates that PKC is not involved in IL1 signalling in these cells. It is possible, though unlikely, that a PKC isoform that is insensitive to these inhibitors is involved in IL1 signalling. PKC-ζ has been shown to be less sensitive than classical PKC isoforms [44], although inhibition would be expected at the higher concentrations used here. It is also possible, although again unlikely given the concentration range used, that the potentiating effect of staurosporine is due to inhibition of a PKC isoform that is less sensitive to Ro 31-8220.

The potentiating effect was also observed when we examined activation of the transcription factor NFκB. Staurosporine, but not Ro 31-8220, activated NFκB, and potentiated NFκB activation and NFκB-driven gene expression in response to IL1α. A 1 h pulse of staurosporine was again effective here. As NFκB is a key transcription factor for IL2 production in EL4 cells [36], it therefore appeared that one of the points of synergy between staurosporine and IL1 was NFκB activation. Since it is usual only for activators of different signalling pathways to synergize, it seems likely that IL1α and staurosporine activate NFκB by different means. In addition, a feature shared by the three previous studies that demonstrated potentiation by staurosporine of responses to IL1 was that each of the agents whose production in response to IL1 was potentiated, namely IL2, IL6 and type 2 cyclo-oxygenase, have genes with important NFκB-binding sites in their 5′ regulatory regions [36,45,46]. Our observations with staurosporine and NFκB would provide an explanation for these studies.

Staurosporine is therefore targeting a process upstream of NFκB that is not activated by Ro 31-8220. From other studies, the concentrations of staurosporine used would be expected to inhibit other protein kinases, including protein kinase A, S6 kinase, cdc2, calcium/calmodulin-dependent protein kinase and tyrosine kinases such as src, lyn and c-Fgr [47]. The Ro 31-8220 concentration used here would not inhibit protein kinase A or calcium/calmodulin-dependent kinase, the other kinases that have been studied for Ro 31-8220 [33]. Higher concentrations of Ro 31-8220, which would have inhibitory effects, were inactive (results not shown). It therefore appears unlikely that such kinases are involved. Tyrosine kinases would also be unlikely to be involved, as we have shown previously that the tyrosine kinase inhibitor genistein does not affect induction of IL2 by IL1α [48], and although herbimycin A is inhibitory we have found that its effects are due to covalent modification of the p50 subunit of NFκB [30]. One possibility we examined was that the effect of staurosporine was due to effects on PKC-ϵ. It has been shown that staurosporine can cause the translocation of PKC-ϵ to the membrane [38]. Furthermore, PKC-ϵ has been shown to be involved in NFκB activation [39]. We demonstrated that staurosporine can induce PKC-ϵ translocation, and also observed translocation of PKC-α and to a lesser extent PKC-β, but not PKC-θ or -ζ. Such translocations are unlikely to correlate with increased activity, however [38], and since Ro 31-8220 was found to be similarly effective, the changes observed were most unlikely to be responsible for the observed effects of staurosporine. The ability of both agents to cause translocation of certain PKC isoforms may be due to their hydrophobicity. This may lead to partitioning of the compounds to the membrane.

Other studies have demonstrated direct effects of staurosporine on a variety of cellular processes. These include up-regulation of epidermal growth factor receptors in PC12 cells [49], induction of an astrocytic phenotype in C6 glioma cells [50], and increased activity of 60 kDa serine/threonine kinase in platelets and bovine chromaffin cells [40,51]. It is possible that such a kinase may be involved in the staurosporine pathway activated in our studies. Two other studies have also found different effects of staurosporine compared with Ro 31-8220. Staurosporine alone was found to increase keratinocyte differentiation [52] and sensitize tumour cells to killing by TNF [33]. This latter study also demonstrated a potentiation of IL6 production. The ability of staurosporine to potentiate TNF action is most interesting, as the IL1 and TNF signalling pathways share many features, including NFκB activation. Finally, staurosporine has been shown to inhibit the activity of an IL1-receptor-associated kinase [3]. Whether this kinase is identical with the recently cloned IL1-receptor-associated kinase is unclear [4]. However, its sensitivity to staurosporine suggests either that it is not involved in NFκB activation or IL2 induction, or alternatively that its activation by IL1 may actually be a negative signal, which is relieved by staurosporine treatment, thereby greatly potentiating IL1 action.

In conclusion, the present study indicates that staurosporine activates NFκB and induces IL2 production in EL4 cells, and synergizes with IL1α in these responses. It highlights the importance of comparing staurosporine with more specific inhibitors of PKC such as Ro 31-8220 when attempting to implicate PKC in signalling pathways, and also explains, at least in part, previous demonstrations of potentiation by staurosporine of responses to IL1 in terms of synergy at the level of NFκB.

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