Contrasting effects of the protein kinase C inhibitor, staurosporine, on cytokine and phorbol ester stimulation of fructose 2,6-bisphosphate and prostaglandin E production by fibroblasts *in vitro*

Comparative studies using interleukin-1 α , tumour necrosis factor α , transforming growth factor β , interferon- γ and 12-*O*-tetradecanoylphorbol 13-acetate

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It is known that both interleukin-1 α (IL-1 α) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) promote increases in intracellular levels of the glycolytic regulatory metabolite fructose 2,6-bisphosphate [Fru(2,6) P_2] and in the production of prostaglandin E (PGE) by subcultured rheumatoid synovial cells (RSC) and human dermal fibroblasts *in vitro*. We report here that the protein kinase C inhibitor staurosporine enhanced the IL-1 α -induced increase in [Fru(2,6) P_2] and PGE production by RSC, whereas in similar concentrations (3–30 nM) this inhibitor decreased the TPA-induced stimulation of these parameters. Staurosporine produced a similar enhancement of the response to IL-1 α by normal human dermal fibroblasts. The increased PGE production provoked by tumour necrosis factor α (TNF α) in RSC was also augmented by staurosporine, but, in contrast, the increases in cellular [Fru(2,6) P_2] induced by transforming growth factor β (TGF β) and interferon- γ (IFN- γ) were diminished. Thus the protein kinase C inhibitor staurosporine discriminates not only between the effects produced by IL-1 α and TPA, but also between those of IL-1 α and two other cytokines (but not between IL-1 α and TNF α). These findings suggest that IL-1 α and probably TNF α act via an intracellular mechanism different from that mediating the action of TPA, TGF- β and IFN- γ , and provide evidence that staurosporine is capable of amplifying the IL-1 signal.

INTRODUCTION

The addition of either recombinant human interleukin-1 α (IL-1 α) or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to subcultured rheumatoid synovial cells (RSC) causes dose-related increases in prostaglandin E (PGE) production and cellular fructose 2,6-bisphosphate [Fru(2,6) P_2] [1]. The latter stimulates the activity of one of the rate-limiting enzymes of glycolysis, and elevation of cellular Fru(2,6) P_2 may be part of the mechanism by which IL-1 stimulates glycolysis in these cells [2]. Phorbol esters, such as TPA, bind to and activate the Ca²⁺/phospholipid-dependent protein kinase C [3], and the similar cellular responses produced by IL-1 and TPA suggested that both factors may act via a similar intracellular mechanism. However, this was inconsistent with the further observation that the combined addition of IL-1 with the maximally effective concentration of TPA produced an additive increase in the cellular Fru(2,6) P_2 , a finding incompatible with the concept that the agents shared a common signal pathway. Furthermore, the combined addition of IL-1 α with interferon- γ (IFN- γ) resulted in a synergistic increase in cellular Fru(2,6) P_2 , whereas TPA with IFN- γ caused only an additive increase [1].

Not only is protein kinase C the major receptor for the tumour-promoting phorbol esters, but it is also one half of the phosphatidylinositol second-messenger signalling system [4]. The latter is used for signal transduction by a large number of hormones and growth factors, including the potent fibroblast mitogen platelet-derived growth factor (PDGF), but its involvement in mediating the cellular responses to IL-1 is uncertain.

With the recent introduction of a class of protein kinase C inhibitors which have much greater potency than previous compounds, we have been able to investigate more directly the proposal that differences exist in the IL-1 α and TPA signal pathways. One such compound, staurosporine, inhibits protein kinase C activity *in vitro* with a K_i of 2.7 nM [5], and we have examined its effect on the IL-1 α - and TPA-induced increases in Fru(2,6) P_2 and PGE production in subcultured RSC. Since these cells are essentially fibroblast-like, and because IL-1 α also increases both these parameters in human dermal fibroblasts (D. J. Taylor, unpublished work), we have compared the responses of RSC with those of a classic fibroblast cell. In addition, because IFN- γ and transforming growth factor β (TGF β) also cause a marked elevation of Fru(2,6) P_2 in RSC [6], we have compared the effect of staurosporine on this response induced by three separate cytokines. Furthermore, since IL-1 α and tumour necrosis factor α (TNF α) produce a very similar range of responses in a variety of cells (for review see [7]), including the stimulation of PGE production in RSC [8], we have also examined whether staurosporine has differential effects on the responses induced by these two cytokines.

MATERIALS AND METHODS

Cytokines and chemicals

All the cytokines were recombinant human forms produced in *Escherichia coli*. IL-1 α (specific activity 4×10^7 units/mg in the thymocyte co-stimulation assay [9]) and TNF α were both ob-

Abbreviations used: IL-1, interleukin-1; TPA, 12-*O*-tetradecanoylphorbol 13-acetate (phorbol 12-myristate 13-acetate, PMA); Fru(2,6) P_2 , fructose 2,6-bisphosphate; PGE, prostaglandin E; RSC, rheumatoid synovial cells; TNF, tumour necrosis factor; IFN, interferon; DMEM, Dulbecco's modified Eagle medium; FCS, foetal-calf serum; PDGF, platelet-derived growth factor; IC₅₀, inhibitor concentration that causes 50% decrease in response.

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tained from Dr. D. Westmacott of Roche Products, Welwyn Garden City, Herts., U.K. IFN- γ (specific activity 2.7×10^7 units/mg) and TGF β were both generously given by Dr H. M. Shephard and Dr. J. Kaumeyer, Genentech, South San Francisco, CA, U.S.A. We have previously confirmed that the appropriate concentration (0.01 %) of dimethyl sulphoxide introduced into the culture media with the TPA (Sigma) had no effect on cell Fru(2,6) P_2 or PGE production [1]. Similarly, the lower concentration (final concn. less than 0.003 %) of this solvent introduced with the staurosporine (Calbiochem) had no effect on the response to IL-1 α .

Cell cultures

Rheumatoid synovium obtained from remedial synovectomies or total knee-replacement operations was dissociated enzymically as previously described [10]. Adherent cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) foetal-calf serum (FCS) and antibiotics and subcultured when confluent. Five preparations of RSC were used between passages 2 and 6, by which time the cells had assumed a uniformly fibroblastic appearance. For the experiments the cells were plated into 6- or 12-well plastic cluster dishes (Costar) and were used when approaching confluence. After addition of the treatments in DMEM/10 % FCS, the cells were incubated in a water-saturated atmosphere of 5 % CO $_2$ in air at 37 °C for 7 h. The medium was collected and subsequently assayed for PGE by conventional radioimmunoassay [11], and the glucose concentration was determined enzymically [12]. The cells were either solubilized with 50 mM-NaOH/1 % (v/v) Triton X-100 for Fru(2,6) P_2 measurement, or precipitated with 6 % (w/v) HClO $_4$ for protein determination by the method of Lowry *et al.* [13]. For experiments under serum-free conditions, the cells cultured in 12-well plates were washed twice in Hanks balanced salt solution before addition of DMEM containing 2 % (w/v) BSA and insulin (5 μ g/ml), sodium selenite (5 ng/ml) and transferrin (5 μ g/ml). After an overnight incubation to ensure consumption of any residual growth factors from the FCS, the cytokines also prepared in this medium were added to the cells.

Human dermal fibroblasts obtained by outgrowth from small pieces of skin removed from the forearm of healthy volunteers were kindly provided by Mrs. J. Wallis of this Department. The dermal fibroblasts were cultured in exactly the same manner as for the RSC.

Fru(2,6) P_2 determination

To measure Fru(2,6) P_2 , the solubilized cells were then scraped into tubes and heated for 10 min at 80 °C to denature protein and stabilize Fru(2,6) P_2 . After cooling in an ice/water bath, the samples were made 20 mM in HEPES, adjusted to neutral pH with 1 M-acetic acid and centrifuged (9000 *g*, 2 min). Fru(2,6) P_2 was assayed in the supernatant by the method of Van Schaftingen *et al.* [14], as modified by Van Schaftingen & Hers [15].

RESULTS

Treatment of RSC with IL-1 α or TPA produced increases in PGE production and cellular Fru(2,6) P_2 that were more marked for the IL-1 α than for TPA. Staurosporine (3–30 nM) enhanced the IL-1 α -mediated increase in RSC PGE production and cellular Fru(2,6) P_2 , but inhibited both these responses in TPA-treated cells (Fig. 1). The IC $_{50}$ values (concentration of inhibitor that decreases the response by 50 %) obtained for staurosporine on the TPA-stimulated Fru(2,6) P_2 and PGE production were 20 and 32 nM respectively. The values were calculated by using a quadratic model to fit the data for TPA minus control at each staurosporine concentration and by noting the concentration

Fig. 1. Effect of staurosporine on the stimulation by IL-1 α and TPA of Fru(2,6) P_2 (a) and PGE production (b) in RSC

Subcultured RSC were incubated for 7 h with DMEM/FCS (□) or 100 ng of TPA/ml (△) or 0.5 ng of IL-1 α /ml (○) in the presence of the indicated concentrations of staurosporine. Points are means $\pm$ S.E.M. of 3–9 experiments: *staurosporine stimulated IL-1 α -induced Fru(2,6) P_2 elevation and PGE production with $P < 0.05$ (Tukey's test). Staurosporine inhibited TPA-induced Fru(2,6) P_2 elevation and PGE production with IC $_{50}$ values of 20 and 32 nM respectively, whereas for IL-1 α both IC $_{50}$ values were > 100 nM.

which represented 50 % of the response produced in the absence of staurosporine. This accommodates the effect of staurosporine on basal Fru(2,6) P_2 and PGE production, and applies to the data plotted in Fig. 3. Indeed, during a 7 h incubation 30 nM-staurosporine caused a 34 % ($n = 8$) decrease in Fru(2,6) P_2 relative to the corresponding control. Extending the incubation period up to 24 h increased the size of the decrease in Fru(2,6) P_2 caused by 30 nM-staurosporine to 60 %, even though glucose uptake during this period was only decreased by approx. 20 %. The effect of staurosporine alone on PGE production was more difficult to quantify, since the basal level of PGE production by these cells was usually below the detectability limit of the assay. However, on two occasions when a quantifiable basal production was obtained, 30 nM-staurosporine decreased this to below detectable levels.

In two of the three RSC preparations examined, staurosporine (20 nM) significantly enhanced the TNF α -mediated increase in PGE production (Table 1). The effect was less than that obtained when staurosporine was added with IL-1 α , as was the overall

Table 1. Effect of staurosporine on the IL-1 α - and TNF α -mediated increase in PGE production by RSC

Three preparations of subcultured RSC were incubated with DMEM/FCS with or without the agent(s) for 7 h. The medium was then removed for PGE determination as described in the Materials and methods section. Values are means $\pm$ S.E.M. of triplicates: * staurosporine caused a significant enhancement of cytokine-stimulated PGE production ($P < 0.05$ by Student's t test). Variability in the detection limit of basal PGE production between experiments results from the different amounts of cell protein present.

Treatment	PGE production (ng/h per mg of protein)		
	RSC 1	RSC 2	RSC 3
DMEM/FCS	< 0.23	< 0.35	< 0.50
Staurosporine (20 nM)	< 0.23	< 0.35	< 0.31
TNF α (30 ng/ml)	2.29 $\pm$ 0.09	11.84 $\pm$ 0.07	6.17 $\pm$ 0.25
TNF α + staurosporine	2.20 $\pm$ 0.14	13.42 $\pm$ 0.21*	8.77 $\pm$ 0.08*
IL-1 α (0.5 ng/ml)	10.08 $\pm$ 0.25	36.74 $\pm$ 0.58	26.17 $\pm$ 0.63
IL-1 α + staurosporine	16.32 $\pm$ 0.42*	47.40 $\pm$ 0.86*	39.19 $\pm$ 0.54*

Table 2. Effect of staurosporine on the IL-1 α -mediated increase in human dermal fibroblast cellular Fru(2,6) P_2 and PGE production

Subcultured human dermal fibroblasts were incubated with DMEM/FCS with or without the agent(s) for 7 h. The medium was then removed for PGE determination, and the cells were processed for Fru(2,6) P_2 measurement as described in the Materials and methods section. Values are means $\pm$ S.E.M. of triplicates: * staurosporine caused a significant enhancement of IL-1 α -stimulated Fru(2,6) P_2 and PGE production ($P < 0.05$ by Student's t test).

Treatment	Fru(2,6) P_2 (pmol/mg of protein)	PGE production (ng/h per mg of protein)
DMEM/FCS	106.1 $\pm$ 2.2	0.32 $\pm$ 0.03
Staurosporine (10 nM)	82.7 $\pm$ 2.9	< 0.19
Staurosporine (30 nM)	77.3 $\pm$ 1.6	< 0.19
IL-1 α (0.5 ng/ml)	311.9 $\pm$ 25.7	0.34 $\pm$ 0.04
IL-1 α + staurosporine (10 nM)	417.4 $\pm$ 6.0*	3.81 $\pm$ 0.11*
IL-1 α + staurosporine (30 nM)	326.2 $\pm$ 6.0	3.64 $\pm$ 0.06*

level of PGE production arising from incubation with TNF α (Table 1). TNF α induced only a marginal increase in Fru(2,6) P_2 in these cells [2], and this was not significantly enhanced by staurosporine (result not shown).

Since subcultured RSC have a fibroblastoid morphology and possess recognized fibroblastic markers [16], the effect of staurosporine on the IL-1 α -mediated increase in Fru(2,6) P_2 and PGE production was compared with that obtained with human dermal fibroblasts. As with the RSC, both 10 nM- and 30 nM-staurosporine enhanced the increased cellular Fru(2,6) P_2 caused by IL-1 α , even though basal Fru(2,6) P_2 was decreased by staurosporine alone (Table 2). Detectable levels of PGE production by dermal fibroblasts were only obtained from this preparation when IL-1 α was added with staurosporine. In additional experiments where IL-1 α alone produced a detectable increase in PGE production ($n = 3$), this was further increased by the simultaneous addition of 10 nM-staurosporine (result not shown).

Two other cytokines, TGF β and IFN- γ , also markedly stimulate Fru(2,6) P_2 in subcultured RSC during 24 h incubations. The time course of Fru(2,6) P_2 elevation by the three cytokines is shown in Fig. 2. It is apparent that, in order to study the effect

Fig. 2. Time course of stimulation by IL-1 α , IFN- γ and TGF- β of Fru(2,6) P_2 in RSC

Subcultured RSC were incubated in DMEM/FCS (○) for the times indicated with 0.5 ng of IL-1 α /ml (●), 5 ng of IFN- γ /ml (■) or 10 ng of TGF- β /ml (▲). Points are means $\pm$ S.E.M. of triplicate determinations from one experiment which was representative of two additional experiments.

Fig. 3. Effect of staurosporine on the stimulation by IL-1 α , IFN- γ and TGF- β of Fru(2,6) P_2 in RSC

Subcultured RSC were incubated in DMEM/FCS for 16 h with 0.5 ng of IL-1 α /ml (○), 5 ng of IFN- γ /ml (■) or 10 ng of TGF- β /ml (▲) in the presence of the indicated concentrations of staurosporine. Points are means of two or three experiments performed on three different cell preparations. The average control, IL-1 α -, IFN- γ - and TGF- β -stimulated Fru(2,6) P_2 levels were 85.2, 267.1, 143.5 and 175.6 pmol/mg of cell protein respectively.

of staurosporine on TGF β and IFN- γ stimulation, an incubation period longer than the 7 h used for IL-1 α and TPA is required. Although a 24 h incubation provided the largest rise in this parameter for all three cytokines, in order to decrease the time-dependent suppression of basal Fru(2,6) P_2 by staurosporine a period of 16 h was chosen for these studies. The effects of increasing staurosporine concentrations on the cytokine-mediated increase in Fru(2,6) P_2 levels are shown in Fig. 3. As observed for the shorter incubation times, the IL-1 α response was enhanced by staurosporine (3 and 10 nM), whereas in contrast those caused by TGF β or IFN- γ were both inhibited. A similar discrimination between the effects of IL-1 and those of TGF- β and IFN- γ was obtained when this experiment was repeated under serum-free conditions (result not shown).

DISCUSSION

The cellular actions of TPA are mediated by the binding to, and subsequent activation of, the enzyme protein kinase C [3], but the nature of the intracellular events that follow the binding of ligand to the IL-1 receptor remains to be elucidated. We have previously provided evidence suggesting that IL-1 and the phorbol ester TPA, despite producing very similar cellular responses in RSC, do not share the same protein kinase signalling pathway [1]. With the recent availability of staurosporine, a potent protein kinase C inhibitor, we have tested our previous proposal, using this agent.

Our findings confirm the effectiveness of staurosporine as a protein kinase C inhibitor, in that the TPA-mediated increases in RSC prostanoid production and cellular Fru(2,6) P_2 were inhibited by nanomolar concentrations of the compound. In marked contrast, staurosporine enhanced both these cellular responses when the stimulating agent was IL-1 α , thus providing further evidence that the IL-1 cell-signalling pathway is unlikely to involve the protein kinase C activated by TPA. The IC₅₀ values obtained with staurosporine for the TPA-stimulated rise in Fru(2,6) P_2 and PGE production of 20 and 32 nM, respectively, are similar to the value of 27 nM reported for the inhibition of phorbol-ester-induced rise in platelet Ca²⁺ [17]. The difference in the IC₅₀ values obtained for the two TPA-mediated responses in RSC may be a consequence of the characteristics of these parameters, one being an accumulated extracellular product (PGE) and the other a single measurement in time of a changing, intracellular, metabolite concentration.

The finding that staurosporine caused a decrease in cellular Fru(2,6) P_2 when added alone to either RSC or human dermal fibroblasts may be a consequence of its inhibitory action on protein kinase C, rather than an unrelated side effect. The addition of medium containing FCS increased the level of Fru(2,6) P_2 (Fig. 2), which probably reflects the presence within the serum of various growth factors. The mitogenic action of one of these factors, PDGF, is known to be mediated in part via activation of protein kinase C [18], and inhibition of this response by staurosporine will thus depress the growth-factor-induced rise in cellular Fru(2,6) P_2 . Indeed, the addition of staurosporine (30 nM) to RSC cultured in serum-free conditions resulted in only a 20% decrease in cellular Fru(2,6) P_2 compared with the corresponding control (result not shown), whereas in the presence of FCS this value was 34%. This indicates that the effect of staurosporine on basal Fru(2,6) P_2 was largely a result of it preventing protein kinase C activation by serum-derived growth factors. However, despite its effect on basal Fru(2,6) P_2 levels, the compound was well tolerated by the cells, with no increase in detachment or change in cell morphology, and even at the highest concentration with extended incubations it only caused a small decrease (20%) in glucose uptake.

These results are the first to describe the enhancement by staurosporine of cellular responses to IL-1 α . The effect was observed over the same concentration range which inhibited similar responses produced by TPA, and was a feature of fibroblasts derived from both rheumatoid synovium and human skin. The extent of the IL-1 α -mediated stimulation of prostanoid production [19], and the more recently reported elevation of cellular Fru(2,6) P_2 [2], varied between the two cell types studied. As reported by other workers [20], IL-1 α produced a larger increase in PGE production in the RSC than in the three dermal fibroblast preparations, whereas for elevation of cellular Fru(2,6) P_2 the situation was the reverse.

The observation that two distinct IL-1-induced responses are amplified by staurosporine suggests that the compound is probably acting on the IL-1 signal response rather than on the individual processes themselves. Our previous studies demonstrated that these two responses are indeed independent, since indomethacin inhibition of PGE production after IL-1 α stimulation did not prevent the accompanying rise in cellular Fru(2,6) P_2 [2], and elevation of Fru(2,6) P_2 by IFN- γ failed to elicit any increase in PGE production [2]. Furthermore, the addition of staurosporine alone to the cells decreased both parameters, which again suggests that the compound is likely to be acting in the area of signal transduction. Since this amplification by staurosporine was demonstrated in both subcultured RSC and normal human dermal fibroblasts, it seems probable that these cells have a similar signal mechanism. How staurosporine amplifies the IL-1 signal has yet to be determined, but if it is related to inhibition of protein kinase C, then the recent observation of a recognized phosphorylation site for this enzyme within the murine IL-1 receptor sequence may provide the mechanism for interaction [21]. This would not be a unique occurrence, since protein kinase C is known to phosphorylate a threonine residue within the epidermal-growth-factor receptor, thereby causing an inhibition of the associated tyrosine kinase activity [22], a phenomenon termed transmodulation. However, at present there is no evidence that the IL-1 receptor is phosphorylated [23], and staurosporine may amplify the IL-1 signal not by relieving inhibition by protein kinase (i.e. transmodulation), but by increasing the level of an as-yet unknown second messenger.

Staurosporine had different effects on the increased cellular responses mediated by other cytokines. For TNF α , which produces a similar range of cellular responses to IL-1 α [7], staurosporine also enhanced PGE production, though not as markedly as it did the increase caused by IL-1 α . This difference may be a consequence of the much-decreased potency of TNF α to stimulate PGE in RSC [2]. The marked inhibition by staurosporine of the stimulation of cellular Fru(2,6) P_2 by TGF β or IFN- γ supports our previous proposal that IL-1 elevates cellular Fru(2,6) P_2 by a mechanism different from that used by IFN- γ or TGF β [6]. This might suggest the involvement of protein kinase C in the mediation of IFN- γ or TGF- β -induced responses, but, in view of the time delay in cellular Fru(2,6) P_2 elevation compared with that mediated by IL-1 α , consideration should be given to the possible induction of autocrine factors whose action is in turn mediated via protein kinase C.

In summary, in subcultured fibroblast-like RSC, staurosporine has proved an effective inhibitor of two cellular responses mediated by the phorbol ester TPA, and also by the cytokines TGF β and IFN- γ . In marked contrast, these same cellular responses mediated by IL-1 α and TNF α were amplified by the compound in both RSC and normal human dermal fibroblasts. Thus our earlier proposal that the IL-1 α signal mechanism is unlikely to involve the protein kinase C activated by TPA has been confirmed, and this can also be said for the TNF α signal

mechanism. The finding that staurosporine consistently enhances the effects of IL-1 α on these cells suggests that this compound is capable of amplifying the signal produced when IL-1 binds to its surface receptor.

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