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Involvement of tyrosine kinase and protein kinase C in platelet-activating-factor-induced *c-fos* gene expression in A-431 cells

Yamini B. TRIPATHI,*‡ Robert W. LIM,* Sagrario FERNANDEZ-GALLARDO,* Jagannadha C. KANDALA,† Ramareddy V. GUNTAKA† and Shivendra D. SHUKLA*§

Departments of * Pharmacology and † Molecular Microbiology and Immunology, University of Missouri–Columbia, School of Medicine, One Hospital Drive, Columbia, MO 65212, U.S.A.

In A-431 cells, platelet-activating factor (PAF) induces the expression of *c-fos* and TIS-1 genes in both the absence and the presence of cycloheximide in a structurally specific and receptor-coupled manner. We have now investigated the molecular mechanisms of this response, particularly in relation to the role of protein kinases. Pretreatment of cells with genistein or methyl-2,5-dihydroxycinnamate (tyrosine kinase inhibitors) or staurosporine (a protein kinase C inhibitor) for 20 min abolished the *c-fos* expression induced by PAF. Interestingly, when genistein was added 90 s after addition of PAF, no inhibition was observed. Similarly, staurosporine did not inhibit *c-fos* expression when added 8 min after PAF addition to the cells. These inhibitions were dose-dependent (IC_{50} for staurosporine was 180 nM, and for genistein 50 μM). Simultaneous addition of PAF and phorbol 12-myristate 13-acetate (PMA) did not give a synergistic effect on *c-fos* expression. Pretreatment of cells with PMA had no effect on [^{3}H]PAF binding, but abolished the PAF-induced gene expression. PAF-stimulated gene expression was desensitized if cells were pretreated with PAF. Interestingly, epidermal growth factor was able to stimulate *c-fos* expression in PAF-desensitized cells, and thus indicated involvement of distinct mechanisms for the two stimuli. Forskolin, an activator of adenylate cyclase, did not induce *c-fos* expression and had no effect on the PAF response. Exposure of cells to PAF for as little as 1 min, followed by its removal, was sufficient to activate the gene expression and demonstrated the rapidity and the exquisite nature of the signalling involved in this process. It is concluded that activation of PAF receptor (a proposed G-protein-coupled receptor) causes rapid production of signals which induce the expression of *c-fos* gene and that this is mediated via tyrosine kinase and protein kinase C.

INTRODUCTION

Platelet-activating factor (PAF) is a potent phospholipid agonist [1,2]. It activates phospholipase C activity in rabbit platelets and produces inositol trisphosphate ($InsP_3$) and diacylglycerol [3,4]. $InsP_3$ releases the intracellular Ca^{2+} [5,6] and diacylglycerol stimulates the protein kinase C (PKC) activity [7]. These two second messengers further cause many cellular responses [8–10]. Previous reports from our laboratory have demonstrated that PKC negatively regulates the PAF-induced phospholipase C activation in rabbit platelets [11]. PKC is involved in transducing signals for a number of agents [12,13], but its role in PAF-induced *c-fos* expression is unknown. We have reported that PAF induces the *c-fos* and TIS-1 (PMA-inducible sequence-1) genes in A-431 cells and that this expression is specific for PAF and appears to be receptor-coupled [14]. The PAF-induced *c-fos* expression has also been observed in human monocytes [15], human neuroblastoma cells [16] and human β-lymphoblastoid cells [17], but the signal-transduction mechanisms involved in this gene expression are not known. The product of *c-fos* acts as a transcriptional factor. In combination with *jun* protein it forms the AP-1 complex, which binds to the specific DNA-consensus sequence (AP-1) of various genes and regulates their transcription [18–21]. The regulatory element of *c-fos* promoter responds to both phorbol esters and calcium ionophores [22–24]. Many agents, including phorbol 12-myristate 13-acetate (PMA) and muscarinic-receptor agonists, stimulate *c-fos* by PKC activation [25–27], whereas intracellular Ca^{2+} is proposed to play a major role in angiotensin II-induced *c-fos* expression [28]. In the present paper we have examined novel characteristics of PAF-induced *c-fos* expression and the involvement of protein kinases. Our results demonstrate that both protein tyrosine kinase and PKC are involved in *c-fos* induction.

MATERIALS AND METHODS

Materials

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and lyso-PAF were supplied by Bachem (Torrance, CA, U.S.A.). Staurosporine was purchased from Kyowa Medex Co. (Tokyo, Japan). [^{32}P]dCTP and genistein were purchased from ICN Radiochemicals (Irvine, CA, U.S.A.) and PMA was obtained from LC Services Corp. (Woburn, MA, U.S.A.). All other chemicals used were of the highest analytical (molecular biological) grade available from Sigma Chemical Co. and Fisher Scientific.

Cell culture

Human A-431 epidermoid carcinoma cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) containing penicillin (110 units/ml), streptomycin (100 μg/ml) and 10% (v/v) fetal-bovine serum. Cells were grown at 37 °C in a humidified atmosphere of air/CO_2 (19:1). Plating density was 6×10^5 cells per 35 mm-diam. dish, and the medium was replaced every 2 days. Experiments were carried on day 5 of plating (80–90% confluent).

Abbreviations used: PAF, platelet-activating factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; DMSO, dimethyl sulphoxide.

‡ Permanent address: Department of Medicinal Chemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.
§ To whom correspondence should be addressed.

RNA extraction and Northern blotting

RNA was isolated by the phenol-extraction method [29]. About 20 μg of RNA was subjected to electrophoresis in agarose gels containing 6% formaldehyde and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, PA, U.S.A.) by diffusion method.

Probe preparation and hybridization

A fragment of the *c-fos* gene was isolated from the plasmid vector by digestion with endonuclease *Eco*R1, which was then run on agarose gel and recovered by electroelution. It was labelled with [^{32}P]dCTP by the random-primer method [30]. pCHOB fragment (a constitutively expressed gene isolated from Chinese-hamster ovary cells) [31] was also labelled similarly and used to determine the relative quantity of RNA loaded in each lane by rehybridizing the blots which had been probed with *c-fos* previously. Blots were prehybridized with a solution containing 50% formamide, 3 × SSC (1 × SSC is 0.15 M-NaCl/15 mM-sodium citrate), 50 mM-Tris/HCl, pH 7.4, 250 μg of denatured salmon testis DNA/ml, 1 mM-EDTA and 0.02% each of BSA, polyvinylpyrrolidone and Ficoll, at 42 °C for 12 h and further hybridized with labelled probe (1 × 10^6 c.p.m./ml) for 40 h at 42 °C in the presence of dextran sulphate [32]. Filters were washed with 2 × SSC/0.2% SDS at 55 °C, followed by another wash with 0.5 × SSC/0.2% SDS. Finally the filters were exposed to Kodak XAR-5 films with intensifying screen at −70 °C. Relative hybridization was assessed by a LKB laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) and compared on the basis of difference between the intensities of *c-fos* and pCHOB (internal standard) in each lane.

RESULTS

In order to define the mechanism of PAF-stimulated *c-fos* gene expression, we have utilized staurosporine, methyl-2,5-dihydroxycinnamate and genistein as pharmacological tools in the present study. Staurosporine is a potent protein kinase inhibitor and probably acts at the ATP-binding site [33,34]. It is more specific to Ca^{2+}-dependent PKC [35]. Genistein is reported to be a specific inhibitor of tyrosine kinase in A-431 cells [36], the cell model used in this study. Methyl-2,5-dihydroxycinnamate is a stable analogue of erbstatin and is a potent tyrosine kinase inhibitor. In addition, several other experimental protocols were designed to probe the steps involved in the gene expression. The results are described below.

Effect of methyl-2,5-dihydroxycinnamate and genistein

The *c-fos* induction by PAF in the presence of cycloheximide and genistein was investigated. The inhibition of PAF-induced *c-fos* expression was dose-dependent (Fig. 1*a*, left panel), and quantification of signals by laser densitometer indicated that IC_{50} was approx. 50 μM. Pretreatment of cells with genistein (200 μM) completely inhibited *c-fos* gene expression, whereas genistein by itself did not induce the expression (results not shown). We also tested whether genistein inhibited PMA-induced *c-fos* expression. A-431 cells were treated with genistein (200 μM) for 20 min and then challenged with PMA (400 nM) for 1 h. The *c-fos* levels were monitored. In two separate experiments genistein inhibited *c-fos* expression by only 10 ± 5% (± S.D.). Under similar experimental protocols, when genistein was replaced by staurosporine (1 μM) it caused about 95% inhibition of PMA-induced *c-fos* expression. These results indicated that genistein had negligible effect on PMA-stimulated (PKC-dependent) induction of *c-fos*. Effect of another tyrosine kinase inhibitor, methyl-2,5-dihydroxycinnamate, a stable analogue of erbstatin, was also studied. This compound also inhibited *c-fos* expression, as shown in Fig. 1(*b*). These results indicated a possible role of tyrosine kinase in this process, However, the step at which tyrosine kinase is involved is not known. It can be envisaged that interaction of PAF with its receptor relays signals, in some stepwise manner, for the activation of tyrosine kinase, phospholipase C and PKC, which in turn may cause induction of the *c-fos* gene. To probe

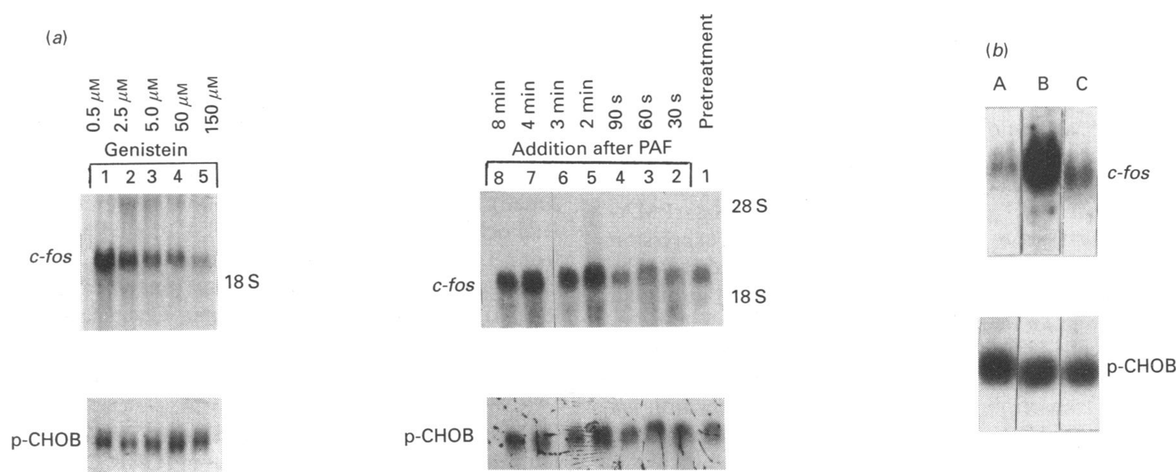

Fig. 1. Effect of genistein and methyl-2,5-dihydroxycinnamate on PAF-induced *c-fos* gene expression

(*a*) A-431 cells, grown up to about 80% confluence, were used. Cells were pretreated with 10 μg of cycloheximide/ml for 30 min. Later, cells were treated with different agents for various time periods, and total RNA was isolated, separated on formaldehyde/agarose gel, transferred to nitrocellulose membrane, cross-linked and hybridized with human *c-fos* genomic probe. Left panel: A-431 cells were pretreated with the different concentrations of genistein for 20 min in serum-free media supplemented with cycloheximide (10 μg/ml), and then PAF (0.1 μM) was added to the same media and incubated for 1 h. Lanes 1–5: genistein concentrations were 0.5 μM, 2.5 μM, 5 μM, 50 μM and 150 μM respectively. Right panel: inhibition response of genistein (100 μM) when added at different times after PAF addition (0.1 μM): lane 8, 8 min; lane 7, 4 min; lane 6, 3 min; lane 5, 120 s; lane 4, 90 s; lane 3, 60 s; lane 2, 30 s; lane 1, pretreatment with genistein for 20 min followed by PAF. (*b*) Experimental conditions were as described above in (*a*) for genistein. A-431 cells were pretreated with methyl-2,5-dihydroxycinnamate (100 μM) for 20 min and then challenged with PAF (0.1 μM) for 1 h. The expression of *c-fos* was monitored as described in the Materials and methods section. A, BSA control; B, PAF; C, 2,5-dihydroxycinnamate followed by PAF. Results of one of two similar experiments are presented; 18 S and 28 S represent sizes of the rRNA.

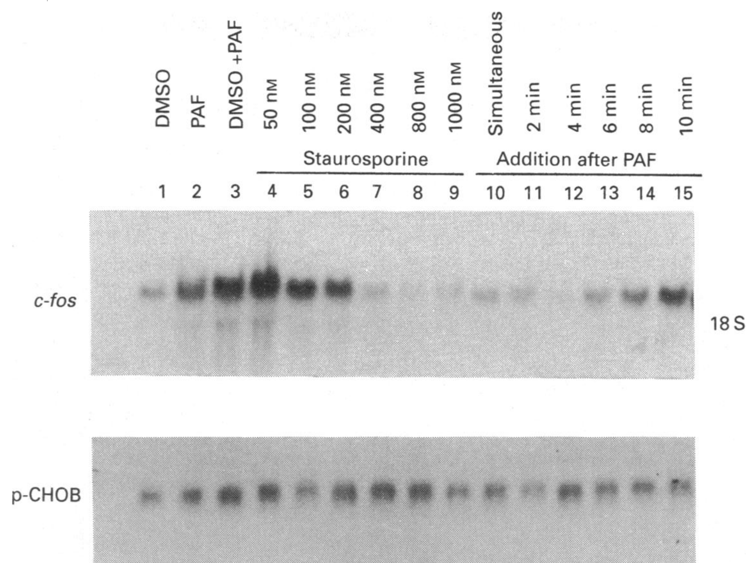

Fig. 2. Staurosporine inhibits the PAF-induced *c-fos* expression in a dose-dependent and time-specific manner

Experimental details were the same as described for Fig. 1. RNA was from A-431 cells treated with staurosporine under different conditions in the presence of cycloheximide (10 μg/ml). Lanes 1–3 are control incubations: lane 1, DMSO (1 μl); lane 2, PAF (0.1 μM); lane 3, PAF+DMSO. Lanes 4–9, cells were pretreated with different doses of staurosporine for 20 min, and then PAF (0.1 μM) was added in the same medium and incubated for 1 h; doses were 50 nM, 100 nM, 200 nM, 400 nM, 800 nM and 1000 nM respectively. Inhibitory effect of staurosporine (1 μM) when added at different time intervals after PAF addition: lane 10, simultaneous addition; lane 11, 2 min; lane 12, 4 min; lane 13, 6 min; lane 14, 8 min; lane 15, 10 min.

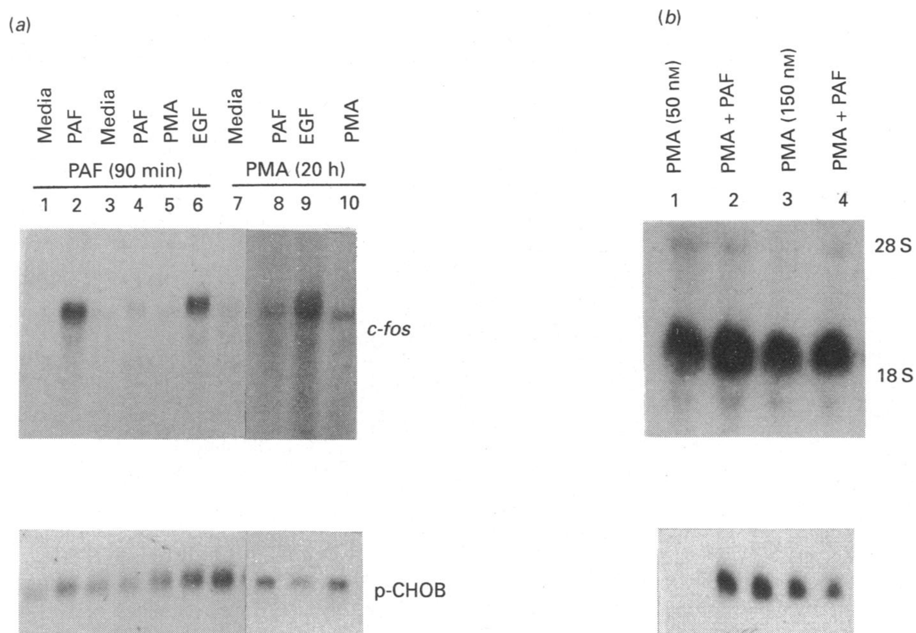

Fig. 3. Effect of pretreatment with PAF and PMA (*a*) and of simultaneous addition of PAF and PMA (*b*)

(*a*) Lane 1, only medium; lane 2, PAF (0.1 μM) in the presence of cycloheximide (10 μg/ml); lanes 3–6, cells were pretreated with PAF (0.1 μM) for 90 min in medium without cycloheximide, then washed with phosphate-buffered saline and again incubated for 1 h with different agents in medium supplemented with cycloheximide (10 μg/ml): lane 3, only medium; lane 4, PAF (0.1 μM); lane 5, PMA (100 nM); lane 6, EGF (200 ng/ml). Cells were pretreated with PMA (400 nM) for 20 h in serum-free medium without cycloheximide. Then the cells were washed and incubated with different agents for 1 h in the medium supplemented with cycloheximide (40 μg/ml): lane 7, only medium; lane 8, PAF (0.1 μM); lane 9, EGF (200 ng/ml); lane 10, PMA (100 nM). (*b*) Cells were preincubated in the medium with cycloheximide (10 μg/ml) for 30 min, and then PAF and PMA were added in different concentrations: lane 1, PMA (50 nM); lane 2, PMA (50 nM)+PAF (0.1 μM); lane 3, PMA (150 nM); lane 4, PMA (150 nM)+PAF (0.1 μM).

this issue, we have attempted to dissect the gene induction pathway temporally by intervening pharmacologically at different time points after PAF challenge. We therefore treated cells with PAF, and at different time intervals genistein (100 μM) was added (Fig. 1*a*, right panel). It was observed that genistein blocked *c-fos* gene expression when added within 60–90 s of PAF

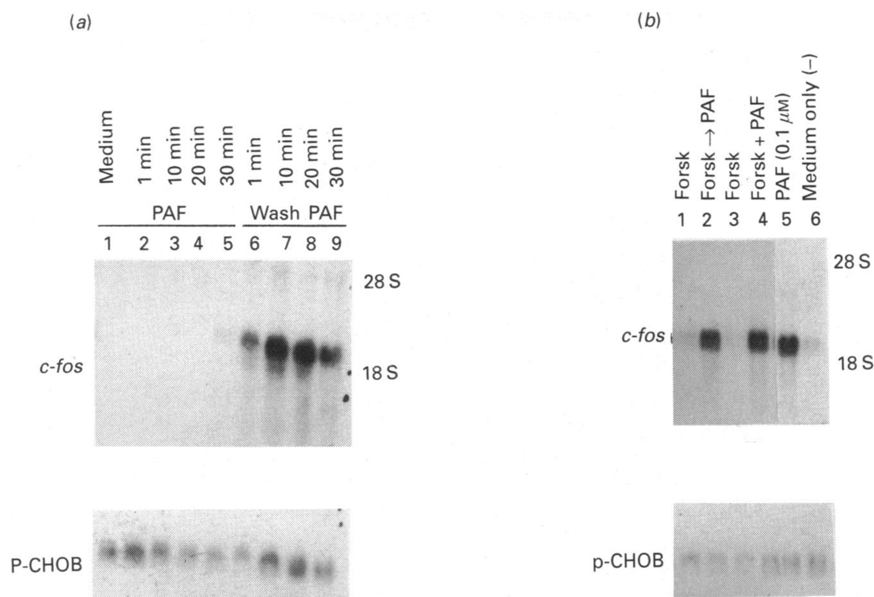

Fig. 4. (*a*) Presence of PAF for the entire incubation time is not necessary for expression of the *c-fos* gene; (*b*) effect of PMA and forskolin on PAF-induced *c-fos* mRNA expression

(*a*) Experimental details were as described for Fig. 1. A-431 cells were incubated with serum-free medium supplemented with cycloheximide (10 μg/ml). PAF (0.1 μM) was added for different time periods (lanes 2–5) and RNA was extracted from cells: lane 1, medium only; lane 2, 1 min; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min. In another set of incubations, cells were treated with PAF (0.1 μM) in the presence of cycloheximide for different time intervals (lanes 6–9) and then washed twice with phosphate-buffered saline to remove PAF. This was followed by addition of 1 ml of fresh medium (with cycloheximide). Cells were further incubated for a total of 60 min. Lane 6, PAF treatment for 1 min followed by wash and reincubation with 1 ml of medium for 59 min; lane 7, PAF treatment for 10 min, wash and reincubation with 1 ml of fresh medium for 50 min; lane 8, PAF treatment for 20 min, wash and reincubation with 1 ml of medium for 40 min; lane 9, PAF treatment for 30 min, wash and reincubation with 1 ml of medium for 30 min. (*b*) Lane 1, forskolin (Forsk; 10 μM, 4 h); lane 2, forskolin (10 μM, 4 h), followed by PAF (0.1 μM) for 1 h; lane 3, forskolin (10 μM, 1 h); lane 4, forskolin (10 μM)+PAF (0.1 μM), 1 h; lane 5, PAF (0.1 μM, 1 h); lane 6, medium supplemented with cycloheximide only.

addition (lanes 2–4), but after this time addition of genistein failed to inhibit the expression (lanes 5–8). These results indicated that a tyrosine-kinase-phosphorylated intermediate is required for the PAF-induced *c-fos* expression and that an early inhibition of tyrosine kinase abolished this response. Tyrosine kinase inhibitors have also been useful as pharmacological tools in other studies (e.g. [37]).

Involvement of PKC and effect of staurosporine

PAF stimulates phospholipase C-mediated phosphoinositide turnover, which results in the production of InsP_3 and diacylglycerol in A-431 cells ([38]; A. T. Thurston & S. D. Shukla, unpublished work). Therefore it seemed appropriate to investigate the involvement of PKC pathway in expression of the *c-fos* gene. Staurosporine, a PKC inhibitor, was utilized in this approach. Cells were pretreated with different concentrations of staurosporine for 20 min and then PAF (0.1 μM) was added. The results (Fig. 2) indicated that staurosporine at concentrations higher than 50 nM inhibited the *c-fos* gene expression. The dose–response curve, generated by laser-densitometric quantification of the bands, indicated that IC_{50} for staurosporine was at 180 nM (lanes 4–9). Thus it appeared that PAF-induced *c-fos* gene expression is dependent on PKC activity. When PAF was added along with dimethyl sulphoxide (DMSO) (vehicle for staurosporine) *c-fos* expression was slightly increased (lane 3) in comparison with PAF alone (lane 2). DMSO by itself slightly elevated the basal level. To characterize further the requirement for PKC activity in PAF-induced *c-fos* expression, we treated cells with PAF, and at different time intervals staurosporine (1 μM) was added. The expression of *c-fos* in these experiments was monitored. The results indicated that addition of staurosporine within 4 min (lane 12) of PAF challenge had an inhibitory effect on the gene expression. This inhibitory effect gradually declined as the time of exposure of cells to PAF increased. If cells were treated with PAF for 8 min or longer (lanes 14 and 15), then any subsequent addition of staurosporine failed to inhibit *c-fos* expression. This indicated that PKC activation plays a role after tyrosine kinase activation in the PAF-stimulated *c-fos* expression. In a control experiment, it was observed that PMA-induced *c-fos* expression was inhibited by staurosporine under similar conditions (results not shown).

It has been documented in many systems that pretreatment of cells with PMA causes down-regulation of PKC activity [39–42]. This protocol was used to test the effect of longer PMA treatments on *c-fos* expression induced by PAF. A-431 cells were treated with PMA (400 nM) for 20 h and then challenged with PAF (0.1 μM) for 1 h in the presence of cycloheximide. Such pretreatments with PMA substantially abolished the PAF induced *c-fos* gene expression (Fig. 3*a*, lane 8) and indicated that *c-fos* gene expression was down-regulated, presumably via down-regulation of PKC. Whereas pretreatment of cells with PMA abolished the PMA-stimulated *c-fos* gene expression (lane 10), expression stimulated by epidermal growth factor (EGF) was unaffected (lane 9).

Whether PMA pretreatment down-regulated the PAF receptor was also evaluated as follows. A-431 cells were treated with PMA (400 nM) for 20 h as above. Binding of [^{3}H]PAF (0.5 nM) in these cells and control cells (i.e. treated with vehicle DMSO) was examined. Non-specific [^{3}H]PAF binding was determined by performing the assay in the presence of 0.1 μM unlabelled PAF, and this value was subtracted from the total binding to obtain the specific [^{3}H]PAF binding. Non-specific binding was about

40% of the total binding. It was observed that specific [^{3}H]PAF binding was unaltered by PMA pretreatment. These results lead to the suggestion that PMA pretreatment did not down-regulate the PAF receptor in A-431 cells. Hence the lack of PAF-induced *c-fos* expression in PMA-pretreated cells is not due to down-regulation of PAF receptors. The involvement of PKC thus appears most likely.

In another series of experiments, A-431 cells were treated with PAF and PMA simultaneously (Fig. 3*b*). It was observed that at lower concentrations of PMA (50 nM), some additive response (15±5%, two experiments) was noted after addition of PAF (0.1 μM; lane 2), but at higher concentrations (150 nM; lane 3) additive response was not apparent (lane 4). To assess further the characteristics of PAF-induced *c-fos* expression, we pretreated the cells with PAF for 2 h in the absence of cycloheximide, washed these cells and re-exposed them to PMA, PAF or EGF for 1 h in the presence of cycloheximide (Fig. 3*a*). Both PAF and PMA failed to induce the *c-fos* gene (lanes 4 and 5), but EGF showed the full induction (lane 6). These experiments demonstrated that PAF pretreatment desensitized the *c-fos* expression induced by PAF or PMA, but not that by EGF. It also indicated that the pathways for PAF and PMA are distinct from that for EGF.

Continuous presence of PAF in the medium is not necessary for gene expression

The results of time-course experiments with genistein and staurosporine indicate a process where the signal is passed from receptor to nucleus in a time-dependent manner. Thus it could be hypothesized that the continuous presence of PAF might not be necessary during the incubation. To address this issue, we incubated the cells with PAF for different time intervals and then washed them with fresh serum-free medium supplemented with cycloheximide (10 μg/ml) and further incubated them in the same medium without added PAF for the remaining part of 1 h. The results indicate (Fig. 4*a*) that even 1 min of incubation with PAF followed by washing is enough to induce *c-fos* gene expression (lane 6).

Effect of forskolin

The above results suggested a possible role for PKC and tyrosine kinase in the PAF-stimulated *c-fos* gene expression. We next ascertained if there was any involvement of the cyclic-AMP-dependent pathway [43,44] in this phenomenon. We therefore treated A-431 cells with forskolin (10 μM), an adenylate cyclase activator, either simultaneously with PAF or for 4 h before the PAF challenge. Our results indicated that forskolin itself did not induce *c-fos* gene expression (Fig. 4*b*) and also had no effect on the PAF-stimulated *c-fos* gene expression when added either before (lane 2) or simultaneously with PAF (lane 4). Under the above condition of forskolin treatment, the cellular cyclic AMP levels rose 10–20-fold, indicating that activation of adenylate cyclase did occur (results not shown). Elevation of intracellular cyclic AMP by itself therefore had no influence on the PAF-induced gene expression in A-431 cells.

DISCUSSION

PAF-induced InsP_3 production has been reported in many cells [45]. It was recently observed that in A-431 cells PAF caused phosphoinositide turnover [38] and expression of *c-fos* and TIS-1 genes in a receptor-specific manner [14,46]. Studies reported here have probed the PAF signalling mechanisms involved in the regulation of *c-fos* gene expression.

Multiple signalling pathways are often involved in stimulus–response coupling for an agent. Among these protein kinase(s) play an important role, and this aspect has been examined in relation to PAF-induced gene expression. In these investigations, activators (PMA) [47] and inhibitors (staurosporine) [35] of PKC, inhibitors of tyrosine kinase (genistein, methyl-2,5-dihydroxycinnamate) [36] and a stimulator of adenylate cyclase to elevate cyclic AMP levels (e.g. forskolin) have been utilized.

The results indicated that the PAF-induction of *c-fos* is mediated through a PKC-dependent mechanism. This was supported by observations that pretreatment of cells with PMA, which is known to down-regulate PKC [39–41], abolished PAF-induced gene expression. The down-regulation of PKC in these experiments is evidenced by our observation that under similar conditions PMA failed to induce the *c-fos* gene, whereas EGF-induced expression was still observed (60–70% of the full response) in the PMA-pretreated cells (Fig. 3*a*). Conversely, in PAF-pretreated cells, PMA and PAF both failed to induce *c-fos*, whereas induction by EGF persisted. This also indicated distinct mechanisms for EGF and PAF. When cells were simultaneously challenged with PAF and PMA, no synergism was noted (Fig. 3*b*), whereas PAF showed a synergistic response with EGF (results not shown). PAF and PMA produce a slight additive response in A-431 cells only at sub-optimal concentrations, indicating an overlap in their pathway. It is worthy of mention here that there is a complex feedback inhibition of *c-fos* expression which many stimuli, including PAF, initiate. Once evoked, this inhibition can affect a number of independent stimuli which induce *c-fos* expression [48]. The third evidence for the involvement of PKC in the expression of *c-fos* is its inhibition by staurosporine, which is a non-specific protein kinase inhibitor at higher concentrations, but at lower concentrations it preferentially inhibits PKC [32,34]. In our system, PAF-induced *c-fos* gene expression was inhibited by staurosporine in a dose-dependent manner (Fig. 2), with an IC_{50} value of 180 nM. This result is consistent with reports in other systems [40, 49]. On the basis of these experimental observations, the involvement of PKC in expression of the *c-fos* gene is suggested.

It has been reported previously that staurosporine potentiates PAF-induced IP_3 production in rabbit platelets [11] and in A-431 cells (A. T. Thurston & S. D. Shukla, unpublished work). But in the present study it inhibited *c-fos* expression. Thus it seems unlikely that InsP_3 generation, another product of phospholipase C activation in these cells [3,4], is directly involved in the induction of *c-fos* by PAF.

In previous papers we have reported that the kinetics of PAF-induced *c-fos* expression [14,46] was similar to those described for other mitogens [49]. Detectable expression was found as early as 20 min after PAF addition [14]. Our findings (Fig. 4*a*) suggest that 1 min of PAF exposure is sufficient to induce the *c-fos* gene to the same level as observed if the incubation time is 1 h. Thus a rapid activation of PAF receptor is apparently sufficient to transduce the signal for further action. Recently, Trejo & Brown [25] have reported that a muscarinic receptor agonist requires 1.5 min to induce maximum increase in *c-fos* and *c-jun* mRNA. The quick action of PAF on *c-fos* expression is also consistent with other findings [21], where *c-fos* transcription starts within 1 min and detectable signals are observed as early as 5 min after ligand incubation [50,51]. In contrast with our findings in A-431 cells, a synergistic response of *c-fos* induction to PAF and PMA was reported for human neuroblastoma cells [16]. On the basis of deletion studies, it was shown that the −65 region of the *c-fos* promoter is mainly responsible for the PAF-induced *c-fos* gene expression. This region is also responsible for stimulation of *c-fos* by agents that act through cyclic AMP activation or by increasing intracellular Ca^{2+} [52]. Our results in A-431 cells, however,

demonstrated that forskolin, which elevated cyclic AMP levels by activating adenylate cyclase, had no effect of its own or on the PAF-induced *c-fos* expression. The role of cyclic AMP in the activation of *c-fos* is ruled out, because in A-431 cells the transfected cyclic-AMP-response element ('CRE') did not exhibit an inducible enhancer activity [53].

Recently, Honda *et al.* [54] have cloned the PAF receptor and showed its homology with the G-protein-coupled receptor family. In addition, studies with platelets [55] have highlighted the involvement of tyrosine kinase in PAF receptor function. We have established here that two different tyrosine kinase inhibitors, genistein (which competes for the ATP-binding site) and methyl-2,5-dihydroxycinnamate (which competes for the substrate-binding site) completely blocked *c-fos* gene expression if cells were pretreated with these inhibitors for 20 min before PAF challenge. However, if genistein is added 90 s after the PAF challenge, the inhibition is not seen. This indicated that within this time PAF stimulated a tyrosine kinase involved in the gene expression. Inhibition of tyrosine kinase after this time was too late to stop the relay of the message. On the other hand, when similar experiments were designed for staurosporine, it was observed that addition of staurosporine was effective in inhibiting *c-fos* induction up to 8 min after PAF challenge. Since both staurosporine and genistein are lipophilic compounds, the permeability of cells to these two drugs should be very rapid (within a few seconds), and they should be readily accessible to the enzymes. In platelets we have observed that addition of genistein 1–2 s before PAF inhibited PAF-stimulated aggregation [55]. This effect was not due to the effect of genistein on PAF-receptor binding [55]. Therefore the difference in the time course of action probably did not result from differential permeability. In keeping with the above assumptions, it is suggested that the activation of PKC occurs at a step temporally distal to tyrosine kinase activation. In a related study, we have observed that PAF causes phosphorylation of several proteins which are immunoprecipitated by anti-phosphotyrosine antibody and that genistein inhibited this response (A. T. Thurston & S. D. Shukla, unpublished work). This indicated that PAF stimulates tyrosine-kinase-mediated phosphorylation of proteins in A-431 cells.

In conclusion, this is the first time that the effect of PAF on A-431 cells has been studied with the objective to delineate signal-transduction mechanisms involved in stimulating primary-response gene expression. We have demonstrated that the expression of *c-fos* mRNA induced by PAF is mediated by both PKC and tyrosine kinase. Furthermore, activation of tyrosine kinase apparently precedes the PKC activation in the signal-transduction pathway leading to this gene expression. As far as we can ascertain, this is the first report where an agonist whose receptor is coupled to G-protein [45,54,56] stimulates *c-fos* expression via tyrosine kinase activation. This suggests involvement of a novel pathway in the PAF-induced gene expression.

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