Protein kinase C activity is not involved in *N*-formylmethionyl-leucyl-phenylalanine-induced phospholipase D activation in human neutrophils, but is essential for concomitant NADPH oxidase activation: studies with a staurosporine analogue with improved selectivity for protein kinase C

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Stimulation of human neutrophils by the receptor agonist *N*-formylmethionyl-leucyl-phenylalanine (fMLP) results in a respiratory burst, catalysed by an NADPH oxidase. Concomitantly, phospholipase D (PLD) is activated. To investigate the role of protein kinase C (PKC) in these neutrophil responses, we have compared the effects of staurosporine and a structural analogue of staurosporine (cgp41251), that reflects a higher selectivity towards PKC [Meyer, Regenass, Fabbro, Alteri, Rösel, Müller, Caravatti and Matter (1989) Int. J. Cancer **43**, 851–856]. Both staurosporine and cgp41251 dose-dependently inhibited the production of superoxide induced by phorbol 12-myristate 13-acetate (PMA). Both compounds also caused inhibition of the fMLP-induced respiratory burst, but with a lower efficacy during the initiation phase of this response. This latter observation cannot be taken as evidence against PKC involvement in the activation of the respiratory burst, because pretreatment of neutrophils with ionomycin before PMA stimulation also results in a lower efficacy of inhibition. Activation of PLD by fMLP was enhanced in the presence of staurosporine, but not in the presence of cgp41251. Enhancement of PLD activation was also observed in the presence of H-89, an inhibitor of cyclic-AMP-dependent protein kinase (PKA). Both staurosporine and H-89 reversed the dibutyryl-cyclic-AMP-induced inhibition of PLD activation, whereas cgp41251 was without effect. These results indicate that the potentiating effect of staurosporine on PLD activation induced by fMLP does not reflect a feedback inhibition by PKC activation, but instead a feedback inhibition by PKA activation. Taken together, our results indicate that in human neutrophils: (i) PKC activity is not essential for fMLP-induced activation of PLD; (ii) PKC activity does play an essential role in the activation of the respiratory burst by fMLP, other than mediating or modulating PLD activation; (iii) there exists a negative-feedback mechanism on fMLP-induced PLD activation by concomitant activation of PKA.

INTRODUCTION

In neutrophils, activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) or dioctanoylglycerol results in the activation of the NADPH oxidase, inducing a so-called respiratory burst (reviewed in [1]). Probably, phosphorylation of p47-*phox*, one of the components of this enzyme system, by activated PKC is a key event in this response [2,3]. However, the role of PKC in the activation of the burst by stimulation of various cell-surface receptors has been questioned [4–7], mostly on the basis of experiments with the potent but non-specific protein kinase inhibitor staurosporine. This compound has been reported to enhance the oxidative burst after stimulation of neutrophils with the receptor agonist *N*-formylmethionyl-leucyl-phenylalanine (fMLP) [8], or to inhibit this response, especially when the second phase is considered [7]. Compound Ro 31-8425, a staurosporine analogue with improved specificity for PKC, does inhibit the respiratory burst after activation of various cell-surface receptors, but at 4–20-fold higher concentrations than required for inhibition of a dioctanoylglycerol-induced response [9].

Recently, the activation of phospholipase D (PLD) resulting in the production of phosphatidic acid has been suggested to play an important role in the activation of the neutrophil respiratory burst [10–12]. The mechanism by which receptor-mediated activation of PLD is regulated is not yet fully understood. A regulatory role for (a) G-protein(s) in PLD activation independent of inducing Ca^{2+} changes is suggested by the observation that fMLP-induced activation of PLD in electropermeabilized neutrophils (in which the free Ca^{2+} concentration is buffered at 1 μM [13]) is dependent on the presence of GTP (G. C. R. Kessels and A. J. Verhoeven, unpublished work). Furthermore, non-hydrolysable GTP analogues are able to stimulate PLD activity in electropermeabilized neutrophils [14] or in electropermeabilized HL-60 cells [15]. The latter study also showed that ATP greatly potentiated this response, suggesting the involvement of a protein kinase. An obvious candidate for this protein kinase would be PKC, because the phorbol ester PMA causes the activation of PLD in several cell types [16,17]. However, studies in which more or less specific inhibitors of PKC were used have revealed rather confusing results [16,18,19]. Studies with various tyrosine kinase inhibitors [20] have suggested that the fMLP-induced activation of PLD depends on the activity of tyrosine kinases in the neutrophil. In the course of our studies on the kinase involvement in PLD activation, we observed that staurosporine (commonly used to inhibit PKC activity) caused a considerable enhancement

Abbreviations used: PLD, phospholipase D; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; dBcAMP, dibutyryl cyclic AMP; PKA, cyclic AMP-dependent protein kinase; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; Me_2SO, dimethyl sulphoxide.

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of the fMLP-induced PLD activity, although staurosporine has been shown to inhibit total neutrophil tyrosine kinase activity almost as potently as PKC activity [21].

To determine in more detail the role of PKC in receptor-mediated respiratory-burst activation and in concomitant PLD activation, we have used a structural analogue of staurosporine (cgp41251) that reflects higher selectivity towards PKC [22]. The results presented here suggest that PKC is not involved in the activation of PLD as induced by the receptor agonist fMLP, because of the failure of compound cgp41251 to influence this response. The potentiating effect of staurosporine does not reflect a feedback inhibition by PKC activation, but instead a feedback inhibition by activation of cyclic AMP-dependent protein kinase (PKA). Since compound cgp41251 does interfere with the fMLP-induced respiratory burst, it can be concluded that PKC plays an essential role in this response other than mediating or modulating PLD activation.

MATERIALS AND METHODS

Materials

Staurosporine derivatives, cgp39360 (parental compound), cgp41251 and cgp42700 were synthesized by CIBA–GEIGY Research Laboratories (Basel, Switzerland). The compounds were dissolved in dimethyl sulphoxide (Me_2SO) at a concentration of 10 mM and stored at −20 °C. Dilutions were freshly made in Me_2SO/water (1:1, v/v), and the final concentration of Me_2SO in the cell incubations never exceeded 0.5%. *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H-89) was purchased from Biomol Research Laboratories (Hamburg, Germany). Stock solutions were made in Me_2SO in a concentration of 30 mM and stored at −80 °C. Dilutions were freshly made in incubation medium. PMA, fMLP, dibutyryl cyclic AMP (dBcAMP) and cytochrome *c* were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Phosphatidylethanol standards were obtained from Avanti Polar Lipids, Birmingham, AL, U.S.A. All other chemicals were of reagent grade.

Cell isolation

Blood was obtained from healthy volunteers. Granulocytes were purified from the buffy coat of 500 ml of blood anticoagulated with 0.4% trisodium citrate (pH 7.4), as described [23]. After isolation, the cells were resuspended in incubation medium containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose and 0.5% (w/v) human serum albumin (pH 7.4), and kept at room temperature until use.

Measurement of PLD activity

PLD activity was measured in neutrophils that had been prelabelled in the phosphatidylcholine pool with [^{3}H]-lysophosphatidylcholine as described previously [14]. In short, 20×10^6 neutrophils/ml of incubation medium without human serum albumin and without $CaCl_2$ were incubated for 60 min in a shaking water bath at 37 °C in the presence of [^{3}H]-lysophosphatidylcholine (2–4 μCi/20×10^6 cells). Subsequently, the cells were washed and resuspended in incubation medium (10^7/ml) containing human serum albumin (0.5%) and $CaCl_2$ (1 mM). After incubation at 37 °C for 5 min in the presence of cytochalasin B (5 μg/ml), inhibitors or solvent were added and, after another 5 min of incubation, the cells were stimulated with fMLP (1 μM) in the presence of ethanol (0.5%, v/v). The production of phosphatidylethanol was used as a measure for PLD activity.

Measurement of superoxide production

Superoxide production was measured by the superoxide dismutase-sensitive reduction of cytochrome *c* in a continuous spectrophotometric assay with a Perkin–Elmer spectrophotometer (model Lambda 2 UV/VIS). The contents of six cuvettes measured in parallel was stirred continuously and thermostatically maintained at 37 °C. Neutrophils were resuspended in incubation medium containing 60 μM cytochrome *c* at a concentration of 10^6 cells/ml. After 5 min of preincubation in the presence of cytochalasin B (5 μg/ml), the cells were pretreated with the inhibitor or Me_2SO for 5 min, and subsequently stimulated with fMLP. When dBcAMP was present during the incubation, the cells were first incubated with dBcAMP before addition of cytochalasin B.

RESULTS

Effect of staurosporine analogues on respiratory-burst activation in human neutrophils

We first investigated the effect of staurosporine and cgp41251 on PMA-induced superoxide production, because this response is generally believed to be mediated by PKC. Both staurosporine and cgp41251 dose-dependently inhibited the production of superoxide induced by PMA (Figures 1a and 1b). The inactive structural analogue cgp42700 had no effect at all (results not shown). The difference in efficacy between staurosporine and its

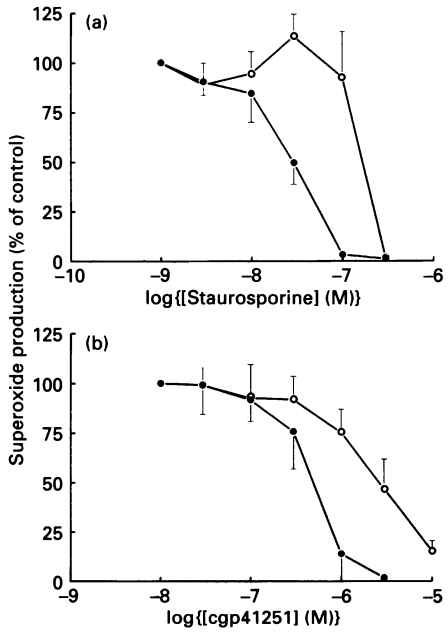

Figure 1 Effect of staurosporine and cgp41251 on superoxide release by human neutrophils stimulated with PMA or fMLP

Neutrophils (10^6/ml) were pretreated with cytochalasin B (5 μg/ml) for 5 min at 37 °C before the addition of staurosporine (**a**) or cgp41251 (**b**). The incubation was continued for another 5 min and the cells were subsequently stimulated with PMA (100 ng/ml; ●) or fMLP (1 μM; ○). With PMA as a stimulus, cytochalasin B (CB) was omitted. Results are the maximum rate of superoxide production after addition of the stimulus, expressed as percentages of the rates obtained in the presence of solvent only. Control rates were: 7.5±1.5 and 13.8±2.7 nmol of superoxide/min per 10^6 cells for PMA and CB/fMLP respectively (means±S.E.M., $n = 4$).

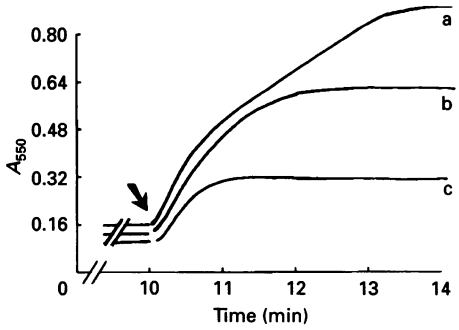

Figure 2 Effect of staurosporine and cgp41251 on the time course of fMLP-induced superoxide production by human neutrophils

Neutrophils (10^6/ml) were pretreated with cytochalasin B (5 μg/ml) for 5 min at 37 °C before the addition of solvent (trace a), 100 nM staurosporine (trace b) or 1 μM cgp41251 (trace c). The incubation was continued for another 5 min and the cells were subsequently stimulated with 1 μM fMLP (arrow). The traces shown are representative of three different experiments performed with neutrophils isolated from different donors.

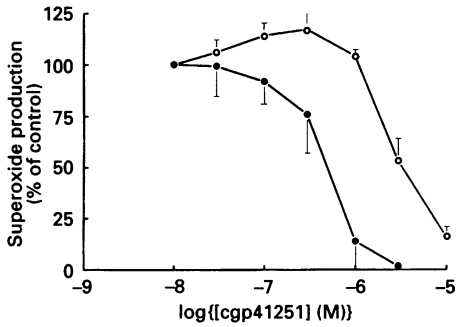

Figure 3 Effect of ionomycin pretreatment on the inhibition of PMA-induced superoxide production by cgp41251

Neutrophils (10^6/ml) were pretreated with 1 μM ionomycin (○) or solvent (●) for 3 min before addition of cgp41251, and the incubation was continued for 5 min. Subsequently the cells were stimulated with PMA (100 ng/ml). Results are expressed as the percentage of the control rate obtained in the absence of inhibitor. Control rates were 8.2 ± 0.4 and 19.0 ± 1.6 nmol of superoxide/min per 10^6 cells for PMA- and ionomycin/PMA-treated neutrophils respectively (means ± S.E.M., $n = 3$).

analogue cgp41251 is in accordance with the reported difference in IC_{50} values for PKC inhibition [22]. Niggli and Keller [24] have shown that both staurosporine and cgp41251 inhibit the PMA-induced phosphorylation of the 47 kDa PKC substrate in intact neutrophils.

We also evaluated the effect of these inhibitors on the fMLP-induced respiratory burst. Staurosporine was more potent in inhibiting the PMA-induced respiratory burst than the fMLP-induced burst (Figure 1a). Similar results were obtained with the more specific inhibitor cgp41251 (Figure 1b). For instance, in the presence of 100 nM staurosporine or 1 μM cgp41251, the PMA-induced burst was completely inhibited, whereas fMLP was able to initiate oxidase activation (Figure 2). However, propagation of the fMLP-induced response was severely inhibited in the presence of either staurosporine or cgp41251 (Figure 2). In fact, the potency of cgp41251 to inhibit the second phase of the fMLP-induced burst was similar to the potency to inhibit the PMA-induced response (results not shown).

The results described above could be taken as evidence for a

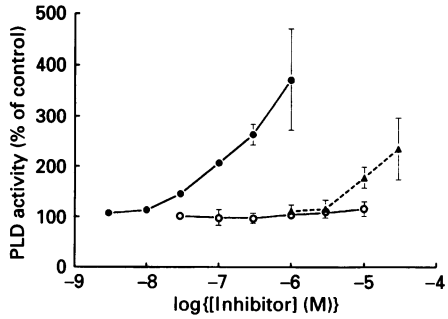

Figure 4 Effect of various protein kinase inhibitors on fMLP-induced activation of PLD

Neutrophils that had been prelabelled in the alkyl-phosphatidylcholine pool with [^{3}H]lysophosphatidylcholine were pretreated with cytochalasin B (5 μg/ml) for 5 min in the presence of ethanol (0.5%). Additionally, staurosporine (●), cgp41251 (○) or H-89 (▲) was added and the incubation was continued for another 5 min. Finally, fMLP (1 μM) was added to stimulate the cells. After 5 min the reactions were stopped, and the lipids were extracted and separated by t.l.c. Results are expressed as PLD activity as percentages of the activity obtained after fMLP stimulation in the presence of solvent only (16288 ± 1018 d.p.m.; mean ± S.E.M., $n = 3$). PLD activity in the absence of fMLP was 2.9% of this value and was not influenced by the inhibitors used.

PKC-independent respiratory burst initiation by fMLP, as has been suggested by Watson et al. [7] on the basis of staurosporine effects. However, we have considered the possibility that the sensitivity of PKC for staurosporine or cgp41251 could be decreased when the cytosolic free $[Ca^{2+}]$ is elevated, as is the case shortly after fMLP stimulation. When neutrophils were pretreated with ionomycin (1 μM) to mimic the initial increase in cytosolic free $[Ca^{2+}]$ after addition of fMLP, the PMA-induced respiratory burst also became less sensitive for cgp41251 (Figure 3) and staurosporine (results not shown). Thus the difference in sensitivity to cgp41251 of the PMA response and the initial phase of the fMLP response might be caused by the rise in $[Ca^{2+}]_i$ occurring in the latter situation and cannot be considered as a clear indication for PKC-independent activation of the respiratory burst.

Effect of staurosporine analogues on fMLP-induced PLD activation

Since PLD activation is thought to play an important role in neutrophil activation [10–14], we investigated the effects of cgp41251 and the parent compound staurosporine on fMLP-induced PLD activation. Compound cgp41251 had no effect on fMLP-induced PLD activity in concentrations up to 10 μM (Figure 4), whereas respiratory-burst activation was greatly attenuated in the presence of this inhibitor (Figure 1b). Thus it seems that PKC activity is not involved in the activation of PLD by fMLP.

The parent compound staurosporine caused a concentration-dependent potentiation of PLD activity after fMLP stimulation (Figure 4). Previously, this result has been found by Reinhold et al. [19]. Considering the lack of effect of cgp41251 on this response, it seemed probable that the enhancement caused by staurosporine was due to an action of this compound other than PKC inhibition. It has been shown that fMLP stimulation of neutrophils also results in elevation of cyclic AMP levels [25]. Possibly a negative control exerted by PKA might be lost in the presence of staurosporine, since PKA is potently inhibited by staurosporine, but much less potently by cgp41251 [22]. Indeed, fMLP-induced activation of PLD was enhanced in the presence of 30 μM H-89 (Figure 4), reported to be a specific inhibitor of

Table 1 Effect of various protein kinase inhibitors on dBcAMP-induced inhibition of PLD activity

Neutrophils prelabelled in the alkyl-phosphatidylcholine pool with [^{3}H]lysophosphatidylcholine were pretreated with staurosporine (1 μM), cgp41251 (10 μM) or H-89 (30 μM) for 5 min, then 2 mM dBcAMP (right column) or solvent (left column) was added and the incubation continued for 5 min. Subsequently, cytochalasin B (5 μg/ml) was included and after 5 min fMLP (1 μM) was added to stimulate the cells. The reactions were stopped 5 min after addition of fMLP. Results are expressed as percentage of the PLD activity in neutrophils stimulated with fMLP not exposed to dBcAMP or inhibitors (17618 ± 1105 d.p.m.; mean ± S.E.M., $n = 3$). PLD activity in the absence of fMLP stimulation was 3.2% of this value and was not affected by dBcAMP.

	PLD activity (%)	
Inhibitor	No dBcAMP	+dBcAMP
None	100	64 ± 18
Staurosporine	370 ± 99	331 ± 83
cgp41251	115 ± 5	70 ± 5
H-89	196 ± 50	179 ± 47

PKA [26]. The effect of even higher concentrations of H-89 was not analysed, because under these conditions several neutrophil responses induced by PMA became sensitive to this inhibitor (results not shown). Control experiments further indicated that both H-89 and staurosporine (but not the staurosporine analogue cgp41251) were able to abolish the inhibitory effect of a pretreatment with dBcAMP on fMLP-induced PLD activation (Table 1).

DISCUSSION

In this study we have investigated the role of PKC in two responses of human neutrophils induced by fMLP: PLD activation and NADPH oxidase activation. For this purpose we have used a staurosporine analogue (cgp41251) with enhanced specificity for PKC [22]. Our results indicate several problems with the interpretation of data previously obtained with staurosporine. The first problem is the inhibition by staurosporine of other kinases, most notably of PKA. Activation of human neutrophils with fMLP has been shown to cause a rapid increase in intracellular cyclic AMP levels [25], and hence activation of PKA. Activation of PKA is a strong inhibitory signal in human neutrophils and could possibly act as a negative-feedback signal even during fMLP stimulation. The problem of inhibition of PKA by staurosporine can be overcome by using cgp41251. In this study the effects of staurosporine and cgp41251 were compared, and the results indicate that fMLP-induced PLD activation is under negative control of PKA and does not require PKC activity. The first hypothesis is supported by the observation that H-89, a specific inhibitor of PKA [26], was able to duplicate the staurosporine effect on PLD activation (Figure 4). The second notion is based on the lack of effect of cgp41251 to interfere with fMLP-induced PLD activation (Figure 3), despite its ability to interfere with respiratory-burst activation (Figure 1b). This lack of effect was not due to the higher cell concentration used in these PLD activity measurements (results not shown). Since cgp41251 only interferes with the catalytic activity of PKC, another role for PKC, for instance as docking protein [27], cannot be excluded.

The inability of staurosporine to prevent fMLP-induced PLD activation seems not in accordance with the purported involvement of a tyrosine kinase in the response [20,28]. However, measurement of total tyrosine kinase activity in neutrophil membranes and cytosol [21] may not be indicative of the ability of staurosporine to inhibit this particular, as yet unidentified, tyrosine kinase.

Another problem in the use of staurosporine is the apparent decrease in potency to inhibit the PMA-induced response when the cytosolic free Ca^{2+} concentration is elevated. This problem is also encountered with the staurosporine analogue cgp41251 (Figure 3). It may perhaps also be responsible for the increase in IC_{50} of other staurosporine derivatives when receptor-mediated stimulation of the respiratory burst is compared with dioctanoylglycerol-induced stimulation [9]. When this decrease in potency of cgp41251 is taken into consideration, our results indicate that PKC activity is required for fMLP-induced NADPH oxidase activation, both in the initial and in the second phase of this response. As noted above, this conclusion was not reached by Watson et al. [7], but is in agreement with earlier observations by Kramer et al. [29] and Dewald et al. [30]. The latter group has postulated the requirement for two activation signals. One signal would be PKC activation and the other signal, according to present knowledge, would be PLD activation. This model can explain the inhibition of the fMLP-induced burst by cgp41251 (at the level of PKC activity) and by Ca^{2+} depletion [13,18], wortmannin [19] or ethanol [31] at the level of PLD activation. Our study clearly shows that PLD activation was not affected in the presence of cgp41251. However, respiratory-burst activation by fMLP was severely inhibited (Figures 1b and 2). Thus it seems that PLD activity alone is not a strong enough signal to activate the respiratory burst, and that PKC activity is required together with PLD to stimulate the respiratory burst by fMLP.

In the present study we analysed fMLP-induced responses in the presence of cytochalasin B. In the absence of this agent, the fMLP receptor becomes rapidly bound to the cytoskeleton [32]. As a consequence, the fMLP-induced respiratory burst in the absence of cytochalasin B is very low or even absent [33]. It should be noted that the enhancement of fMLP-induced PLD activation caused by H-89 observed in the presence of cytochalasin B (Figure 4) did not significantly affect fMLP-induced superoxide generation (results not shown). Apparently, the great enhancement of PLD activation after fMLP stimulation due to the presence of cytochalasin B [20] is already sufficient to allow maximal NADPH oxidase activity. However, when cytochalasin B was omitted from the incubation medium, the low oxidative response after fMLP stimulation was enhanced 3-fold in the presence of H-89 (results not shown), similar to the enhancement previously observed with staurosporine [8].

The present study also suggests an important regulatory role of cyclic AMP generation during fMLP-induced activation of PLD. Our results obtained with H-89 (and with staurosporine) suggest that an increase in intracellular cyclic AMP as induced by fMLP activates PKA, which in its turn exerts a negative influence on PLD activation during fMLP stimulation. Recently, Tyagi et al. [34] and Agwu et al. [35] reported an inhibitory effect of dBcAMP pretreatment on fMLP-induced PLD activity. As yet, the mechanism by which PKA activation interferes with the activation of PLD has not been elucidated. Most probably, this regulation is at the level of the receptor or the receptor-coupled G-protein, because PMA-induced PLD activation was not influenced by cyclic AMP [31,32]. In summary, this study shows that in human neutrophils data obtained with staurosporine cannot be properly interpreted, due to its ability to inhibit not only PKC activity, but also PKA activity. The use of cgp41251, a staurosporine analogue with improved selectivity, overcomes this problem, but even with this compound caution is warranted because the efficacy of inhibition of PKC is dependent on the intracellular Ca^{2+} concentration. With this caveat in mind, our

results indicate an important role for PKC activation at all stages of the fMLP-induced respiratory burst, which is not at the level of fMLP-induced PLD activity.

We thank Professor Dr. D. Roos and A. T. J. Tool for critically reading the manuscript. A.J.V. is a Research Fellow of the Royal Dutch Academy of Sciences.

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Received 1 October 1992/26 January 1993; accepted 10 February 1993