STUDIES WITH PROTEIN KINASE C INHIBITORS PRESENTLY AVAILABLE CANNOT ELUCIDATE

THE ROLE OF PROTEIN KINASE C IN THE ACTIVATION OF NADPH OXIDASE

Roland Seifert[1]* and Christoph Schächtele[2]

[1] Institut für Pharmakologie, Freie Universität Berlin,
Thielallee 69/73, D-1000 Berlin 33, F.R.G.

[2] Goedecke Forschungsinstitut, Biochemische Pharmakologie,
Mooswaldallee 1/9, D-7800 Freiburg, F.R.G.

The effects of various protein kinase C (PKC) inhibitors on NADPH oxidase (NO) activation by the phorbol ester PMA and by the chemotactic peptide FMLP were studied. H-7 reduced the effects of both stimuli in human neutrophils (HN) and HL-60 cells by 13-63%. Polymyxin B did not inhibit NO activation by PMA and FMLP in HN and reduced the effects of both stimuli in HL-60 cells by 27-55%. Retinal and retinoic acid enhanced the effects of PMA and FMLP in HL-60 cells and of FMLP in HN up to 4.5-fold. In contrast, retinoic acid inhibited the effect of PMA in HN. In the presence of cytochalasin B, retinal inhibited the effect of FMLP in HN, whereas retinoic acid inhibited NO activation by FMLP in both cell types. The dual PKC/calmodulin inhibitors trifluoperazine and W-7 abolished NO activation by PMA and FMLP in HN and HL-60 cells. Thus, the effects of PKC inhibitors on NO activation exhibit (1) cell type specificity, (2) stimulus dependency and (3) no correlation with *in vitro* inhibition of PKC. Our results suggest that studies with PKC inhibitors presently available cannot clarify the role of PKC in NO activation.   © 1988 Academic Press, Inc.

Human neutrophils possess an NADPH oxidase which catalyzes O_2^- formation and can be activated by cell-permeable diacylglycerol and PMA *via* PKC [1,2] and by the chemotactic peptide, FMLP [3]. The mechanism by which FMLP activates O_2^- formation is not known. There is a debate concerning the involvement of PKC in cellular activation by FMLP [3]. In recent studies, certain PKC

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* To whom correspondance should be addressed.

ABBREVIATIONS:
CB, cytochalasin B; FMLP, *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; O_2^-, superoxide anion; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; TFP, trifluoperazine; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

inhibitors were reported to inhibit PMA- but not receptor mediated O_2^- forma-
tion, suggesting that NADPH oxidase activation by FMLP may be independent of
PKC [4,5]. Furthermore, PKC-independent activation of NADPH oxidase in cell-
free systems was demonstrated by us and others [6,7]. In contrast, a PKC inhi-
bitor prevented the chemotactic response of neutrophils towards FMLP [8].

The difficulties in the interpretion of studies with PKC inhibitors in
intact cells have been discussed in our recent study [9]. We reported that
various PKC inhibitors failed to inhibit receptor-mediated platelet aggrega-
tion and differentially inhibited activation by a cell-permeable diacylglyce-
rol. We concluded that the failure of PKC inhibitors to suppress cellular
activation does not necessarily exclude a role of PKC in this process. There-
fore, studies with PKC inhibitors should desirably include (1) various stim-
uli, (2) chemically unrelated PKC inhibitors and, if possible, (3) different
cell types.

We investigated the effects of the PKC inhibitors, H-7 [10], polymyxin B
[11], retinal [12,13], retinoic acid [14], and of the dual PKC/calmodulin in-
hibitors, TFP and W-7 [15,16], on O_2^- formation in human neutrophils as well
as in differentiated HL-60 cells. NADPH oxidase was stimulated by PMA, FMLP
and FMLP plus CB, the latter agent potentiating the effect of FMLP by affect-
ing the state of actin polymerization [17]. We report here on differential,
stimulus-dependent inhibition or stimulation of O_2^- formation by PKC inhibi-
tors, suggesting that studies with PKC inhibitors presently available are not
suitable to elucidate the role of PKC in NADPH oxidase activation.

MATERIALS AND METHODS

H-7, W-7, TFP, polymyxin B, retinal (all trans), retinoic acid (type XX,
all trans), PMA, FMLP, CB, superoxide dismutase and ferricytochrome C (type
III) were obtained from Sigma Chemie (Taufkirchen, F.R.G.). Dibutyryl cyclic
AMP was purchased from Pharma Waldhof (Düsseldorf, F.R.G.). All cell culture
media and Ficoll-Hypaque were obtained from Biochrom (Berlin, F.R.G.). Stock
solutions of W-7 (20 mM), retinoids (10 mM), PMA (10 mg/ml), FMLP (10 mM) and
CB (1 mg/ml) were prepared in dimethyl sulfoxide. Retinoids were stored in the
dark at 4°C. Polymyxin B and H-7 (30 mM) were dissolved in the buffer de-
scribed below.
Heparinized blood was obtained by venous puncture from healthy volun-
teers of both sexes who had taken no drugs for at least 3 weeks. Neutrophils
were isolated by dextran sedimentation and centrifugation through Ficoll-Hy-

paque [7]. Cell preparations consisted of more than 98% viable neutrophils as judged by trypan blue dye exclusion.

HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% (v/v) horse serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere with 7% CO_2 at 37°C. To induce differentiation, cells were seeded at a density of 10^6 cells/ml and were cultured for 48 h in the medium supplemented with 0.2 mM dibutyryl cyclic AMP [18]. Pappenheim stains showed that the differentiated HL-60 cells consisted of more than 95% myelocytes or cells beyond this stage.

NADPH oxidase-catalyzed O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase. Reaction mixtures (1 ml) contained 2.5-5.0 x 10^6 cells, 100 µM ferricytochrome C, 0.5% dimethyl sulfoxide and a buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM HEPES, pH 7.4. Reaction mixtures were preincubated for 2 min in the absence or presence of CB and inhibitors at 37°C. O_2^- formation was initiated by the addition of PMA or FMLP. V_{max} of O_2^- formation was calculated from the linear parts of absorbance curves.

RESULTS

The effects of various PKC inhibitors on O_2^- formation in neutrophils and HL-60 cells are summarized in Tables 1 and 2. In preliminary experiments, the concentrations of PMA, FMLP and CB used in this study were found to be maximally effective to stimulate NADPH oxidase (data not shown). In order to prevent cell damage by dimethyl sulfoxide, W-7, TFP and retinoids had to be added to assay mixtures in a maximum volume of 0.5% (v/v). Thus, the maximum

Table 1: Activation of NADPH oxidase by PMA in human neutrophils and in HL-60 cells and its modulation by various inhibitors of protein kinase C

Inhibitor		NADPH oxidase activity (in % of control)			
		neutrophils		HL-60 cells	
None		100	(8)	100	(8)
H-7	(300 µM)	46 ± 7	(6)	37 ± 7	(8)
Polymyxin B	(300 µM)	93 ± 8	(6)	73 ± 11	(8)
Retinal	(50 µM)	97 ± 33	(9)	287 ± 70	(6)
Retinoic acid	(50 µM)	45 ± 20	(7)	206 ± 68	(7)
TFP	(100 µM)	0	(3)	0	(3)
W-7	(100 µM)	0	(3)	0	(3)

NADPH oxidase-catalyzed O_2^- formation was measured as described under MATERIALS AND METHODS. V_{max} of O_2^- formation in the presence of various PKC inhibitors is referred to that in the absence of these compounds. In the absence of inhibitors neutrophils and HL-60 cells generated 69.0 ± 17.6 and 15.0 ± 3.7 nmoles of $O_2^-/10^7$ cells/min upon stimulation with PMA (100 ng/ml). The numbers in parentheses indicate the number of individual blood donors and batches of HL-60 cells investigated. Data are the mean ± S.E.M.

Table 2: Activation of NADPH oxidase by FMLP in human neutrophils and in HL-60 cells and
its modulation by various inhibitors of protein kinase C

Inhibitor		NADPH oxidase activity (in % of control)						
		neutrophils				HL-60 cells		
		- CB		+ CB		- CB		+ CB	
None		100	(8)	100	(8)	100	(8)	100	(8)
H-7	(300 µM)	87 ± 12	(5)	106 ± 24	(5)	58 ± 18	(4)	70 ± 11	(4)
Polymyxin B	(300 µM)	97 ± 17	(5)	96 ± 20	(5)	45 ± 17	(4)	58 ± 7	(6)
Retinal	(50 µM)	158 ± 15	(5)	41 ± 22	(4)	445 ± 131	(3)	185 ± 33	(4)
Retinoic acid	(50 µM)	244 ± 100	(3)	55 ± 21	(4)	334 ± 47	(3)	76 ± 20	(5)
TFP	(100 µM)	0	(3)	0	(3)	0	(3)	0	(3)
W-7	(100 µM)	0	(3)	0	(3)	0	(3)	0	(3)

NADPH oxidase-catalyzed O_2^- formation was measured as described under MATERIALS AND METHODS.
V_{max} of O_2^- formation in the presence of various PKC inhibitors is referred to that in the
absence of these compounds. In the absence of inhibitors, neutrophils generated 17.5 ± 5.4
and 53.8 ± 13.5 nmoles of $O_2^-/10^7$ cells/min upon stimulation with FMLP (1 µM) and FMLP (1 µM)
plus CB (1 µg/ml), respectively. HL-60 cells produced 7.3 ± 2/5 and 21.9 ± 4.8 nmoles of
$O_2^-/10^7$ cells/min upon exposure to these stimuli. The numbers in parentheses indicate the
number of individual blood donors and batches of HL-60 cells investigated. Data are the mean
± S.E.M.

concentrations of these compounds obtainable in the assays were in the range

of those required for half-maximal inhibition of PKC *in vitro* [12-16]. The

concentrations of H-7 and polymyxin B present in the assays were 50- and 150-

fold higher than those required for half-maximal inhibition of PKC *in vitro*

[10,11].

H-7 inhibited PMA-induced O_2^- formation in neutrophils and in HL-60

cells by 54 and 63%, respectively. Polymyxin B reduced the effect of PMA in

neutrophils only marginally and inhibited the one of PMA in HL-60 cells by

27%. Retinal did not affect NADPH oxidase activation by PMA in neutrophils and

potentiated the effect of PMA in HL-60 cells. In contrast, retinoic acid in-

hibited the effect of PMA in neutrophils by 55% and enhanced the effect of PMA

in HL-60 cells more than twice. TFP and W-7 completely prevented PMA-induced

O_2^- formation in both cell types.

In neutrophils, H-7 did not inhibit NADPH oxidase activation by FMLP +

CB and reduced the effect of FMLP in the absence of CB by 13%. In contrast, H-

7 reduced the FMLP-induced O_2^- formation in HL-60 cells by 30 and 42% in the

presence and absence of CB, respectively. Polymyxin B did not inhibit NADPH oxidase activation by FMLP ± CB in neutrophils, but reduced the effect of FMLP ± CB in HL-60 cells by 42 and 55%, respectively. The effects of retinoids on receptor-mediated O_2^- formation depended on the absence or presence of CB in the assay mixtures: In the absence of CB, retinal enhanced O_2^- formation by FMLP in both cell types up to 4.5-fold. In the presence of CB, retinal inhibited FMLP-induced O_2^- formation in neutrophils and stimulated the one in HL-60 cells. Retinoic acid potentiated O_2^- formation induced by FMLP in both cell types. In contrast, retinoic acid inhibited receptor-mediated O_2^- formation in the presence of CB. As was the case with NADPH oxidase activation by PMA, the one induced by FMLP ± CB in both cell types was completely prevented by TFP and W-7.

DISCUSSION

We demonstrate differential inhibition and stimulation of O_2^- formation by PKC inhibitors, using various stimuli and two related cellular systems, possessing similar transmembrane signalling mechanisms. In agreement with previous studies, we found that H-7 and polymyxin B did not substantially inhibit receptor-mediated activation processes in neutrophils [5,6]. However, these compounds substantially inhibited FMLP-induced O_2^- formation in HL-60 cells. These differences may be explained, at least in part, by different permeation of these compounds into the cells and support the view that the effects of PKC inhibitors exhibit cell type specificity [9]. However, neither with H-7 nor with polymyxin B complete inhibition of O_2^- formation was observed regardless of the stimulus. Polymyxin B was recently reported not to inhibit PMA-induced protein phosphorylation in HL-60 cells [19]. One explanation for these results may be the possibility that H-7- and polymyxin B-insensitive PKC molecules mediate cellular activation which may play a role in both PMA- and FMLP-induced activations of NADPH oxidase. However, even inhibition of receptor-mediated NADPH oxidase activation by these compounds in HL-60 cells does not necessarily imply that PKC is involved in this signalling process: At the high concen-

trations used in our study, polymxin B may also inhibit ion channels [20] and calmodulin-dependent processes [11]. In comparison, H-7 inhibits cyclic nucleotide- and calmodulin-dependent kinases [10], which may contribute to the inhibition of O_2^- formation.

Even more difficult to interprete are the results concerning retinoid-modulation of O_2^- formation. In agreement with previous reports, retinoic acid inhibited O_2^- formation in neutrophils induced either with PMA or FMLP plus CB [21,22]. However, retinoids potentiated receptor-mediated activation of NADPH oxidase in the absence of CB. In addition, retinoids enhanced NADPH oxidase activation by PMA in HL-60 cells, indicating that retinoids do not generally antagonize the effects of phorbol esters [21]. Retinoids do not only inhibit but also stimulate PKC *in vitro* under certain experimental conditions [13,14, 23]. In addition, retinoids were reported to activate NADPH oxidase in neutro-phils [24]. We found that retinoic acid and retinal did not induce O_2^- forma-tion in HL-60 cells (data not shown). In neutrophils retinal at concentrations up to 100 μM also did not activate NADPH oxidase, whereas retinoic acid (50 μM) induced the formation of 16.9 ± 6.9 nmoles of O_2^-/10^7 cells/min (n=6). Thus, retinoids may act as activators or inhibitors of PKC *in vivo*, with their effects varying with the cell type and the experimental conditions. Finally, the finding that retinoids potentiated receptor-mediated activation of NADPH oxidase in the absence of CB suggests that these compounds exhibit CB-like properties [25].

In contrast to H-7, polymyxin B and retinoids, the dual PKC/calmodulin inhibitors, TFP and W-7, completely inhibited O_2^- formation without stimulus or cell type specificity. Thus, it may be tempting to speculate that calmodu-lin- rather than PKC-mediated reactions are involved in NADPH oxidase activa-tion by PMA and FMLP [26]. There is, however, no additional evidence that PMA activates NADPH oxidase *via* calmodulin [2]. Interestingly, it was recently re-ported that TFP and W-7 but not H-7 strongly inhibited the PKC- and calmodu-lin-independent activation of NADPH oxidase in cell-free systems [7,27]. These data suggest that W-7 and TFP may be potent inhibitors of NADPH oxidase *per*

se. This interpretation is also supported by our present results, showing that these compounds exhibited very untypical inhibitory profiles on NADPH oxidase activation in comparison to other PKC inhibitors.

In conclusion, it appears difficult to correlate inhibition or stimulation of O_2^- formation by various classes of PKC inhibitors with modulation of PKC activity *in vivo*. Thus, studies with the PKC inhibitors presently available can apparently not elucidate the precise role of PKC in the course of NADPH oxidase activation. These results, however, do not exclude the possibility that potent and specific PKC inhibitors may provide valuable tools to elucidate the role of PKC in cellular activation processes.

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