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STAUROSPORINE, A PROTEIN KINASE INHIBITOR, UP-REGULATES THE STIMULATION OF HUMAN NEUTROPHIL RESPIRATORY BURST BY N-FORMYL PEPTIDES AND PLATELET ACTIVATING FACTOR

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Staurosporine (STAR), a potent protein kinase C (PKC) antagonist, was found to modulate the chemoattractant-induced respiratory burst of human polymorphonuclear leukocytes (PMNs) according to drug concentration. Low STAR concentrations from 10 to 200 nM potentiated the N-formyl-methionyl-leucyl-phenylalanine (fMLP) and platelet activating factor (Paf)-induced respiratory burst, affecting both the initial rate and the total amount of superoxide anion generated. The maximal increase occurred in the presence of 100 nM STAR and optimal fMLP concentrations and reached 60-100 % of control values. Above 250 nM, STAR inhibited the respiratory burst with an IC_{50} of 360 and 320 nM for fMLP and Paf, respectively. The respiratory burst induced by PKC activators such as phorbol myristate acetate or phorbol 12, 13 dibutyrate was inhibited effectively by STAR, with a low IC_{50} (25 nM) for both stimuli. Thus, the use of low STAR concentrations points to two possible roles of PKC in the regulation of NADPH oxidase activity, i.e. a positive regulation in phorbol ester-treated cells and a negative regulation in chemoattractant-stimulated PMNs .

The calcium and phospholipid-dependent protein kinase C (PKC) is a key enzyme in the regulation of numerous cellular activities (1, 2). At low calcium concentrations PKC activity is strongly stimulated by diacylglycerol and phorbol ester tumor promoters (1, 3). A number of inhibitors have been used to investigate the roles of PKC in cellular activities including isoquinoline family members (4, 5), sphingosine (6), chlorpromazine (7) and palmitoylcarnitine (8). Unfortunately, the use of these agents in biological systems is unsatisfactory because of their low potency and limited selectivity. Recently, a new class of PKC inhibitors with high potencies has been described. Staurosporine (STAR), a microbial alkaloid from *Streptomyces* species inhibits PKC activity at nanomolar concentrations (10). The exact mechanism of action of this drug remains to be

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Abbreviations used : PMN, polymorphonuclear leukocytes; STAR, staurosporine; fMLP, N-formyl-metionyl-leucyl-phenylalanine; Paf, platelet-activating factor; PKC, protein kinase C; PMA, phorbol myristate acetate; PDBu, phorbol 12, 13 dibutyrate; IC_{50}, concentration of drug that reduces control values by 50%; $O_2^{-\cdot}$, superoxide anion.

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elucidated. STAR does not alter the binding of phorbol esters to the regulatory domain of PKC (10) but is known to inhibit the proteolitically-generated fragment of the enzyme (9, 10), most likely by interfering with ATP binding (11).

In human PMNs, the phorbol ester-induced generation of superoxide anion (respiratory burst) is effectively inhibited by STAR (12), whereas stimulation of this function by chemoattractants, e.g. fMLP has been found unaltered (12) or decreased (13). In this report, we show that STAR induces up- or down-regulation of chemoattractant-stimulated PMN respiratory burst, depending on the drug concentration. Potentiation affected both the initial rate and total amount of oxidase activation and occurred at low STAR concentrations that block the effects of PMA and PDBu. These results suggest different roles of PKC in the regulation of NADPH oxidase activity.

MATERIALS AND METHODS

The sources of staurosporine, monopoly resolving medium and other reagents have been given elsewere (14).

Isolation of PMNs. Heparinized human venous blood obtained from healthy volunteers was centrifuged over a cushion of a mixture of ficoll and hypaque (monopoly resolving medium) as described previously (14). The purified PMNs (97%) were subjected to hypotonic lysis, washed and resuspended in HBSS.

PMN respiratory burst. The production of superoxide anion was continuously recorded by monitoring the superoxide dismutase-inhibitable reduction of cytochrome C (15) using a Uvikon 860 spectrophotometer equipped with a thermally controlled cuvette holder and a magnetic stirrer. Aliquots of 2x106 cells were preincubated in the absence (control) or in the presence of staurosporine for 10 min at 37°C before stimulation. The final concentration of the drug solvent (DMSO) in the medium did not exceed 0.1% and had no effect on PMN respiratory burst. Results are expressed as a percentage of control values ± SEM and are the means of at least 4 experiments.

Statistics. Statistically significant differences between experiments performed in the absence and presence of staurosporine were identified using Student's paired t test.

RESULTS AND DISCUSSION

Figure 1A shows a comparison of STAR effects on initial rate and total amount of superoxide anion ($O_2^{-}\cdot$) production by PMNs stimulated with 2 μM Paf. At concentrations ranging from 10 to 180 nM, STAR induced a similar biphasic potentiation of both respiratory burst parameters. The maximal increase was observed in the presence of 100 nM STAR and reached 97 ± 25 % and 130 ± 15 % of control values for the initial rate and total production of $O_2^{-}\cdot$, respectively. Both parameters were inhibited in a dose-dependent manner by STAR concentrations above 180 nM. The STAR concentrations reducing 50 % of initial rate and total $O_2^{-}\cdot$production by control cells (IC_{50}) were 316 ± 57 and 350 ± 14 nM, respectively. When respiratory burst was stimulated by 100 nM fMLP, STAR also induced diverging biological effects (fig. 1B). High STAR concentrations (above 200 nM) decreased both respiratory burst parameters similarly with IC_{50} values of 365 ± 34 and 360 ± 34 nM for initial rate and total amount of $O_2^{-}\cdot$, respectively. In contrast, low STAR concentrations potentiated respiratory burst with a maximal effect also observed with 100 nM STAR. However, the increase in the

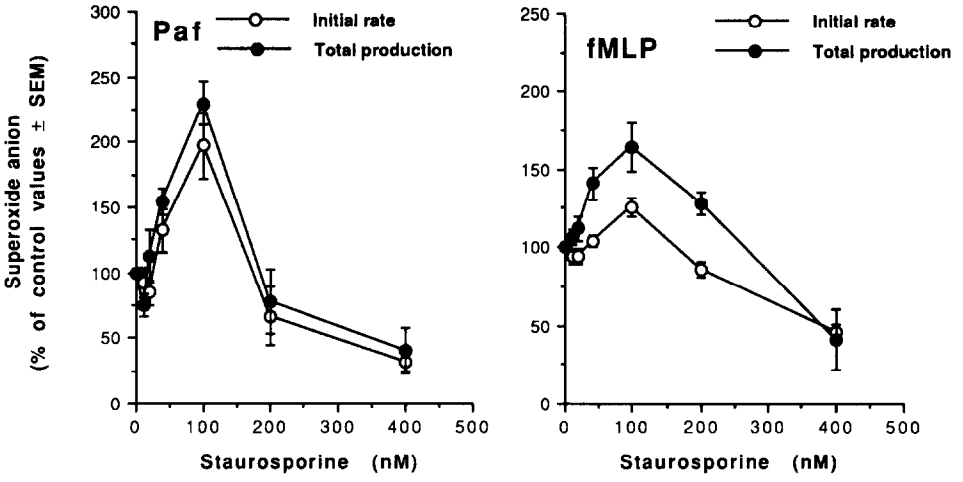

Figure 1. Effect of staurosporine on Paf- and fMLP-induced PMN respiratory burst. PMNs were pretreated in the absence (control) or presence of staurosporine at the indicated concentrations for 10 min and stimulated with 2 μM Paf (left half) or 0.1 μM fMLP (right half). The initial rate and the total amount of $O_2^{-}\cdot$ generated are expressed as % of control values. The initial rate and total amount of $O_2^{-}\cdot$ produced by 10^6 cells in the absence of staurosporine were 1.20 ± 0.15 nmoles/min and 0.52 ± 0.09 nmoles respectively for Paf and 2.40 ± 0.32 nmoles/min and 4.30 ± 0.85 nmoles respectively, for fMLP.

initial rate was only 26 ± 6 % of control values, whereas the rise in total $O_2^{-}\cdot$ production was 64 ± 16 %, a value close to that found with Paf (fig. 1A).

These data suggest that in chemoattractant-treated PMNs both activating and terminating processes of the respiratory burst are down-regulated by a mechanism mediated by protein kinases. However, as STAR was significantly ($P<0.05$) less potent on the initial rate than on the total amount of $O_2^{-}\cdot$ generated by fMLP-stimulated PMNs, we compared its effects on the respiratory burst stimulated by various fMLP concentrations (fig. 2). In the absence of STAR, both respiratory burst parameters rose proportionally to the fMLP concentration up to 0.5 μM (fig.2A). At higher concentrations the total amount of $O_2^{-}\cdot$ declined ($P<0.05$), whereas the initial rate remained unchanged. At optimal and higher concentration of fMLP, the STAR-induced enhancement of both respiratory burst parameters was similar (fig. 2B) but was inversely related to fMLP concentration. At lower fMLP concentrations, enhancement of the total amount of $O_2^{-}\cdot$ remained maximal, whereas that of the initial rate was proportional to fMLP concentration. In contrast to chemoattractants, the respiratory burst induced by PMA and PDBu, both PKC activators, was blocked by low STAR concentrations (fig. 3). However, with PDBu both respiratory burst parameters were similarly reduced (IC_{50} : 26 ± 5 and 27± 6 nM), whereas with PMA the initial rate was significantly ($P<0.05$) less inhibited (IC_{50} : 53 ± 6 nM) than the total amount of $O_2^{-}\cdot$ (IC_{50} : 27 ± 7 nM). Thus, in PMA-stimulated PMNs, the initiation and or activation of the respiratory burst may occur through a mechanism which differs slightly from that following PDBu stimulation.

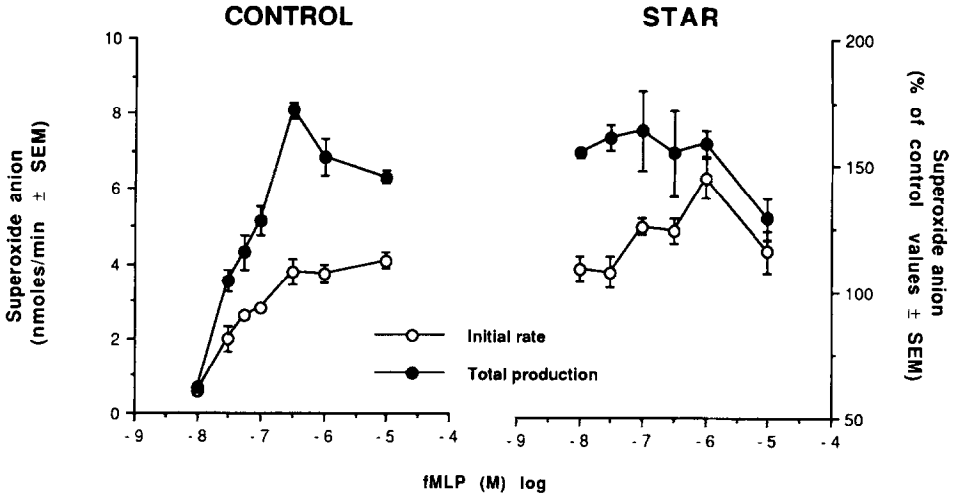

Figure 2. Effect of fMLP on the respiratory burst of control and staurosporine-treated PMNs. PMNs were incubated in the absence (left half) or presence of 100 nM staurosporine (right half) and stimulated with various fMLP concentrations. The initial rate and total production of O_2^{-}· by 10^6 PMNs are expressed as nanomoles O_2^{-}· (control values) or as % of control values.

STAR has been widely used to investigate the regulatory role of PKC. However, it is now evident that this agent alters the activity of other kinases (16), meaning that the biological effects induced by STAR cannot always be interpreted as being due to the inhibition of PKC. The ability of STAR to prevent the phorbol ester-stimulated PMN respiratory burst (fig. 3) confirms and extends previous results obtained with this class of

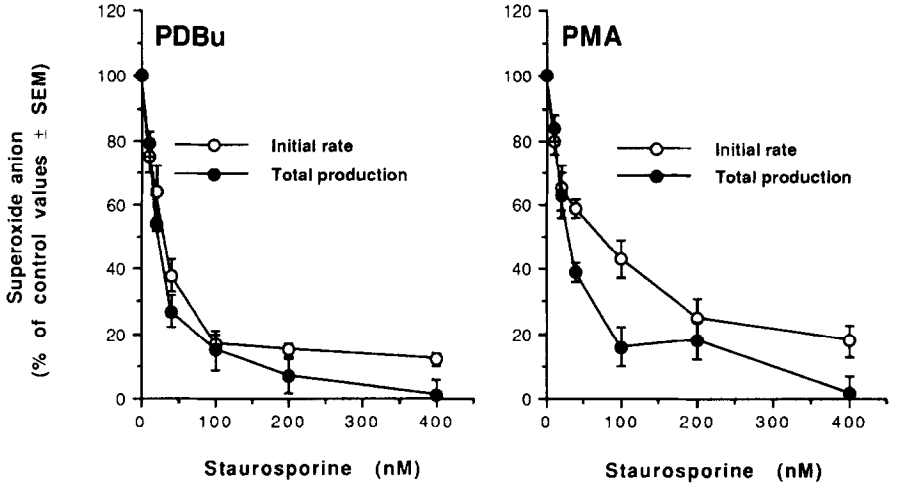

Figure 3. Effect of staurosporine on PDBu- and PMA-induced PMN respiratory burst. PMNs were pretreated with or without (control) various staurosporine concentrations for 10 min and stimulated with 500 ng/ml PDBu (left half) or 100 ng/ml PMA (right half). The initial and total O_2^{-}· produced by 10^6 PMNs during 10 min are expressed as % of control values. The initial rate and total amount of O_2^{-}· produced by 10^6 cells in the absence of staurosporine were 4.30 ± 0.85 nmoles and 38.0 ± 6.50 nmoles for PDBu and 6.90 ± 0.99 nmoles/min and 50.7 ± 4.80 nmoles for PMA.

inhibitor (12, 17, 18). Since phorbol esters selectively activate PKC (1, 2, 19, 20), the blockading effects of low concentrations of STAR support the hypothesis that PMA and PDBu mediate the activation of NADPH oxidase through a PKC-dependent process (21, 22). The behaviour of the respiratory burst following chemoattractant stimulation reveals two distinct properties of STAR, i.e. inhibitory effects seen with relatively high drug concentrations, as described with other STAR analogues (18) and a biphasic potentiation observed with low STAR concentrations (fig. 1). This latter effect was maximal in the presence of the optimal fMLP concentration (fig. 2B) and declined with higher fMLP concentrations. This may explain why under certain conditions, STAR failed to affect the fMLP-induced respiratory burst (12). The diverging STAR effects described here have not been reported with other PKC inhibitors and may be due to the ability of STAR to alter potently PK activities (16) or to a possible exposure of other pools of PKC. From these results it is tempting to postulate that the potentiated respiratory burst observed with low STAR concentrations results from an inhibition of PKC activity whereas the reduction in the respiratory burst induced by high STAR concentrations occurs through the inhibition of other PK activities. The observation that potentiation occurs at low STAR concentrations that block the effect of PMA raises the possibility that chemoattractants trigger NADPH oxidase activation through a different pathway from that induced by phorbol esters. The termination process of the respiratory burst induced by both types of stimuli may also differ since the STAR concentrations that inhibited fMLP- or Paf-induced PMN responses were approximately 10 times greater than those required with phorbol esters (fig. 1B and 3).

Interestingly, potentiation affected both the initial rate and total production of $O_2^{-}\cdot$ and was optimal in the presence of maximally active fMLP concentrations, suggesting the drug may alter early transductional events induced by chemoattractant receptors. In platelets, STAR enhanced the production of inositol trisphosphate induced by Paf (23). fMLP and Paf stimulate the hydrolysis of phosphatidyl inositol 4-5 biphosphate (PIP2) through the activation of a guanine nucleotide regulatory protein (G protein) that couples chemoattractant receptors to phospholipase C (24-27). Increased generation of inositol trisphosphate and diacylglycerol may thus account for the enhancement of the chemoattractant-induced respiratory burst. The STAR potentiation of phospholipase C in platelets is associated with an inhibition of protein phosphorylation (23), suggesting that activation of protein kinases mediates down-regulation of the transductional pathway. This hypothesis is supported by the observation that pretreatment of PMNs with high PMA doses inhibits calcium mobilization (28) and PIP2 hydrolysis induced by chemoattractants, through a mechanism affecting G-protein-phospholipase C coupling (29). It is possible that STAR potentiates the chemoattractant-induced respiratory burst by preventing autoregulation of transductional events mediated by PKC activation.

In summary, treatment of human PMNs with STAR induced a positive and negative regulation of fMLP- and Paf-induced respiratory burst, according to the drug concentration. Potentiation occurred at STAR concentrations that block phorbol ester-

induced biological effects and involved both initial rate and total amount of $O_2^{-\cdot}$ generated. These results suggest at least two different roles of PKC in the regulation of NADPH oxidase activity, i.e. a positive regulation in phorbol ester-treated cells and a negative regulation in chemoattractant-stimulated cells

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