Stimulation of polymorphonuclear neutrophils (PMN) by phorbol esters or formyl peptides (fMLP) generates large quantities of superoxide anion, the so-called respiratory burst (RB), a phenomenon associated with intense phosphorylation of a 47-kD protein (p47 phox). Staurosporine, a potent protein kinase C (PKC) antagonist, inhibits both responses when PMN are stimulated by phorbol myristate acetate (PMA), suggesting a positive role of PKC. In this study, we reassessed these PMN responses in fMLP-stimulated cells and found that staurosporine had opposite effects depending on the duration of PMN treatment with staurosporine. Short PMN incubation (0.5 to 3 minutes) with 25 to 100 nmol/L staurosporine inhibited the fMLP-induced RB, whereas longer treatment (15 to 20 minutes) enhanced it by up to about 200% relative to controls. In contrast, the PMA-mediated RB was depressed by staurosporine in a time-dependent manner. A primed fMLP-induced RB was also observed after long (15 minutes) PMN treatment with 5 to 100 nmol/L H-7, whereas shorter treatment (5 minutes) resulted in a small decrease in RB. By contrast, the tyrosine kinase inhibitor genistein (2 to 80 μmol/L) depressed fMLP-induced RB whatever the duration of PMN treatment. Analysis of 32P-phosphorylated RB showed that the duration treatment with staurosporine is a depressive effect, staurosporine markedly potentiated fMLP-stimulated RB as a function of the duration of PMN treatment. The restoration of p47 phox phosphorylation was reduced in a time-dependent manner. Furthermore, the staurosporine-primed RB and the staurosporine-induced recovery of phosphorylation were inhibited by sphingosine but not by genistein. Thus, in addition to its known depressive effect, staurosporine markedly potentiated fMLP-stimulated RB as a function of the duration of PMN treatment. The restoration of p47 phox phosphorylation suggests that staurosporine may alter the interactions between different protein kinases, producing marked time-dependent changes in signalling pathways. These data emphasize the care that should be taken in interpreting data obtained using this kinase inhibitor that may, however, be helpful in analyzing in signalling pathways.

© 1993 by The American Society of Hematology.
ries, Puteaux, France) as described previously. The purified PMN were subjected to hypotonic lysis, washed, and resuspended in Hank's Balanced Salt Solution (HBSS) with 1.2 mmol/L CaCl₂.

**PMN RB.** The production of superoxide anion was continuously recorded by monitoring the superoxide dismutase-inhibitable reduction of cytochrome C, using a UVIKON 860 spectrophotometer (KONTRON Instruments, Zurich, Switzerland) equipped with a thermostated (37°C) cuvette holder and a magnetic stirrer. Suspensions of 2 × 10⁶ PMN in 2 mL HBSS were incubated in the absence (control) or presence of kinase inhibitors for various times before stimulation. The final concentration of drug solvent did not exceed 0.1% and had no discernable effect on RB. Results are expressed in nanomoles of superoxide per minute and per 10⁶ PMN, calculated in initial rate conditions, ie, from the linear part of the curve. The viability of PMN, which had been treated with the drugs and stimulated, was checked using the Trypan-blue exclusion method. Except for sphingosine, which was associated with approximately 20% of dead cells (versus 7% to 8% in controls), the drugs were not cytotoxic.

**Stimulation of protein phosphorylation in [³²P]-labeled PMN.** PMN (50 × 10⁶/mL) suspended in loading buffer (20 mmol/L HEPES, 12 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L D-glucose) were incubated for 60 minutes at 37°C in the presence of 0.5 mg/mL [³²P]-phosphoric acid. The cells were then washed 3 times with loading buffer and resuspended in loading buffer supplemented with 1.2 mmol/L CaCl₂, 0.49 mmol/L MgCl₂, and 0.40 mmol/L MgSO₄. [³²P]-labeled PMN (2.5 × 10⁶/mL) were treated with 50 nmol/L staurosporine for various times at 37°C and stimulated with either 100 nmol/L fMLP for 2 minutes or 100 ng/mL PMA for 3 minutes. They were then diluted in 10 mL of ice-cold loading buffer and collected by centrifugation at 4°C for 10 minutes at 4°C. The pellets were resuspended in lysis buffer composed of 20 mmol/L Tris HCl, pH 7.5, 0.25 mol/L sucrose, 2 mmol/L phenylmethylsulphonyl fluoride, 0.2 mmol/L leupeptine, 50 mmol/L 2-mercaptoethanol, 2 mmol/L EDTA, and 2 mmol/L EGTA and disrupted by sonication at 25 W (4 bursts of 10 seconds each) using a Fisher model 20/200 SV TC4C (C.I.T., Alcatel-Pons, France). Postnuclear supernatants were centrifuged at 100,000g for 60 minutes in a Beckman TL100 Beckman Instruments, Palo Alto, CA) and the soluble fraction was separated from the pellets (particulate fractions). Protein was assayed with BIORAD assay (Ivry-sur-Seine, France) and precipitated with an equal volume of 20% trichloracetic acid. Precipitates were washed twice with 10% volume of 20% trichloracetic acid. Precipitates were washed twice with 0.1 mol/L NaOH. Samples (25 µg) were electrophoresed on a polyacrylamide gel containing 0.05% SDS. The gels were stained, fixed, and autoradiographed with intensifying screens with Kodak X-OMAT films (Eastman-Kodak, Rochester, NY). Phosphorylated proteins were predominantly present in the soluble fraction. It is possible that some particle-bound protein was released during the sonication step, and the results are thus those obtained with the soluble fractions.

**Identification of p47 phox.** Protein A sepharose was prepared by swelling the beads in distilled water for 30 minutes. The beads were sedimented and resuspended in lysis buffer containing 5% BSA, 0.01% sodium azide, and a soluble fraction of unlabeled PMN (1%), and rocked for 1 hour at 4°C. They were then washed 3 times with distilled water and resuspended in an equal volume of water. Immunoprecipitation of p47 phox was performed by incubating soluble fraction of 10⁷ [³²P]-labeled PMN with 25 µg of MoAb to p47 phox for 2 hours at 4°C and then with 100 µL of protein A-sepharose beads for 2 hours at 4°C. The immune complex was sedimented by centrifugation (10,000g for 3 minutes), washed 3 times with 0.5 mL of lysis buffer, and washed twice with 0.5 mL of 10 mmol/L Tris, pH 7.4. Finally, the pellet was resuspended in 100 µL of electrophoresis buffer, boiled for 3 minutes, and electrophoresed as described above. Autoradiography films were scanned using a densitometer (OneScanner; Apple Computer, Paris, France) with version 3.05 Image 1.45 gel analysis software.

**Statistical analysis.** Values are given as means ± SEM of at least three duplicate experiments. Statistically significant differences between experiments were identified using Student's paired t-test.

**RESULTS**

**Effect of the duration of PMN treatment with staurosporine on fMLP- and PMA-induced RB.** The effect of staurosporine on superoxide anion production by PMN stimulated with 100 nmol/L fMLP was analyzed as a function of the time of treatment (Fig 1, left panel). Short treatment (0.5, 1, and 3 minutes) with staurosporine concentrations from 25 to 100 nmol/L inhibited the fMLP-induced RB, proportionally to the concentration of staurosporine. Surprisingly, when treatment with staurosporine was increased to 10 minutes, the inhibition was progressively lost, and longer treatments (15 and 20 minutes) enhanced the fMLP-induced RB. The maximal effect was observed with PMN treated with 50 nmol/L of staurosporine for 20 minutes (213% ± 12% of control values; P < .05). Staurosporine concentrations of 25 or 100 nmol/L were less effective (178 ± 6; P < .05 and 181 ± 15; P < .05). When PMN treatment exceeded 20 minutes, the enhancing effect was progressively lost, and no effect was detectable after 1 hour of treatment (data not shown). Staurosporine concentrations from 25 to 100 nmol/L induced a concentration-dependent inhibition of the PMA-mediated RB, as previously reported. Maximal inhibition was reached after 30 seconds of PMN treatment with 100 nmol/L staurosporine and did not change with longer treatment (Fig 1, right panel), finding consistent with a positive influence of PKC in this model.

**Effect of the duration of PMN treatment with H-7 and genistein on fMLP- and PMA-induced RB.** The above results show that staurosporine primed the fMLP-induced RB when the duration of cell treatment by staurosporine exceeded 10 minutes. To determine whether such a priming effect was unique to staurosporine, we analyzed the effect of two other kinase antagonists: H-7, an isooquinoline sulfonamide that interferes with the adenosine triphosphate-bind-
after treatment with H-7 for 15 minutes (Fig 2, right panel), the RB was depressed in a concentration-dependent manner with an IC50 of approximately 100 μmol/L, in agreement with other reports.30-32

Pretreatment of PMN for 3 and 15 minutes with 1 to 80 pmol/L genistein resulted in a concentration-dependent inhibition of the fMLP-induced RB, with an IC50 of approximately 10 μmol/L (Fig 2, middle panel). These drug concentrations did not alter the PMA-mediated RB (Fig 2, right panel), in agreement with other data.33 However, a higher concentration of genistein of 200 pmol/L reduced the RB by only 20%.

The production of superoxide anion by a cell-free system consisting of a particulate fraction from PMA-stimulated PMN was not altered by staurosporine concentrations from 25 to 1,000 nmol/L (results not shown). The result indicates that staurosporine does not affect the catalytic activity of reconstituted NADPH oxidase. The above differential effects of staurosporine probably occur through alterations in signal transduction pathways.

Staurosporine both inhibited and potentiated the fMLP-induced RB when cells were pretreated for short (3 minutes) and long (15 minutes) periods, respectively. A possible mechanism for the enhanced staurosporine effect is that inhibition of PKC is reversed after 15 minutes. If this were the case, further short PMN treatment with kinase antagonists should reduce the priming effects of staurosporine. Alternatively, different kinases may be involved. To gain insight into these points, we set up a model in which PMN were first treated with staurosporine in priming conditions (15 minutes) and then in inhibiting conditions (3 minutes) with freshly added kinase inhibitors such as sphingosine, staurosporine, and genistein before stimulation with fMLP. Figure 3 compares the effects of the three drugs on both primed and unprimed fMLP-induced RB. When PMN were not treated by staurosporine, genistein (Fig 3, left panel) depressed the fMLP-induced RB in a concentration-dependent manner, with a complete inhibition at 80 pmol/L. Genistein also significantly reduced the total primed RB. However, the difference between the primed and unprimed productions, which may represent the response mediated by staurosporine, was not decreased by genistein. In contrast, sphingosine and staurosporine reduced (P < .05) the difference between the primed and unprimed RB, suggesting a possible regulation by PKC of the enhancing effect of staurosporine (Fig 3, middle and right panels).

Staurosporine modulates the phosphorylation of the 47-kD protein in fMLP-stimulated PMN. In the generally accepted model for RB stimulation, PKC-dependent phosphorylation of proteins is required for the RB.1•16 Among these, a protein of 47 kD has been identified.10,11,13 The influence of the duration of treatment with staurosporine on protein phosphorylation in fMLP-stimulated 32P-labeled PMN is reported in Fig 4. FMLP increased the phosphoryla-
STAUROSPORINE PRIMES PMN RESPIRATORY BURST

Fig 3. Effect of genistein, sphingosine, and staurosporine treatment on the fMLP-induced RB of (○) control and (●) staurosporine-treated PMN. PMN were treated in the absence or presence of 50 nmol/L staurosporine for 20 minutes. Three minutes before stimulation with 100 nmol/L fMLP, PMN were again treated with various concentrations of genistein (left panel), staurosporine (middle panel), or sphingosine (right panel). The results represent the initial rate of superoxide production and are expressed as nanomoles per minute per 10⁶ PMN.

Fig 4. Effect of staurosporine on protein phosphorylation in fMLP-stimulated PMN. [³²P]-labeled PMN (2.5 × 10⁶/mL) were treated for the indicated times with 50 nmol/L STAR and were then stimulated with 100 nmol/L fMLP for 2 minutes. The results represent the phosphorylated proteins analyzed by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography. The duration of PMN treatment with staurosporine is indicated below each lane. Similar results were obtained in three other experiments.

When PMN pretreated with staurosporine for 20 minutes were again submitted to short treatment (3 minutes), inhibiting conditions (Fig 6, lane 2), with freshly added kinase antagonists (sphingosine, staurosporine, and genistein), only staurosporine (50 nmol/L) and sphingosine (1 μmol/L) inhibited the restoration of the p47 phosphorylation mediated by long staurosporine treatment of PMN (Fig 6, lanes 6 and 7), whereas genistein (50 μmol/L) had no effect (Fig 6, lane 5).

Modulation of the phosphorylation of p47 phox by staurosporine. To determine whether altered phosphorylation of p47 was due to the NADPH oxidase factor p47 phox, we analyzed phosphorylation of immune complexes obtained by treating soluble fractions of [³²P]-labeled PMN with antibody against p47 phox. The results in Fig 7 show that, in comparison with resting PMN, PMA and fMLP stimulated an activity that immunoprecipitated with anti-p47 phox. This activity was absent when the soluble fraction of PMA-stimulated PMN was treated with an irrelevant antibody of
2894

COMBADIÈRE ET AL

the same isotype as anti-p47 phox. When PMN were treated with staurosporine for short times (5 to 10 minutes) p47 phox phosphorylation induced by fMLP was inhibited, whereas phosphorylation was partially restored after longer cell treatments (Fig 8, upper panel). On the basis of densitometric analysis, the inhibition of phosphorylation was almost complete, whereas the restoration reached approximately 50% of the control value (Fig 8, lower panel).

DISCUSSION

The novel observation described in this study is that fMLP stimulation of the PMN RB is respectively prevented and primed by short (<5 minutes) and long treatment (15 to 20 minutes) with staurosporine. In contrast, the PMA-induced RB was always depressed. The inhibition of the RB induced by fMLP or PMA was associated with a decrease in the phosphorylation of p47, identified as p47 phox, whereas the primed RB was associated with partial restoration of p47 phox phosphorylation. This restoration and the primed RB were inhibited by sphingosine and staurosporine but not by genistein, suggesting that the recovery of p47 phox phosphorylation may contribute to RB priming by staurosporine.

Staurosporine is a potent PKC inhibitor and has been...
STAUROSPORINE PRIMES PMN RESPIRATORY BURST

Fig 7. Identification of p47phox in fMLP- and PMA-stimulated PMN. [32P]-labeled PMN were treated in the absence (lane 1) or in the presence of 100 nmol/L fMLP (lane 3) or 80 nmol/L PMA (lanes 2 and 4) for 2 minutes. Soluble fractions of these PMN were treated with an MoAb against p47phox (lanes 1 through 3) or an irrelevant MoAb of the same isotype (lane 4) and precipitated. Results represent the phosphorylation recovered in the precipitates after electrophoresis and autoradiography.

widely used to analyze the role of this enzyme in cell signaling and cell functions. Although staurosporine is not a specific PKC inhibitor,38,39 several studies have clearly shown that it prevents the PMN RB induced by phorbol esters,14,15,18,21 indicating a positive role of PKC in this function. By contrast, controversial alterations of receptor-mediated RB stimulation have been described with staurosporine and H-7. For instance, the fMLP-induced RB of PMN has been reported to be depressed,15,18,19 unaltered,20 and potentiated.21-23 One possible explanation for these different effects may be related to the duration of cell treatment with staurosporine, as described here (Fig 1). We have reported that staurosporine also primes the platelet-activating factor (PAF)-induced RB.31 In unpublished studies, we found a similar enhancing effect of staurosporine on leukotriene B4 (LTB4)-induced and complement fraction C5a-induced but not ionomycine-induced PMN RB. Unlike PMA and ionomycine, fMLP, PAF, LTB4, and C5a bind to membrane receptors and stimulate PMN through a mechanism involving G protein-dependent activation of phospholipases.40 These data raise the possibility that staurosporine priming of the RB may be due to metabolic alterations affecting early events triggered by chemoattractant receptor stimulation. That a potent, primed RB was also mediated by H-7 appears to be in contradiction with other data obtained with the same concentration30,32; however, it has been observed with rabbit PMN.17 The reasons for these discrepancies are unclear, but they may be partly due to differences in cell treatment, i.e., the use of cytochalasin B,32 stimulant concentrations, and experimental conditions.30 Unlike H-7 and staurosporine, more selective PKC inhibitors such as GF 109203X41 and cherythrine42 failed to prime fMLP-induced RB in our experimental conditions (results not shown). From these data, it is tempting to postulate that staurosporine may primarily alter early signaling events in a manner not related to PKC.

The NADPH oxidase system responsible for generating superoxide, consists of both cytoplasmic and membrane components and is disassembled in unstimulated cells.29,30 On stimulation, some cytoplasmic components translocate...
to the plasma membrane, a phenomenon that has been implicated in the activation of the oxidase.

Phosphorylation of p47 phox by PKC plays a key role in this activation. Short PMN treatment with staurosporine drastically inhibited both superoxide production and phosphorylation of the p47 induced by fMLP and PMA (Figs 4 and 5), in keeping with earlier reports. Interestingly, the fMLP but not the PMA-induced RB was also prevented by short and long treatment of PMN with genistein (Fig 6), which indicates that genistein may not alter PKC activation in these conditions. The results are consistent with the assumption that tyrosine kinase activation plays an important role in the signalling induced by fMLP.

Unlike short incubation, long treatment (15 to 20 minutes) of PMN with staurosporine resulted in a twofold enhancement of the fMLP-induced RB and in partial restoration of phosphorylation of the p47 phox (Figs 1 and 8). However, the maximal restoration of phosphorylation reached only about 50% of control values (Fig 8), whereas the primed RB was enhanced by approximately 200%, indicating a dissociation between RB and enhanced phosphorylation of p47 phox. Such a dissociation has also been described in unprimed PMN stimulated with retinal and DiC8, as well as with fMLP and concanavalin A. However, a link between the two responses is suggested by the fact that PKC inhibitors (sphingosine and staurosporine) reduced primed RB and partially inhibited the restoration of phosphorylation (Fig 8), suggesting that recovery of p47 phox phosphorylation may contribute to the primed RB.

Potential mechanisms for the recovery of the phosphorylation of p47 phox theoretically include processes that may (1) reverse the PKC inhibition set up during short treatment with staurosporine, (2) inhibit phosphatase activation, or (3) involve kinases weakly sensitive (or insensitive) to staurosporine. In preliminary experiments with a phosphatase inhibitor, okadaic acid, which also primes fMLP-induced RB, restoration of phosphorylation in staurosporine-treated PMN persisted (results not shown). Although the results need to be confirmed with other phosphatase inhibitors, they suggest that the inhibition of serine and threonine phosphatase activation may not play an important role in the staurosporine-priming effect. The observation that long treatment of PMN with staurosporine failed to enhance the PMA-induced RB and phosphorylation of the p47 (Fig 5) indicates that staurosporine does not induce metabolic processes that reverse PKC inhibition in this model. However, in fMLP-stimulated PMN, such a process may exist, because staurosporine and sphingosine were able to partially inhibit staurosporine-mediated priming of the RB and the restoration of p47 phox phosphorylation. The residual RB that was not inhibited by staurosporine or sphingosine may be due to kinase-independent processes or to kinases weakly sensitive (or insensitive) to these inhibitors.

The involvement of PKC-independent mechanisms in RB has been suggested with intact PMN treated with retinal and DiC8, cell-free systems derived from PMN and reconstituted models. Such cell-free systems have shown several apparent discrepancies between the superoxide production by intact cells and PMN membranes. They are usually composed of membranes, cytosolic protein factors, magnesium, and an anionic lipid, eg, SDS or fatty acids such as arachidonic acid and phosphatidate. It has been suggested that the presence of an anionic lipid can mimic phosphorylation of p47 phox for its role in superoxide production. We recently reported that in human PMN treated by staurosporine, phosphatidic acid (PA) levels increased in a time- and concentration-dependent manner, peaking at 15 to 20 minutes. Enhanced PA levels appear to be due to both phospholipase-D activation and inhibition of PA dephosphorylation and also reach a level close to that induced by fMLP in PMN not treated with cytochalasin B (results not shown). In other studies, staurosporine potentiated fMLP-induced PA production and arachidonic acid release. PA production has been correlated with RB. These observations raise the possibility that long treatment (15 minutes) with staurosporine followed by stimulation with fMLP results in a sufficient amount of mediators (PA and arachidonic acid) to achieve an exaggerated RB through a mechanism that does not involve phosphorylation. Alternatively, the mediators could reverse the inhibition of a PKC-dependent pathway, as suggested by the restoration of p47 phox phosphorylation.

In addition to enhancing PMN RB stimulation, staurosporine stimulates actin polymerization, degranulation, and calcium mobilization. In human basophils, staurosporine and sphingosine prime fMLP-induced degranulation. In other cell types, staurosporine promotes changes in morphology and differentiation and mimics nerve growth factor for the induction of neuropeptide gene expression. In an in vitro system, staurosporine promotes the association of PKC to vesicles. We have shown that staurosporine enhances the expression of phorbol dibutyrate binding sites in PMN, probably due to translocation of PKC. Whereas the molecular mechanism of these stimulating effects of staurosporine remains to be clarified, these data indicate that staurosporine has numerous stimulating effects that may not be related to kinase alterations.

In conclusion, the stimulation of PMN RB by fMLP was both inhibited and primed when PMN were treated for short and long periods with staurosporine, respectively. This is in contrast with the PMA-mediated RB, which was always depressed. Inhibited and potentiated RB were associated with decreased and partial restoration of p47 phox phosphorylation, respectively, suggesting a possible role of phosphorylation recovery in primed RB. These results suggest that staurosporine may alter the interactions between different kinases, resulting in time-dependent changes in signalling pathways and primed functions. This study also emphasizes the care that must be taken in interpreting alterations of cellular responses by kinase inhibitors.

ACKNOWLEDGMENT

We thank Dr B.M. Babior for providing us with antibody against p47 phox.

REFERENCES


34. Segal AW, Heyworth PG, Cockcroft S, Barrowman HM: Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein. Nature 316:547, 1985


42. Herbert JM, Augusteau JM, Goleye J, Maffrand JP: Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochim Biophys Res Commun 172:993, 1990
Stimulation of the human neutrophil respiratory burst by formyl peptides is primed by a protein kinase inhibitor, staurosporine

C Combadiere, J el Benna, E Pedruzi, J Hakim and A Perianin