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SPERMINE DOWN-REGULATES SUPEROXIDE GENERATION INDUCED BY fMET-LEU-PHE IN ELECTROPERMEABILIZED HUMAN NEUTROPHILS

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Effect of spermine, a naturally occurring polyamine, was investigated on superoxide generation in intact and electropermeabilized human neutrophils. Spermine suppressed N-formyl-methionyl leucyl phenylalanine (fMLP)-induced superoxide generation in permeabilized cells by reducing the rate and shortening the duration time. The inhibition was specific for spermine comparing with its precursor amines, spermidine and putrescine. The inhibition was not observed when cells were preincubated with spermine without permeabilization. Concanavalin A-induced superoxide generation was also down-regulated by spermine in permeabilized cells, but the activation induced by non receptor-mediated agonist (dioctanoylglycerol, phorbol myristate acetate, and arachidonate) was not affected by spermine. On the other hand, GTP-γ-S-induced activation of superoxide generation was substantially suppressed by spermine. These results indicate that spermine inhibition occurs at a step prior to protein kinase C in signal transduction or in a pathway which is independent of the kinase.

Superoxide generating NADPH oxidase of phagocytes such as neutrophils and macrophages plays an important role in host defense against microbial infection (1,2). The mechanisms for the activation and regulation of NADPH oxidase are not fully elucidated (3,4). In particular the sequence of events induced by receptor-mediated agonist, e.g. N-formyl-Met-Leu-Phe (fMLP), is still controversial. The involvement of G-protein, protein kinase C, tyrosine kinases have been suggested in fMLP-induced response as well as several phospholipases (PL) including PLC, PLD, and PLA_2 (3,4). Also, some molecules with low molecular weight are proposed as second messengers, which include diacylglycerol, phosphatidic acid, and arachidonic acid.

Regarding diacylglycerol generation in response to fMLP, the contribution of PLC-mediated phosphoinositides breakdown, which is generally accepted in other cells, is now questioned and the action of PLD and phosphatidate phosphohydrolase has been more plausible (5).

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Abbreviations: fMLP, N-formyl-methionyl-leucyl-phenylalnine; PMA, 12',13'-phorbol myristate acetate; GTP-γ-S, guanosine 5'-[γ-thio]triphosphate; PL, phospholipase.

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Organic cations have been shown to inhibit the superoxide generation in neutrophils. Miyahara et al. (6) showed that alkylamines such as palmitylamine strongly inhibit superoxide generation induced in guinea-pig neutrophils. Lambeth and his colleage (7) showed that sphinganine, a biogenic long chain base, inhibits superoxide generation induced by fMLP or other stimulants. The inhibition by cellular amines of fMLP-induced response is of particular interest with respect to the termination process, which follows the activation, and is much less understood.

Spermine is a naturally occurring polyamine present in abundant in most animal cells. The polyamine is well known to serve as a DNA stabilizer in nuclei and as a promotor in cell growth or maturation (8,9). More recently, a direct effect of spermine has been reported on several particular enzymes. Sakai et al. (10) showed that spermine stimulates a tyrosine kinase activity from porcine spleen. It was reported that spermine inhibits superoxide dismutase in hamster spertozoma (11) and also inhibits PLC-catalyzed phosphoinositides hydrolysis in a tumor cell line (12). Moreover, the amine is implicated as a regulator of phoshatidate phosphohydrolase activity in rat liver (13).

In the present study, we examined the effect of spermine on the activation of NADPH oxidase in electropermeabilized neutrophils, and found that it suppressed receptor-mediated activation of the enzyme. The mechanism of inhibition was disccussed in relation to signal transduction pathway.

EXPERIMENTAL PROCEDURES

Materials

N-formyl-Met-Leu-Phe (fMLP), 2-dioctanoyl-*rac*-glycerol, sodium arachidonate, spermine (tetrahydrochloride), spermidine (trihydrochloride), cytochrome *c* (horse heart, type VI), and superoxide dismutase (bovine erythrocyte) were obtained from Sigma. Concanavalin A and putrescine (dihydrochloride) were purchased from Wako Pure Chemicals, Osaka. All other reagents were the best grade commercially available.

Isolation and permeabilization of Human Neutrophils-

Neutrophils were separated from human peripheral blood as described previously (14). Isolated cells were suspended in buffer A (136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 5.5 mM glucose). Cells (1 x 10^7) were taken into 1 ml of ice-cold permeabilization buffer (140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.193 mM $CaCl_2$, 1mM ATP, 10 mM glucose, 10 mM Hepes, pH 7.0) containing 2.5 mM NADPH with or without spermine, and electropermeabilized with a Bio-Rad Gene Pulser according to the method of Gristein et al.(15) with some modifications. Cells were subjected to one discharge of 2 kV / 0.4 cm from a 25 μF capacitator. Under the conditions, more than 90% cells were permeabilized at pulse and most (85%) of them are restored within 1 min, ascertained by trypan blue exclusion.

Assay for Superoxide Production-

Intact or permeabilized cells (1 x 10^6 each) were transfered to sample and reference cuvettes which contain 20 μM cytochrome c in 2 ml of buffer A, and incubated at 37°C for 1 min with stirring. Superoxide production was initiated by addition of fMLP or other agonist to sample

cuvette, and measured by cytochrme *c* reduction spectrophotometrically as described previously (16). In each case, the cytochrome *c* reduction observed was verified to be due to superoxide formation by separate control experiment using superoxide dismutase.

RESULTS

Fig. 1 shows the fMLP-induced superoxide generation in neutrophils which are electrically permeabilized in the absence (trace a) or presence of 2 mM spermine (trace b). Permeabilization with spermine caused a marked decrease in maximal rate (70% inhibition) and a shortening of duration time (from 2.1 to 1.1 min). The viability and responsiveness of these cells was ascertained by subsequent stimulation with phorbol myristate acetate (PMA), the stimulation by which was found to be largely insensitive to spermine (Fig. 2). Fig. 2 also shows that the inhibition of fMLP-induced activation was dose-dependent to spermine concentration added at

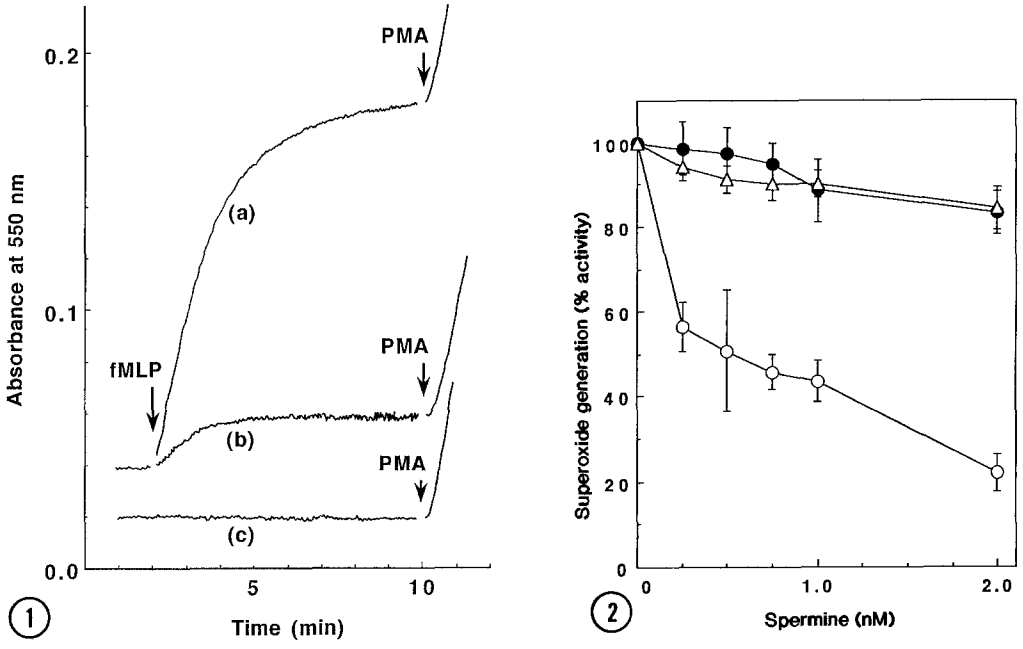

Fig. 1. Effect of spermine on superoxide generation in permeabilized human neutrophils. Cells were electropermeabilized in the absence (a, c) or presence (b) of 2 mM spermine as described under "Experimental Procedures". Permeabilized cells were transferred to the assay mixture and superoxide generation was assayed by cytochrome *c* reduction. Arrows indicate the addition of fMLP (4 μM) or PMA (0.8 μM). The traces are representative of at least three experiments.

Fig. 2. Effect of spermine on the rate of superoxide generation in intact and permeabilized neutrophils. Cells were permeabilized in the presence of various concentrations of spermine, and activated by fMLP (○) or PMA (●). As a control, non permeabilized cells were also preincubated with various concentrations of spermine for the same period as permeabilized cells and activated by fMLP (Δ). The control activities for intact (fMLP-induced), permeabilized (fMLP-induced), and permeabilized (PMA-induced) cells are 127.9 ± 18.4, 137.2 ± 5.0, and 168.8 ± 14.3 nmol /min /10^7 cells, respectively. Each point is the average of three seperate experiments.

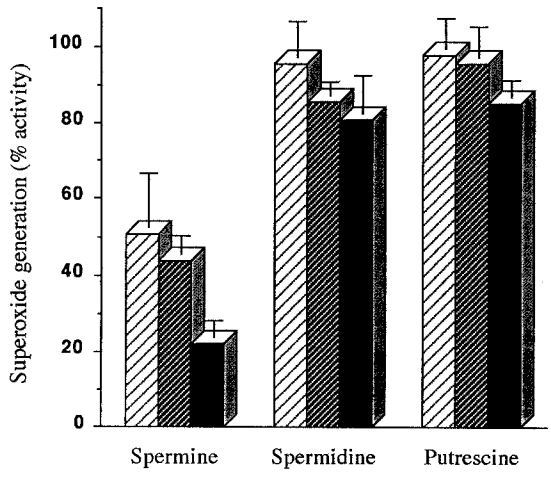

Fig. 3. Effect of several polyamines on superoxide generation in permeabilized neutrophils. Cells were permeabilized in the presence of spermine, spermidine, or putrescine at 0.5 (▨), 1 (▩), or 2 mM (■). The permeabilization and assay conditions are described in "Expermental Procedures". The control activity without polyamine is 137.2 ± 5.0 nmol /min /10^7 cells. Data are expressed as means ± SD from three separate experiments.

permeabilization. In addition, the inhibition by spermine was not observed in intact (non-permeabilized) cells, indicating that the site of action of spermine is intracellular.

Fig. 3 shows the effect of several polyamines, spermine and its metabolic precursors, having different chain length and different number of amino groups. Of these amines, spermine was most effective in inhibition and the other two showed only a modest inhibitory effect even at the highest concentration used, indicating that the inhibition is specific for spermine.

As described above, spermine depressed fMLP-induced,but not PMA-induced, superoxide generation. So, we next used several agonists for activation in examining the effect of sperimine on superoxide generation (Table I). Concanavalin A-induced activation was also inhibited by spermine (37 % inhibition at 1 mM spermine), but the activation by non-receptor mediated agonists (dioctanoylglycerol, arachidonate, and PMA) was not affected. GTP-γ-S, which has shown to induce the activation in electropermeabilized cells (17), elicited the superoxide generation and the activation was suppressed by spermine to a similar extent in fMLP activation .

DISCUSSION

In this paper we demonstrate the inhibition by spermine of receptor-mediated activation of superoxide generation in neutrophils. As mentioned above, the inhibition by lipophilic amines such as alkylamines or sphinganine has been reported, but the inhibition by spermine described here is different from those in the following points. (i) Unlike those amines, spermine is effective

Table I. Effect of spermine on O_2^- generation induced by various stimulants. Neutrophils permeabilized in the absence or presence of 1 mM spermine were activated by different stimulants and assyed for superoxide generation. The data are expressed as means ± SD from three different experiments.

Agonist (concentration)	O_2^- generation (nmol /min /10^7 cells)		% activity
	Control	+ 1 mM Spermine	
fMLP (4 μM)	137.2 ± 5.0	59.8 ± 6.8	44
Concanavaline A (2mM)	83.4 ± 15.0	52.7 ± 6.0	63
PMA (0.8 μM)	190.7 ± 7.0	168.8 ± 14.3	89
Dioctanoylglycerol (100 μM)	145.0 ± 21.9	130.8 ± 12.2	90
Arachidonate (50 μM)	136.5 ± 14.2	127.3 ± 9.8	93
GTP-γ-S (200 μM)[a)]	91.7 ± 10.4	37.9 ± 1.9	41

a) Cells were electropermeabilized in the presence of GTP-γ-S with or without spermine, and immediately assayed for superoxide generation.

only with permeabilization. This, reflecting the impermeability of spermine to plasma membrane, clearly indicates that the site of action is not on the cell surface but is intracellular. (ii) While the inhibition by lipophilic amines occurs independently of agonists used, spermine inhibition was observed only in the receptor-mediated activation.

As a mechanism for alkylamine inhibition, the change in membrane charges by added amine has been postulated (6), although the details of mechanism was not elucidated. Ohtsuka et al. (18) suggest that this change may lead to an impairment of the interaction between cytosol and membrane component to constitute active NADPH oxidase. On the other hand, the sphinganine inhibition on neutrophil activation is interpreted as its strong inhibitory action on protein kinase C (7).

In spermine inhibition, the fact that non-receptor-mediated activation was unaffected indicates that the inhibition is neither on NADPH oxidase itself, nor a result from changes of membrane charges as suggested for alkylamines (6,18). Instead, the site of action should be on a step(s) in signal transduction pathway. Since the stimulation by protein kinase C activators was not inhibited by spermine, the inhibition site is not on protein kinase C, but a step prior to the kinase or a step in a protein kinase C-independent pathway (15,17,19). In summary, we suppose that the site of action is somewhere between receptor linked G-protein and diacylglycerol generation. In this respect, a recent report by Brindley's group that spermine inhibits phosphatidate phosphohydrolase (20) is of interest.

The bioligical relevance of the findings reported here is not clear, but it is noteworthy that only spermine, and not precursor amines, has a substantial effect. The subcellular distribution of spermine is still ambiguous due to the possible redistribution during fractionation (21), but the amine is present at high concentrations in cells (0.05 - 2 mM) (22) and the intracellular level, which is strictly controlled, can vary on cell conditions (9) or by hormone actions (24). Thus, it is tempting to speculate a role of spermine in the regulation (e.g. termination) of NADPH oxidase in neutrophils.

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REFERENCES

1. Babior, B.M. (1978) N.Engl.J.Med. **298**, 659-668.
2. Badwey, J.A. and Karnovsky, M.L. (1980) Annu.Rev.Biochem. **49**, 695-724.
3. Rossi, F. (1986) Biochim.Biophys.Acta **853**, 65-89.
4. Bellavite, P. (1988) Free Radical Biol.Med. **4**, 225-261.
5. Billah, M.M., Eckel, S., Mullmann, T.J., Egan, R.W. and Siegel, M.I. (1989) J.Biol.Chem. **264**, 17069-17077.
6. Miyahara, M., Watanabe, S., Okimasu, E. and Ustumi, K. (1987) Biochim.Biophys.Acta **929**, 253-262.
7. Wilson, E., Olcott, M.C., Bell, R.M., Merrill, A.H. and Lambeth, J.D. (1986) J.Biol.Chem. **261**, 12616-12623.
8. Tabor, C.W. and Tabor, H. (1978) Annu.Rev.Biochem. **45**, 285-306.
9. Pegg, A.E. and McCann, P.P. (1982) Am.J.Physiol. **243**, C212-222.
10. Sakai, K., Sada, K., Tanaka, Y., Kobayashi, T., Nakamura, S. and Yamamura, H. (1988) Biochem.Biophys.Res.Commun. **154**, 883-889.
11. Singh, A., Kumar G, P., Laloraya, M., Verma, S. and Nivsarkar, M. (1991) Biochem.Biophys.Res.Commun. **177**, 420-426.
12. Wojcikiewicz, R.J.H. and Fain, J.N. (1988) Biochem.J. **255**, 1015-1021.
13. Brindley, D.N. (1987) Phosphatidate Phosphohydrolase, pp. 1-77. CRC Press, Boca Raton.
14. Tamura, M., Tamura, T., Tyagi, S.R. and Lambeth, J.D. (1988) J.Biol.Chem. **263**, 17621-17626.
15. Grinstein, S. and Furuya, W. (1988) J.Biol.Chem. **263**, 1779-1783.
16. Tamura, M., Tamura, T., Burnham, D.N., Uhlinger, D.J. and Lambeth, J.D. (1989) Arch.Biochem.Biophys. **275**, 23-32.
17. Nasmith, P.E., Mills, G.B. and Grinstein, S. (1989) Biochem.J. **257**, 893-897.

18. Ohtsuka, T., Hiura, M., Ozawa, M., Okamura, M. and Ishibashi, S. (1990) Arch.Biochem.Biophys. **280**, 74-79.
19. Watoson, F., Robinson, J. and Edwards, S.W. (1991) J.Biol.Chem. **266**, 7432-739.
20. Jamal, Z., Martin, A., Gomez-Munoz, A. and Brindley, D.N. (1991) J.Biol.Chem. **266**, 2988-2996.
21. Tabor, C.W. and Tabor, H. (1984) Annu.Rev.Biochem. **53**, 749-790.
22. Bachrach, U. (1973) Function of Naturally Occuring Polyamines, pp. 5-20. Academic Press, New York.
23. Feige, J.J., Madani, C. and Chambaz, E.M. (1986) Endocrinology **118**, 1059-1066.