Selective Inhibition of Stimulation Responses of Neutrophils by Membrane Modulators

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ABSTRACT. Polymorphonuclear leukocytes undergo a series of morphological and biochemical changes in response to various chemical stimuli. Transmembrane potential change is an early event that follows stimulation and membrane depolarization may act as a trigger for superoxide generation. To determine if there is a correlation between membrane depolarization and superoxide generation, we investigated the effects of different membrane modulators on stimulus-dependent depolarization. The membrane modulators mepacrine, chlorpromazine and cepharanthine inhibited the superoxide generation produced by chemotactic peptide, FMLP, and/or digitonin in neutrophils. Inhibitory profiles of the activation parameters, however, demonstrate that membrane depolarization is not associated with superoxide generation: FMLP-induced depolarization was inhibited by the modulators tested and was accompanied by the suppression of superoxide generation, but the depolarization produced by digitonin was stimulated somewhat by these drugs. Our results indicate that receptor-mediated membrane depolarization is not a necessary event for the activation of superoxide generation by digitonin.

When stimulated with both soluble and particulate stimuli, polymorphonuclear leukocytes (PMN) display a dramatic series of biochemical and morphological changes (1, 20). These changes are induced by modifying the membrane surfaces; there is no accompanying entrance of the chemical stimuli. But the exact mechanism by which surface modulation activates these leukocyte functions is poorly understood.

Previous investigators have suggested that PMN has characteristics very similar to those of excitable cells such as muscle and nerve cells. Therefore, a change in transmembrane potential may play an important part in phagocytotic processes, and it has been suggested to be the initial event in metabolic activation (4, 7, 8, 19, 25). A high degree of correlation of stimulus-dependent changes in the transmembrane potential to the production of superoxide generation also has been demonstrated (6, 10, 19, 25). Thus membrane depolarization has been suggested to trigger the

Abbreviations used: FMLP, formylmethionyl-leucyl-phenylalanine; PMN, polymorphonuclear leukocytes; KRP, Krebs-Ringer-Phosphate; Con A, concanavalin A; diS-C₃-(5), 3,3'-dipropylthiadicarbocyanine iodide; CPZ, chlorpromazine.

activation of respiratory bursts (3). The actual role of membrane potential and the causal sequence between it and superoxide generation, however, has still to be determined.

To better understand the process of superoxide generation by membrane perturbants, we studied the effect of various membrane modulators on membrane potential change and superoxide generation of PMN induced by chemotactic peptides and digitonin. We here report that superoxide generation of PMN is a reaction independent of membrane potential change and that the sensitivity of membrane potential change to membrane modulators in PMNs differs for each stimuli.

MATERIALS AND METHODS

Chemicals. All reagents were purchased from Sigma Chem. Co. (St. Louis, Mo. USA) except where indicated. Cyanine dye, 3,3'-dipropyl-2,2'-thiodicarbocyanine iodide [diS-C₃-(5)], was donated by Kankoshikiso Kenkyusho Ltd. (Okayama, Japan). Cepharanthine and its related derivatives were donated by Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). The drugs tested were dissolved in ethanol, the amount of ethanol added being less than 0.5% of the final concentration.

Preparation of PMN. PMNs were obtained from the peritoneal cavities of a guinea pigs weighing 300–400 g. The peritoneal fluid was collected 16 h after an injection of 30–40 ml of 2% neutrose dissolved in physiological saline solution. It then was washed twice with Ca²⁺-free Krebs-Ringer-Phosphate (KRP) solution by centrifugation at 500 × g for 2 min at room temperature.

Measurement of membrane potential. Membrane potential was measured by the method of Hoffman *et al.* (5, 22) in a fluorospectrophotometer (Shimadzu RF-510). Changes in the fluorescence intensity of the cyanine dye were monitored at 670 nm after the dye has been excited at 622 nm. The final concentration of cyanine dye was 2.5×10^{-6} M. The temperature of the incubation mixture was maintained at 37°C and the suspension maintained by magnetic stirring.

Membrane permeability. Release of potassium ion from the cells was measured at 37°C with a potassium ion electrode in medium containing 0.15 M choline chloride-10 mM Tris-HCl buffer (pH 7.4).

Oxygen consumption. Oxygen consumption was measured at 37°C with a Clark-type oxygen electrode in a semiclosed cuvette.

Superoxide generation. Superoxide generation was monitored continuously as the superoxide dismutase-inhibitable cytochrome c reduction using the method of Nakagawara *et al.* (13). The incubation mixture was KRP solution containing 1 mM CaCl₂ 0.1 mM NaN₃, 10 μM of cytochrome c, 10 mM glucose and 10⁶ cells/ml of PMN. The change in absorbance at 550–540 nm was monitored by a dualbeam spectrophotometer (Shimadzu-UV 300).

RESULTS AND DISCUSSION

Membrane potential changes in PMNs induced by various stimuli. As reported previously (15, 22), concanavalin A (Con A) and glucose induce changes in the fluorescence intensity of PMN and Ehrlich ascites tumor cells that had been equilibrated with cyanine dye. Detailed analyses of these changes in fluorescence intensity were made by Seligman and Gallin (18). The addition of formylmethionyl-leucyl-

Fig. 1. Changes in the fluorescence of diS-C₃-(5) in a PMN suspension on the addition of various stimuli. A: Guinea pig PMNs (1.4×10^6 cells/ml) were suspended in KRP solution containing 1 mM CaCl₂ and 2.5 μ M diS-C₃-(5) at 37°C. Downward deflections in the trace indicate the uptake of cyanine dye by the cells. B: Transitional changes in fluorescence intensity produced by 50 μ M FMLP (the decrease in fluorescence intensity indicates hyperpolarization). C: Rate of fluorescence change as a function of different concentrations of FMLP.

phenylalanine (FMLP) to PMN produced a biphasic response (Figs. 1A and B). The first phase of that response was a fluorescence decrease (hyperpolarization) and the second a fluorescence increase (depolarization). Similar changes in the fluorescence of PMNs were found after treatment with various types of stimuli; Con A, Ca²⁺ ionophore A23187, digitonin and fatty acids. Most of the fluorescence changes produced by these stimuli were transitional, and both the rate and velocity of the changes were stimuli specific and concentration dependent (Fig. 1C).

Relation between membrane potential change and K⁺ extrusion. The relation between K⁺ release from PMNs and the fluorescence change in diS-C₃-(5) following the addition of different stimuli to the medium is shown in Fig. 2. Replacement of choline in the medium with Na⁺ slightly decreased the time required to reach the steady-state diS-C₃-(5) uptake, but did not modify the amount of cation accumulated. Conversely, replacement of Na⁺ with K⁺ depressed the steady-state uptake of diS-C₃-(5), an indication that the resting potential depends entirely on the K⁺ gradient across the membrane. When PMNs were exposed to FMLP, an immediate release of K⁺ from the cells was detected that was concomitant with a transitional increase in fluorescence intensity. But when exposed to digitonin, after an initial lag period of more than 10 sec, a large amount of intracellular K⁺ was released and there was

Fig. 2. Relation between K^+ release from PMNs and the fluorescence change of diS- Ca_3 -(5) on the addition of various stimuli. PMNs (2×10^6 cells/ml) were incubated in medium containing 0.15 M choline chloride, 10 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$ and 10 mM glucose at $37^\circ C$. K^+ release was monitored with a K^+ electrode. Other conditions were the same as described in Fig. 1.

a remarkable increase in fluorescence intensity. The initial decrease in fluorescence is consistent with membrane hyperpolarization produced by increased potassium permeability and the later increase in fluorescence is consistent with membrane depolarization caused by a decrease in the K^+ gradient or in K^+ permeability.

Calcium ionophore, A23187 also stimulated K^+ release and the fluorescence change that accompanied superoxide generation. The potency of the ionophore to release intracellular K^+ was greater than that of FMLP.

Recently, Mottola and Romeo (11) suggested that a stimulus increases the displacement of Ca^{2+} from the surface membrane of PMNs, and that is partially responsible for the increased permeability of the membrane to other ions, thus inducing a change in the transmembrane diffusion potential. From their evidence it is clear that the stimulus-dependent membrane potential change is closely correlated with the ion permeability of the plasma membrane.

Inhibitory effect of membrane modulators on changes in membrane potential induced by FMLP and digitonin. Recent research on stimulation mechanism has produced

much information about the series of molecular events that take place on the plasma membrane following its interaction with stimuli. The physical state of phospholipids determines their susceptibility to various enzyme activities in the membrane. Thus interaction of the cell membrane with stimuli could be affected by drugs that interact with the lipids or by various modulators of the physical state of the biological membrane (23).

We used specific inhibitors of phospholipase A₂ that belong to a group of drugs known as modulators of the physical state of the membrane; mepacrine (21), chlorpromazine (CPZ) (12) and cepharanthine (24). Mepacrine (10^{-4} M) totally suppressed the depolarization of the cell membrane induced by FMLP (Fig. 3A). The degree of suppression was concentration dependent (Fig. 3B) and the half-maximum inhibition was at $20\text{ }\mu\text{M}$ mepacrine. FMLP-dependent K⁺ release also was inhibited by the same concentration of mepacrine (data not shown). Similar inhibitory actions were found with cepharanthine (24) and CPZ (16) (Fig. 4), the respective concentrations for half-maximum inhibition being $100\text{ }\mu\text{M}$ and $20\text{ }\mu\text{M}$. Many of the biscolaurine alkaloid derivatives of cepharanthine, berbamine, tetrandrine, isotrilobine and isotetrandrine showed inhibitory actions similar to the action of cepharanthine. With high concentrations of these membrane modulators, the release of K⁺ from neutrophils also was induced (data not shown).

Our results show that the drugs tested in these experiments caused modification of the cell membrane and that the permeability of small ions across the plasma membrane was greatly modulated. In contrast, digitonin-induced membrane-depolarization (Fig. 5) and K⁺-release were enhanced by these drugs in a concentration-

Fig. 3. Effect of mepacrine on the fluorescence change of diS-C₃(5) in PMN induced by FMLP. A: Experimental conditions were the same as described in Fig. 1 except that 5 mM CaCl₂ was present. B: % inhibition of fluorescence change as a function of different concentration of mepacrine.

Fig. 4. Effects of various membrane modulators on the fluorescence change of the diS-C₃-(5) of PMN induced by FMLP. Experimental conditions were the same as described in Fig. 3.

dependent manner. No effects of the drugs were observed on depolarization with the calcium ionophore, A23187 (data not shown). Indomethacin, an inhibitor of cyclooxygenase, had no effect on the stimuli-induced membrane depolarization (Fig. 4). The exact mechanism of the differential effect of these drugs on the membrane potential change induced by FMLP or digitonin is still not clear; probably the different responses of potential change to modulators is correlated with changes in membrane permeability.

Inhibitory effects of membrane modulators on the superoxide generation of PMN. The effects of membrane modulators on superoxide generation of PMN, induced by treatment with FMLP or digitonin, are shown in Figs. 6 and 7. The oxygen burst was concomitant with superoxide generation. The rate of superoxide generation was 30 nmoles O₂/min/10⁷ cells when stimulated with 5 × 10⁻⁸ M FMLP. Similar superoxide generation was induced with 5 × 10⁻⁶ M digitonin. Mepacrine and cepharanthine inhibited the stimulus-dependent superoxide generation and the oxygen burst as does CPZ (12): Mepacrine or CPZ incubated for 3 min before stimulation with FMLP or digitonin inhibited superoxide generation of PMNs in a cooperative manner (the hill coefficients for mepacrine and CPZ were 1.68 and 1.92 in FMLP stimulation, and 1.12 and 1.42 in digitonin stimulation). Concentrations for half-maximum inhibition were 20 μM mepacrine and 10 μM CPZ for FMLP, and 18 μM mepacrine and 5 μM CPZ for digitonin. Thus, in cells in which

Fig. 5. Effects of various membrane modulators on the fluorescence change of the diS-C₃-(5) of PMN induced by digitonin. Experimental conditions were the same as described in Fig. 4 except that the stimulus was 5 μ M digitonin.

depolarization could be demonstrated, it was linked neither to changes in superoxide generation nor to respiratory bursts. These results agree with the findings of several investigators who used in different systems (2, 8, 9, 11, 17, 21).

Recently a Ca²⁺-activated and phospholipid-dependent protein kinase (protein kinase C) has been shown to function in the activation of several responses in various types of cells (14). Many drugs, including the membrane modulators CPZ and cepharanthine, inhibit the activity of this enzyme, presumably by affecting its interaction with phospholipid (12). This evidence together with our results suggest that protein kinase C could be correlated with superoxide generation through unidentified intermediate activation steps between ligand binding and NADPH-oxidase expression, but not with stimulus-dependent depolarization and K⁺-release.

Our results also indicate that common and different steps are involved in the transport of different signals as well as for different responses.

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Fig. 6. Effects of mepacrine and CPZ on superoxide generation by PMN as stimulated by FMLP. A: Superoxide generation was monitored continuously by following the reduction of ferri-cytochrome c in KRP solution. Medium containing 0.1 mM NaN_3 , 1 mM CaCl_2 , 10 mM glucose and 10 μM cytochrome c as described in MATERIALS AND METHODS. CPZ inhibited superoxide generation when added to cells already stimulated. B: Dose responses for the inhibition of superoxide generation were graphed as Hills plots as described elsewhere (15). Cells first were treated with CPZ or mepacrine for 3 min before stimulation.

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Fig. 7. Effect of mepacrine and CPZ on superoxide generation by digitonin. Experimental conditions were the same as described in Fig. 6.

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