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INHIBITORY EFFECTS OF VARIOUS DRUGS ON PHORBOL MYRISTATE ACETATE AND *n*-FORMYL METHIONYL LEUCYL PHENYLALANINE INDUCED O_2^- PRODUCTION IN POLYMORPHONUCLEAR LEUKOCYTES

KATSUHIKO TANIGUCHI and KOICHIRO TAKANAKA*
Department of Toxicology, Niigata College of Pharmacy, Kamishinei-cho, Niigata, Japan

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Abstract—To clarify the mechanisms of O_2^- formation by polymorphonuclear leukocytes (PMNs), the effects of clinically employed drugs on PMNs were investigated by measuring changes in membrane potential and rates of O_2^- production. These variables were effectively diminished with antihistaminic agents, adrenergic β-antagonists, and antiarrhythmic drugs when guinea pig peritoneal PMNs were stimulated by either phorbol myristate acetate (PMA) or *n*-formyl-methionyl-leucyl-phenylalanine (FMLP). The order of potency of the inhibitory effects of these chemicals on the PMA-induced O_2^- formation was as follows: azelastine (IC_{50} = 4.1 μM) < clemastine < *dl*-propranolol < chlorpheniramine maleate < dichlorisoproterenol < quinidine < diphenhydramine < indomethacin (IC_{50} > 400 μM). Similar phenomena were observed when FMLP was employed instead of PMA, but the FMLP-stimulated O_2^- production was effectively inhibited by indomethacin. Changes in membrane potential, using the cyanin dye method, also indicated that most of these drugs cancelled functional changes of plasma membrane of PMNs. From these observations, it was demonstrated that changes in membrane potential by the stimuli were essential for the initiation of O_2^- generation from plasma membrane of PMNs, although the initiation mechanisms were not identical for the two stimuli.

Polymorphonuclear leukocytes (PMNs) are known to play a central role in host defensive systems against invading microorganisms. Many studies in the past decade have clarified the mechanisms of bactericidal function of PMNs [1–3] as well as its involvement in the inflammatory process [4–6]. The generation of reduced oxygen products, such as O_2^-, $OH^\cdot$, and H_2O_2, and the release of lysosomal enzymes are known to be responsible for them [7, 8].

When the isolated PMNs are treated with a variety of soluble or particulate agents [9, 10], molecular and functional changes take place in the plasma membrane of PMNs including Na^+ influx [11–13], changes of the membrane potential [11, 14, 15], mobilization of Ca^{2+} [14, 16, 17], and phospholipid turnover [18, 19]. However, the precise nature and sequence of these events responsible for the trigger mechanisms of the O_2^- generation are now being discussed [11, 20, 21].

Recent studies have provided evidence which indicates that the above-mentioned phenomena in the membrane commonly occur in different types of cells, such as platelets [22] isolated nerve endings [23, 24] and mast cells [25, 26]. Therefore, we assume that some clinically employed drugs for these cells might affect the functions of PMNs, if there are some common triggering mechanisms in the plasma membrane. In this study, attempts were made to investigate the effects of antihistaminic drugs, adrenergic β-antagonists, and anti-inflammatory drugs on the O_2^- generation from PMNs activated with two different types of stimuli. The initiation mechanisms of the respiratory burst are also discussed on the basis of the changes of membrane potential.

* Address all correspondence to: Dr. Koichiro Takanaka, Department of Toxicology, Niigata College of Pharmacy, 5829 Kamishinei-cho, Niigata 950–21, Japan.

MATERIALS AND METHODS

Preparation of neutrophils. Guinea pigs were injected intraperitoneally with 20 ml of a 2% solution of casein in sterile saline. After 18 hr, peritoneal exudates containing the PMNs were collected and filtered through three layers of cheesecloth. The cell suspension was centrifuged (400 *g*, 5 min), and the pellet was resuspended in Hanks' solution (Nissui Seiyaku Co., Tokyo, Japan) (5 ml). Two volumes of ice-cold distilled water were added, and the suspension was left to stand for 30 sec to lyse contaminating erythrocytes. After restoring the osmolality with 1.8% NaCl solution, the suspension was centrifuged (400 *g*, 2 min). The pelleted cells were resuspended in Hanks' solution in a plastic tube to give a final concentration of 5×10^7 cells/ml. This procedure yielded leukocytes containing over 95% PMNs.

Stimuli on PMNs. Phorbol myristate acetate (PMA) and *n*-formyl-methionyl-leucyl-phenylalanine (FMLP) were obtained from the Sigma Chemi-

cal Co. (St. Louis, MO, U.S.A.). They were dissolved in 1 mg/ml dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Osaka, Japan) and diluted with medium prior to each experiment.

Drugs. Sotalol (4′-[1-hydroxy-2-(isopropylamino) ethyl] methanesulfonanilide) and dichlorisoproterenol were gifts from Dr. T. Nagatomo (Niigata College of Pharmacy, Niigata, Japan). Azelastine (4-(*p*-chlorobenzyl)-2-[*N*-methylazepinyl-(4)]-1-(2*H*)-phthalazinone hydrochloride), clemastine (1-methyl-2-[-2-(methyl-*p*-chlorodiphenylmethyloxy)ethyl]pyrrolidine), and chlorpheniramine maleate (γ - (4 - chlorophenyl) - *N* - *N* - dimethyl - 2 - pyridine - propanamine maleate) were obtained from the Eisai Co. (Tokyo, Japan). Diphenhydramine (2-diphenylmethoxy-*N*-*N*-dimethylethanamine), quinidine and indomethacin (1 -*p* -chlorobenzoyl) -5-methoxy -2-methylindole -3-acetic acid) were purchased from the Sigma Chemical Co. Indomethacin and clemastine were dissolved in DMSO and then diluted with medium.

Measurement of superoxide anion (O_2^-) generation. Superoxide anion production was measured by following the reduction of ferricytochrome *c* (Type III, Sigma Chemical Co.) as described by Babior *et al.* [1]. The reaction mixture containing 2.45 ml of Hank's solution, 20 μl of 2 mM ferricytochrome *c*, 20 μl of cell suspension (10^6 cells) and various drugs was preincubated at 37° for 5 min in a cuvette with a 1 cm light path, and then 5 μl of stimulus (10 μg/ml) was added. The increase in optical density at 550 nm was followed with reference to 540 nm using a dual beam spectrophotometer (Hitachi, model 557). The rate of O_2^- production was calculated from the initial rate of ferricytochrome *c* reduction (mM $\varepsilon = 21$).

Measurement of membrane potential change. The fluorescence intensity of 3,3-dipropylthiobicarbocyanin iode [de-C-(5)] (Nihon Kanko Shikiso Co., Okayama, Japan) was followed at 510 nm with exitation wave length at 460 nm, using a spectrofluorophotometer (Simazu, type RF-510). The reaction mixture contained 2.45 ml of Hanks' solution, 20 μl of cell suspension (10^6 cells), 5 μl of the cyanin dye (125 μM) and various inhibitors and was preincubated in a cuvette at 37°. When the fluorescence intensity reached equilibrium, 5 μl (10 μg/ml) of stimulus was applied.

RESULTS

As summarized in Table 1, various clinically employed drugs affected O_2^- production by PMNs. The degree of inhibition was dependent on the concentration of the drugs and the type of stimuli. When PMNs were preincubated for 5 min with the chemicals before the stimulus, the lowest IC_{50} concentrations were observed with antihistaminic agents, such as clemastine and azelastine. Concentrations of these chemicals over 10–50 μM almost completely inhibited the production of O_2^- from PMNs stimulated by PMA or FMLP. PMA-induced O_2^- formation was inhibited depending on the categories of drugs: histaminic antagonists (azelastine, clemastine, diphenhydramine, chlorpheniramine maleate), adrenergic β-antagonists (*dl*-propranolol, dichlorisoproterenol), an antiarrhythmic drug, quinidine, and the nonsteroidal antiinflammatory agent indomethacin. The above-mentioned chemicals affected almost equally well the release of O_2^- stimulated by FMLP instead of PMA, although FMLP-challenged PMNs generally required higher concentrations of these chemicals (Table 1). As shown in Fig. 1, the PMA-induced O_2^- production was diminished by over 90% in the presence of the various drugs within a concentration range of 10–500 μM. Approximately one-fifth of the 90% inhibitory concentration of each drug did not show much inhibitory effect. It is also seen in Fig. 2 that over 90% inhibition of O_2^- production by FMLP was obtained in the

Table 1. Effects of various drugs on PMA- and FMLP-induced O_2^- production by peritoneal PMNs*

Drugs	IC_{50} (μM) PMA-induced	IC_{50} (μM) FMLP-induced
Indomethacin	>400	90
Diphenhydramine	300	210
Azelastine	4.1	17
Clemastine	6.2	18
Chlorpheniramine maleate	50	125
dl-Propranolol	12	70
Dichlorisoproterenol	80	
Sotalol	>300	>300
Quinidine	150	150

* Absolute amounts of O_2^- produced by PMA and FMLP were 4.9 ± 1.0 nmoles/min/10^6 cells and 3.8 ± 0.8 nmoles/min/10^6 cells respectively. Control (blank) of cytochrome reduction had been estimated by the additions of SOD, indicating that the rates were always less than 1%.

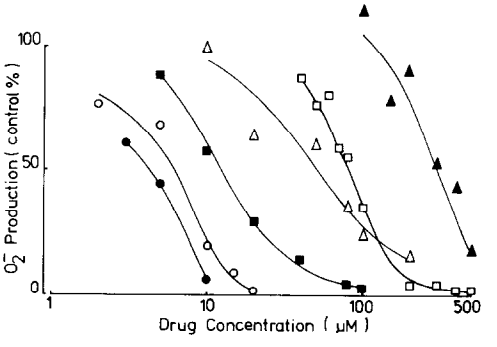

Fig. 1. Effects of various drugs on PMA-induced O_2^- production by peritoneal PMNs. PMNs (10^6) were preincubated for 5 min at 37° with the indicated concentrations of the various drugs and cytochrome *c*. PMA (0.04 μg) was then added and O_2^- production was determined by cytochrome *c* reduction as described in Materials and Methods. The results indicate the percentage of O_2^- production by nontreated cells. Key: (●——●) azelastine, (○——○) clemastine, (▲——▲) diphenhydramine, (△——△) chlorpheniramine maleate, (■——■) *dl*-propranolol, and (□——□) dichlorisoproterenol.

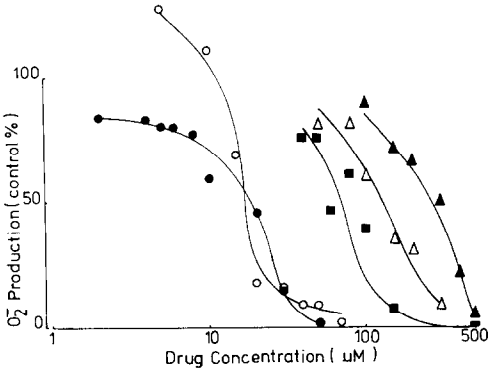

Fig. 2. Effects of various drugs on FMLP-induced O_2^- production by peritoneal PMNs. PMNs (1×10^6) were preincubated for 5 min at 37° with the indicated concentrations of the various drugs and cytochrome *c*. FMLP (0.04 μg) was then added, and O_2^- production was determined by cytochrome *c* reduction as described in Materials and Methods. The results indicate the percentage of O_2^- production by nontreated cells. Key: (●——●) azelastine, (○——○) clemastine, (▲——▲) diphenhydramine, (△——△) chlorpheniramine maleate, and (■——■) *dl*-propranolol.

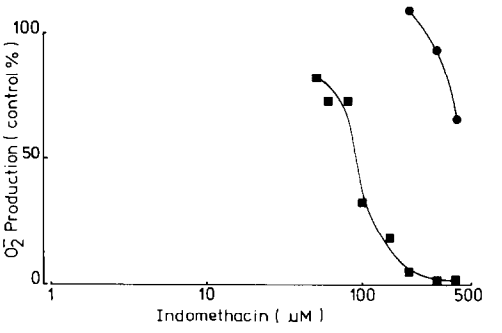

Fig. 3. Effects of indomethacin on PMA- and FMLP-induced O_2^- production by peritoneal PMNs. PMNs (1×10^6) were preincubated for 5 min at 37° with the indicated concentrations of indomethacin and cytochrome *c*. PMA or FMLP was then added, and O_2^- production was determined by cytochrome *c* reduction. The results indicate the percentage of O_2^- production by nontreated cells. Key: (●——●) PMA-induced, and (■——■) FMLP-induced.

presence of 50–500 μM concentrations of these drugs. In most cases, the shapes of the curves were similar for the various drugs and the type of stimulus. One remarkable difference between the O_2^- production stimulated with PMA and FMLP was in the case of indomethacin. As mentioned above, the FMLP-challenged PMNs required higher concentrations of the chemicals except for indomethacin. Results in Fig. 3 clearly demonstrate that 100 μM indomethacin effectively inhibited O_2^- production when the cells were stimulated with FMLP, but over 400 μM indomethacin was required to inhibit PMA-induced O_2^- formation. To investigate the inhibition site of the drugs, the changes in membrane potential were followed using the cyanin dye method. A typical example of the changes in fluorescence is shown in Fig. 4. Azelastine did not alter the membrane potential in PMNs by itself but did diminish the PMA-induced changes. FMLP-challenged PMNs showed similar phenomena, indicating that the membrane alterations were suppressed by the chemical. However, the addition of indomethacin (200 μM) affected only FMLP-induced changes in fluorescence intensity, but the PMA stimulation was not diminished with the same concentration, as shown in Fig. 5.

These results indicate that the respiratory burst correlated with the membrane alterations. In other words, if the changes in membrane potential were inhibited, the release of O_2^- from PMNs would not

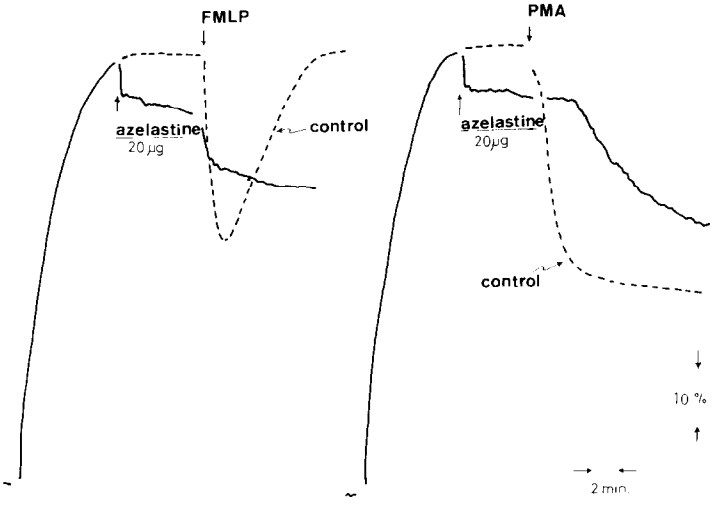

Fig. 4. Depressive effects of azelastine on changes of membrane potential. PMNs (10^6) in Hanks' solution were pretreated with 0.25 nM de-C-(5) for 5 min at 37° and then challenged with PMA or FMLP.

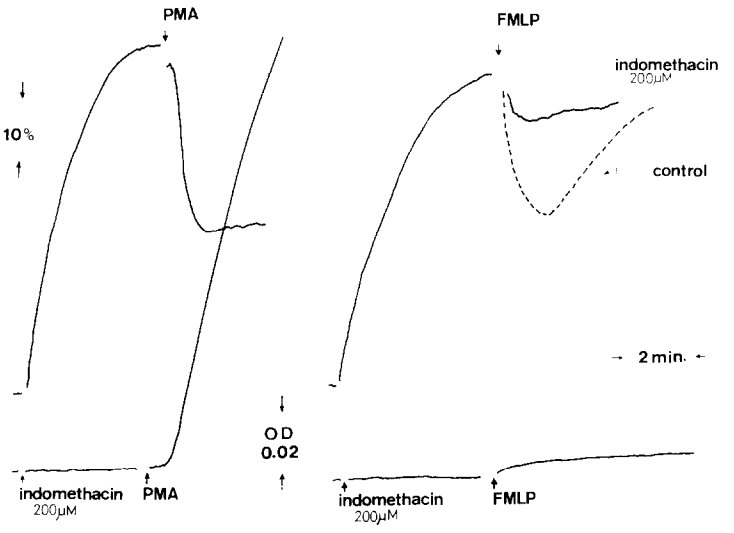

Fig. 5. Effect of indomethacin on the changes in membrane potential and O_2^- generation. The changes in membrane potential shown by the upper lines were followed as described in Fig. 4. The membrane potential was measured under the same conditions except that de-C-(5) (16 μM) was added instead of cytochrome *c*.

take place on the plasma membrane. We further observed (data to be published elsewhere) that various phospholipase or protease inhibitors suppressed both metabolic burst as well as changes in membrane potential. From these results, it may be decided that changes in membrane potential are essential for the initiation of O_2^- production in PMNs.

DISCUSSION

The production of O_2^- by PMNs is a consequence of two separate processes: activation (trigger) and enzyme activity. Since the enzymes and the activation mechanisms are known to be associated with the plasma membrane of the granulocytes, the molecular and functional changes in the plasma membrane might be affected by some drugs which are aimed for other membrane of target cells. As demonstrated earlier, chlorpromazine (50 μM) completely inhibits the O_2^- production by PMNs [27]. In this study, both the rates of O_2^- release and the changes in membrane potential were equally well diminished by the antihistaminic agents, the adrenergic β-antagonists, or the antiarrhythmic drug. These results indicate that they do not directly inhibit the O_2^--generating enzymes (NAD(P)H oxidase) but may affect the activation systems located in the plasma membrane. Alternatively, the depression of O_2^- production would diminish the changes in membrane potential. However, since it has been demonstrated that superoxide dismutase, catalase and dihydroxybenzoic acid have no effect on PMA-induced deporalization of PMNs [28], this possibility is excluded here. That is, the effects of drugs on the changes in membrane potential may not be a consequence of the inhibition of O_2^- production.

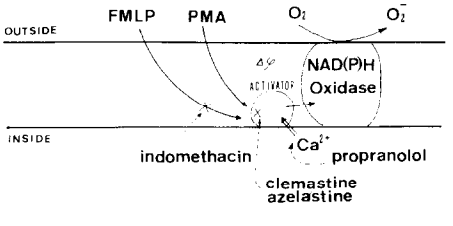

Fig. 6. Sites of drug action on the plasma membrane located NAD(P)H oxidase activity.

The stimulations of several types of cells, including macrophages [29, 30] and mast cells [26], are accompanied by the changes in their membrane potential. Furthermore, it has been suggested that the trigger mechanism of the respiratory burst of PMN involves the following ionic and molecular events in the plasma membrane; (i) Na^+ influx [11–13], (ii) Ca^{2+} mobilization [14, 16], and (iii) phospholipid turnover [18, 19]. Similar events were demonstrated for histamine release from mast cells [22] or for activation for platelets [26]. Therefore, it seems likely that the inhibition of the O_2^- generation of PMNs with the drugs employed here is due to the interference of one or more of these processes. The antihistaminic agents are generally classified as antagonists or histamine release inhibitors. Since the release inhibitors are known to affect the intracellular Ca^{2+} concentration in mast cells [31, 32], the drugs could inhibit the mobilization of Ca^{2+} in PMNs [15]. This explains why the antagonist (diphenhydramine) required over a 10-fold higher concentration. Some of the adrenergic β-blockers may also inhibit Ca^{2+} mobilization or Na^+ influx, although the site of the action may not be the adrenergic β-receptors. As seen in Table 1, *dl*-propranolol was effective at much lower concentrations than the other β-blockers (*d*- and *l*-bodies did not show differences). Another point in this study was to compare differences among the stimuli, such as PMA and FMLP.

Under identical conditions, FMLP- or PMA-stimulated PMNs required almost similar concentrations of the chemicals to inhibit the O_2^- generation. Indomethacin, however, was demonstrated [33] to be an effective inhibitor only when cells were challenged with FMLP. The PMA-stimulated PMNs, on the other hand, were hardly inhibited by indomethacin, indicating that the site of action of the indomethacin is not involved in the FMLP stimulation process. It has been demonstrated recently that the stimulation process of PMNs by FMLP and PMA is quite different in terms of membrane phospholipid metabolism [18] and mobilization of Ca^{2+}.

From these observations, we infer that PMA stimulates some site(s) relatively close to NAD(P)H oxidase, but that the FMLP involves a more complicated process including the action of phospholipase and protease activation [34]. This has been illustrated in Fig. 6. where the change of the membrane potential is shown as $\Delta\psi$.

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