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Transmembrane Potential Changes During Phagocytosis in Rat Alveolar Macrophages

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ABSTRACT Studies were carried out to measure changes in the transmembrane potential of rat alveolar macrophages during exposure of the cells to zymosan particles or to the membrane perturbant, phorbol-12-myristate-13-acetate (PMA), and to determine if changes in membrane potential are related to superoxide anion release. Exposure of the cells to either zymosan or PMA leads to membrane depolarization, which precedes superoxide anion release. Furthermore, the magnitude of the depolarization is dependent upon the concentration of either zymosan or PMA. During exposure of the alveolar macrophages to increasing levels of zymosan, there is an increase in the amount of superoxide released as well as an increase in the magnitude of the depolarization. Incubation of the cells in medium containing 150 mM K^+, a medium which causes membrane depolarization, leads to superoxide release from resting cells and a decrease in the amount of superoxide released from cells exposed to zymosan. These results indicate that release of superoxide anion from rat alveolar macrophages is related to membrane depolarization and suggest that the transmembrane potential change may act as a signal to initiate the phagocytotic responses of the cells.

Alveolar macrophages are cells found in the alveoli and small airways of the lungs. Their function is to ingest foreign debris and bacteria that enter the respiratory tract. During the process of phagocytosis in alveolar macrophages, various forms of reactive oxygen are released by the cells. For example, superoxide anion (Drath and Karnovsky, '75), hydrogen peroxide (Gee et al., '70), and possibly singlet oxygen and hydroxyl radical (Miles et al., '78) are released from alveolar macrophages during exposure of the cells to foreign particles or bacteria. Although the function of these reactive forms of oxygen has not been definitely established, there is some evidence to suggest that they are related to the antibacterial activity of the cells (Johnston, '78). At the present time, very little is known about the mechanisms involved in the release of active oxygen from alveolar macrophages.

The results of some studies done with polymorphonuclear leukocytes (PMN) suggest a relationship between the release of superoxide anion and changes in the transmembrane potential. For example, membrane perturbants, such as phorbol myristate acetate (PMA) and concanavalin A, cause release of superoxide anion from neutrophils (Lehmeyer et al., '79; Kitagawa et al., '80) and changes in the transmembrane potential of the cells (Utsumi et al., '77; Korchak and Weissman, '78; Jones et al., '80; Whitin et al., '80). Exposure of PMN to chemotactic factors also leads to membrane depolarization (Seligman et al., '80) and to superoxide release (Lehmeyer et al., '79). In addition, there are changes in the permeability of the cell membranes of these phagocytic cells to ions during stimulation by chemotactic factors. There is an increase in sodium influx (Simchowitz and Spilberg, '79; Naccache et al., '77), a decrease in potassium influx (Dunham et al., '74), and an increase in calcium influx (Boucek and Synderman, '76) during stimulation of human neutrophils. Also, changes in transmembrane potential have been reported following stimulation of macrophages from peripheral blood (Gallin and Gallin, '77; Gallin et al., '78). Although there is considerable evidence to support a relationship between transmembrane potential changes, ionic permeability of the membranes, and release of superoxide in neutrophils and macrophages from blood, no

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studies of this nature have been done in alveolar macrophages.

Recently, we measured the resting transmembrane potential of rat alveolar macrophages and reported a value of −37 mV (Castranova et al., '79). In the present study, measurements of transmembrane potential changes were made during the process of phagocytosis in rat alveolar macrophages by using the fluorescent probe, Di-S-C_3(5). The objectives of this investigation were 1) to measure changes in the membrane potential following exposure of the cells to foreign particles or in response to the membrane perturbant, PMA, and 2) to determine if changes in transmembrane potential are related to superoxide anion release from the cells. A preliminary report of these results has appeared previously (Miles et al., '80).

MATERIALS AND METHODS

Alveolar macrophages were harvested from male Long-Evans hooded rats (230–300 gm) by tracheal lavage (Myrvik et al., '61; Castranova et al., '79). The animals were first anesthetized with sodium pentobarbital (0.2 gm/kg body weight) and then exsanguinated by cutting the abdominal aorta. Pulmonary lavage was accomplished by washing the lungs from each animal 12 times in situ with a total volume of 80 ml of an ice-cold Ca^{++}-free solution containing 145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 5 mM glucose (pH=7.4). The cells were separated from the lavage fluid by centrifugation at 500 x g for 5 minutes. The alveolar macrophages from several animals were then pooled and washed twice by alternate centrifugation and resuspension in a medium of the following composition: 140 mM NaCl, 5 mM KCl, 10 mM Na-Hepes (N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid), and 5 mM glucose (pH=7.4). After the alveolar macrophages were washed, the cells were resuspended in a small amount of Hepes-buffered medium. The total number of cells in the suspension was determined by using a Coulter Model Z_B electronic cell counter (Coulter Instrument Co., Hialeah, FL). Aliquots of this suspension were used in all the experiments. The Hepes-buffered medium described above was used for all experiments except those done in media of various K^+ concentrations. In those experiments, NaCl was replaced with equal amounts of KCl so that normal osmolarity was maintained.

The effects of exposure of the cells to the foreign particles, zymosan, and to the membrane perturbant, phorbol-12-myristate-13-acetate (PMA), on the transmembrane potential of alveolar macrophages were determined. Zymosan, an extract from the cell walls of *Saccharomyces cerevisiae* yeast (Sigma Chemical Co., St. Louis, MO), was prepared by boiling 50 mg in 3 ml of Hepes-buffered medium for 15 minutes. Then the zymosan was separated from the medium by centrifugation, and a stock suspension was prepared by resuspending the pellet in fresh Hepes-buffered medium. This procedure seemed to prevent the zymosan particles from sticking together. We have shown previously that opsonization of zymosan is not required to stimulate alveolar macrophages (Miles et al., '78; Castranova et al., '80). A stock solution of PMA (Sigma Chemical Co.) was prepared by dissolving a small amount in .05 ml of dimethyl sulfoxide (DMSO) and then making up to volume with water. We tested the effects of this small amount of DMSO on transmembrane potential and superoxide anion release in alveolar macrophages and found no effect.

Changes in the transmembrane potential of alveolar macrophages were measured by using the fluorescent probe, Di-S-C_3(5) (Hoffman and Laris, '74; Castranova et al., '79). This dye has been used previously to measure the resting membrane potential of rat alveolar macrophages (Castranova et al., '79). The fluorescence was measured with a Perkin-Elmer Model MPF-3 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CN), which was set for excitation and emission wavelengths of 622 nm and 665 nm, respectively. Each measurement was made by first equilibrating 4 x 10^6 cells with Di-S-C_3(5) (0.66 μg/ml) in a final volume of 3 ml in a cuvette. After the baseline fluorescence reached a steady level, aliquots of either zymosan or PMA were added to the suspension, and the resultant changes in fluorescence were recorded. All measurements were made at 22°C.

We also measured the effects of zymosan and PMA on fluorescence in the absence of cells. At high levels of zymosan (>2 mg), there is a decrease in the baseline fluorescence. This shift in fluorescence is in the same direction as that produced by hyperpolarization of the cells. Since zymosan has an effect on fluorescence, before each experiment the zymosan was first equilibrated with the Di-S-C_3(5) by incubating the particles in medium containing dye for 1 hour at 22°C. In addition, we corrected all measurements for these small shifts in baseline produced by higher levels of the particles. PMA has no effect on baseline fluorescence.

Superoxide anion release from alveolar macrophages was measured as reduction of cytochrome c according to a method published previously (Babior et al., '73; Miles et al., '78). Alveolar macrophages (4 x 10^6 cells) were incubated at 22°C in 5 ml of Hepes-buffered medium containing 0.12 mM cytochrome c (type VI; Sigma Chemical Co.) and either 4 mg of zymosan or 10 μg/ml PMA. We determined these incubation conditions to be optimal for measurement of superoxide release. After incubation the cells were immediately separated from the incubation medium by centrifugation at 6,000 x g for 30 seconds in a refrigerated centrifuge. Optical densities of the supernatants obtained following incubation were measured at 550 nm with a Gilford Model 300-N spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH) and compared to those obtained with no incubation. Experiments were also done in the presence and absence of superoxide dismutase (SOD; Sigma Chemical Co.), an enzyme which catalyzes the conversion of superoxide to hydrogen peroxide and oxygen (McCord and Fridovich, '69; Fridovich, '72), in order to be certain that the reduction of cytochrome c was due to superoxide. When SOD (0.4 mg/ml) was added to the incubation mixtures, there were no changes in optical density. In addition, measurements of superoxide release in resting cells, i.e., cells not exposed to zymosan, were made. At 22°C there is no release of superoxide from resting cells. In these experiments superoxide anion production is expressed in optical density units.

RESULTS

Effects of zymosan particles and PMA on superoxide anion release and transmembrane potential

Exposure of rat alveolar macrophages either to zymosan particles or to the membrane perturbant, phorbol myristate acetate (PMA), will cause superoxide anion release. The time courses for release are shown in Figure 1. The amounts of zymosan (4 mg) and PMA (10 μg/ml) used in these experiments are the amounts that yield maximal superoxide release. Note that zymosan is a more effective stimulus to superoxide release than is PMA; i.e., the maximal amount of superoxide anion released in response to zymosan is about 50% greater than that induced by PMA. In addition, the time courses are different. Zymosan-induced superoxide release becomes maximal in 40 minutes with a half-time of about 10 minutes. The PMA-induced response is much slower; i.e., maximal release requires 80 minutes, and the half-time is approximately 30 minutes.

The effects of zymosan and PMA on the transmembrane potential of alveolar macrophages are shown in Figures 2A and 2B, respectively. The amounts of zymosan and PMA used in these experiments are also those that yield maximal levels of superoxide anion release. It has been shown that the fluorescence of Di-S-$C_3(5)$ increases during membrane depolarization and decreases during hyperpolarization (Hoffman and Laris, '74; Castranova et al., '79). Addition of either zymosan or PMA to alveolar macrophages causes an increase in fluorescence, indicating that membrane depolarization occurs. The time courses of these fluorescence changes are shown in Figure 2. The depolarization induced by exposure to zymosan reaches a maximal level in about 5 seconds and is maintained at a steady level for at least 1 hour. The depolarization induced by PMA is much slower; i.e., it takes about 3 minutes to reach the maximal level. Furthermore, the depolarization initiated by PMA is not sustained but, rather, tends to decrease with time. The results of these experiments demonstrate that exposure of the cells both to particles and to a membrane perturbant causes membrane depolarization. In addition, the depolarization is much faster when induced by zymosan, just as is superoxide anion release.

The effects of various concentrations of zymosan and PMA on transmembrane potential are shown in Figures 3A and 3B, respectively. In each case the magnitude of the depolarization increases as the concentration of either zymosan or PMA is increased. At the highest concentrations tested, there is a tendency toward saturation; i.e., greater levels of zymosan or PMA do not cause greater levels of depolarization. The magnitude of the depolarization is approximately three-fold greater in the presence of zymosan than with PMA. It is interesting that zymosan is also more effective in promoting superoxide anion release than is PMA (Fig. 1). During the course of these experiments, we discovered that the relationship between the amount of zymosan used and membrane depolarization appeared to be similar to the relationship between the amount of zymosan and superoxide release. Therefore, the remainder of the experiments described in this communication were designed to study the relationship between membrane depolarization and superoxide anion release in alveolar macrophages exposed to zymosan.

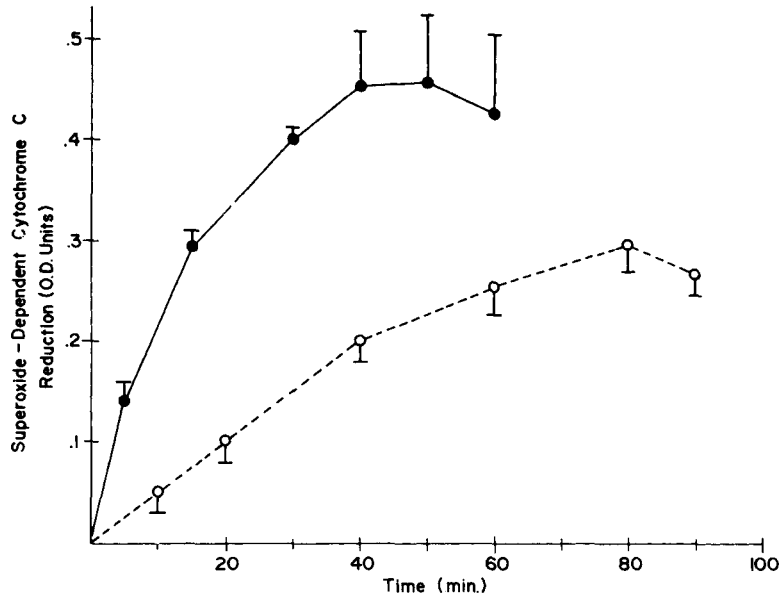

Fig. 1. Time course of superoxide anion release induced by zymosan (•——•) and by PMA (o---o). The incubation mixture consisted of 4 x 10^6 cells and either 4 mg of zymosan or 50 μg of PMA in a final volume of 5 ml Hepes-buffered medium containing 0.12 mM cytochrome c. The points are mean values for five to seven experiments, and the bars represent the standard errors of the means.

Relationship between membrane depolarization and superoxide anion release

Changes in transmembrane potential and superoxide anion release in response to five different amounts of zymosan, 0.1, 0.4, 0.5, 2.0, and 4.0 mg, were measured in the same samples of alveolar macrophages. The relationship between membrane depolarization and superoxide release is shown in Figure 4. Note that as the level of depolarization increases, the amount of superoxide anion released by the alveolar macrophages also increases. Thus, these data demonstrate that a relationship exists between membrane potential changes and superoxide anion release; i.e., depolarization is accompanied by superoxide production.

We have demonstrated previously that increases in the external K^+ concentration cause depolarization of rat alveolar macrophages (Castranova et al., '79). Therefore, if depolarization of the cell membrane leads to release of superoxide anion, an increase in the external K^+ concentration should also cause superoxide release. The results of such experiments are shown in Figure 5. These results demonstrate that as the extracellular K^+ level is increased, the release of superoxide anion from the alveolar macrophages also increases. The magnitude of the release is much smaller than that caused by exposure to zymosan; i.e., superoxide release in the presence of 150 mM K^+ is only about 20% of that induced by zymosan. However, the magnitude of the depolarization in the presence of 150 mM K^+ is also only approximately 20% of that initiated by zymosan. In another series of experiments we studied the effects of zymosan on superoxide release in cells that had been previously depolarized with K^+. The results are shown in Table 1. Note that there is less superoxide released from cells exposed to zymosan

TABLE 1. Effects of depolarization on zymosan-induced superoxide release

Treatment	$[K^+]_o$(mM)	Superoxide release (O.D. Units)
Cells + zymosan	5	.463(±.028)
Cells + zymosan	150	.358(±.043)

Superoxide anion release was measured following incubation of the samples at 22°C for 50 minutes. Each sample contained 4 x 10^6 alveolar macrophages and 4 mg zymosan in a final volume of 10 ml of incubation medium. The incubation medium was a Hepes-buffered solution that contained either 5 mM K^+ or 150 mM K^+ (i.e., K^+ replacement of Na^+). The numbers shown are mean values for six experiments ± the standard errors of the means.

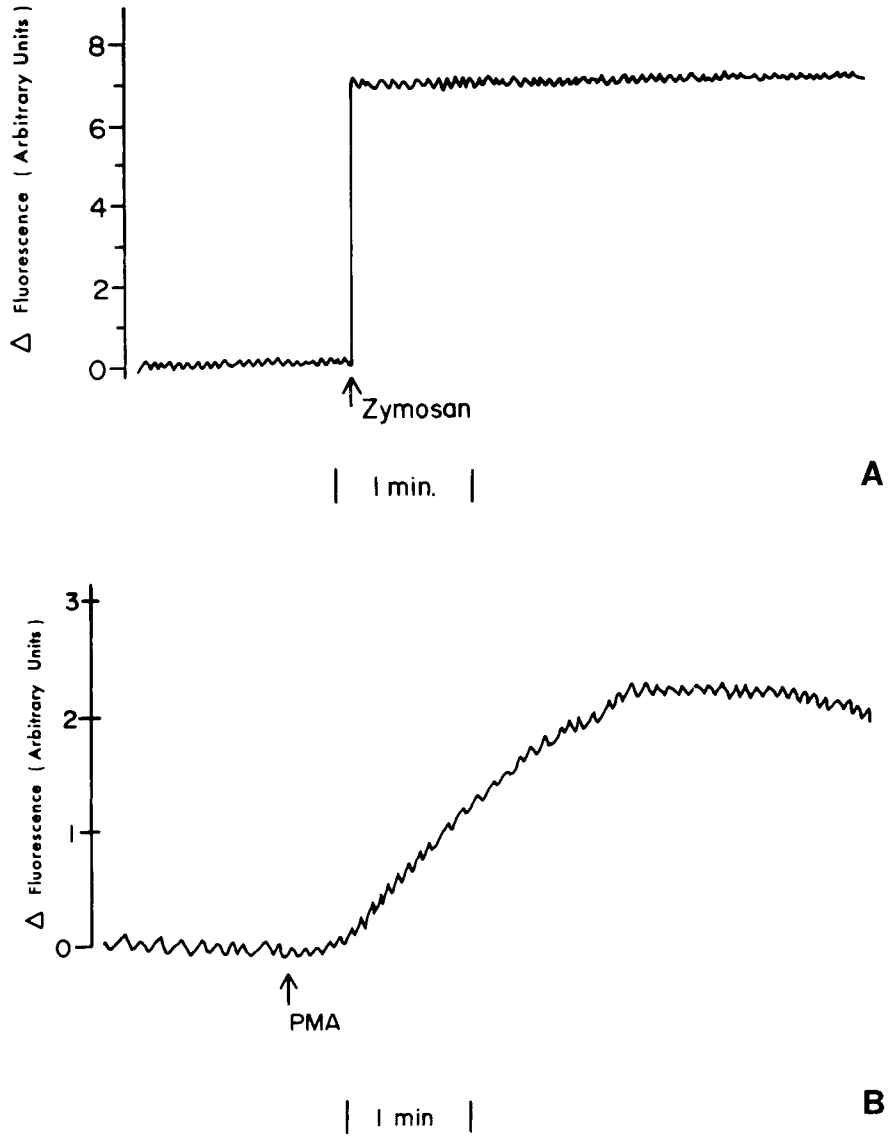

Fig. 2. A) Time course of the membrane depolarization induced by exposure of alveolar macrophages to zymosan particles. The incubation mixture contained alveolar macrophages (4×10^6) and Di-S-C_3(5) (0.66 μg/ml) in a final volume of 3 ml of Hepes-buffered medium. Zymosan (4 mg) was added to the incubation medium where indicated. B) Time course of the membrane depolarization induced by exposure of alveolar macrophages to PMA. The incubation mixture contained alveolar macrophages (4×10^6) and Di-S-C_3(5) (0.66 μg/ml) in a final volume of 3 ml of Hepes-buffered medium. PMA (10 μg/ml) was added to the incubation medium where indicated.

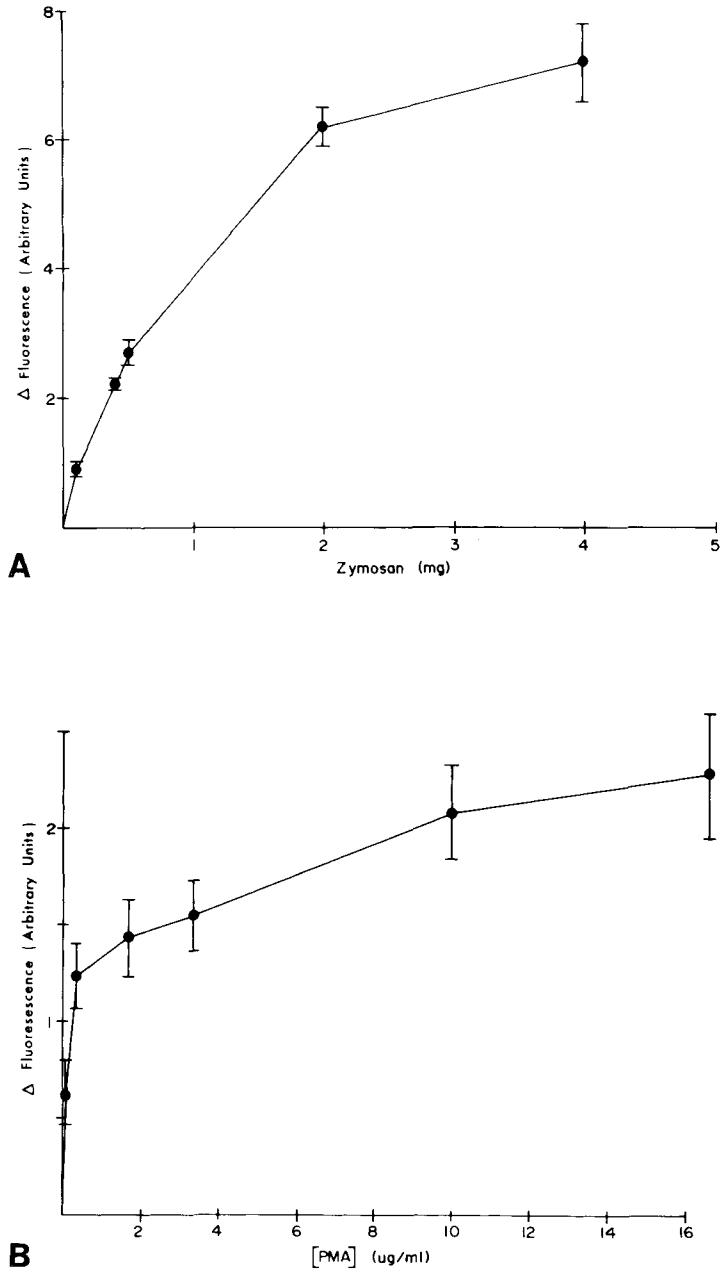

Fig. 3. A) Relationship between membrane depolarization and the amount of zymosan to which alveolar macrophages were exposed. The incubation mixture contained alveolar macrophages (4 x 10^6), Di-S-C_3(5) (0.66 μg/ml), and the appropriate amount of zymosan in a final volume of 3 ml of Hepes-buffered medium. The points are mean values for six experiments, and the bars represent the standard errors of the means. B) Relationship between membrane depolarization and the amount of PMA to which alveolar macrophages were exposed. The incubation mixture was identical to that described for Figure 2A, except that PMA was used instead of zymosan. The points are mean values for six experiments, and the bars represent the standard errors of the means.

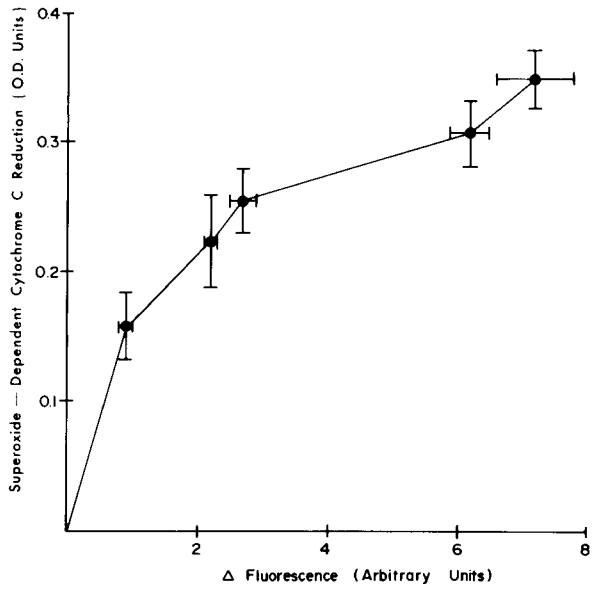

Fig. 4. Relationship between membrane depolarization and the amount of superoxide anion released from alveolar macrophages during exposure to zymosan particles. Superoxide release and changes in transmembrane potential were measured as described in Methods during exposure of the same cell samples to five different amounts of zymosan (0.1, 0.4, 0.5, 2.0, and 4.0 mg). Each point represents the superoxide release and depolarization obtained during exposure to one amount of zymosan. The points are mean values for six experiments, and the bars represent the standard errors of the means.

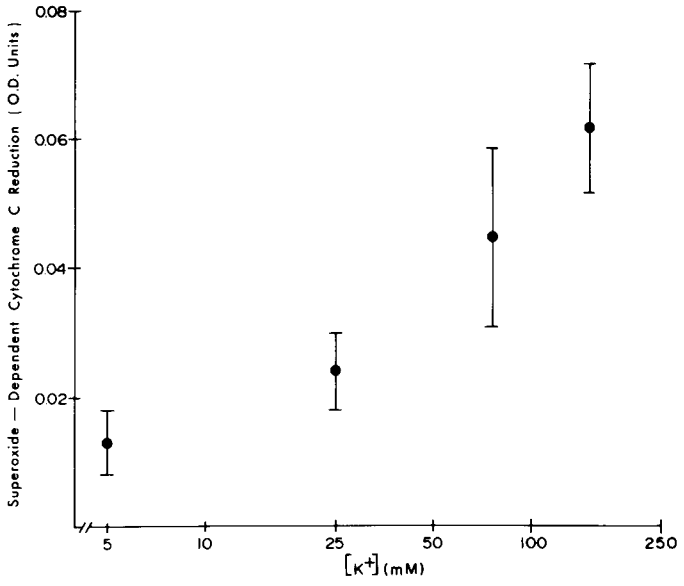

Fig. 5. Superoxide anion release as a function of the extracellular K^+ concentration. Superoxide release was measured as described in Methods. The various levels of K^+ were obtained by replacing NaCl with KCl in the Hepes-buffered medium. The points are mean values for six experiments, and the bars represent the standard errors of the means.

when the cells have already been depolarized with K^+. The results of these experiments with K^+ indicate that membrane depolarization of alveolar macrophages is associated with superoxide anion release.

DISCUSSION

The results of these experiments indicate that superoxide anion release from rat alveolar macrophages is related to depolarization of the cell membrane and suggest that the change in transmembrane potential may act as a signal for superoxide release. There are four lines of evidence to support this conclusion: 1) Membrane depolarization precedes superoxide anion release. The half-times for changes in transmembrane potential induced by either zymosan or PMA are less than 5 seconds and 1.5 minutes, respectively (Fig. 2). These half-times are much shorter than those required for release of superoxide anion, i.e., about 10 minutes for zymosan-induced release and 30 minutes for the PMA-stimulated response (Fig. 1). 2) The magnitude of superoxide anion release induced by zymosan appears to be related to the magnitude of the change in membrane potential (Fig. 4). In addition, both the depolarization and superoxide release induced by zymosan are greater than those responses induced by PMA (Figs. 1, 2). 3) Depolarization of alveolar macrophage membranes with K^+ leads to release of superoxide, even in the absence of zymosan or PMA (Fig. 5). 4) Superoxide anion release in response to zymosan is decreased in cells previously depolarized with K^+ (Table 1).

Changes in the transmembrane potential of polymorphonuclear leukocytes (PMN) have also been associated with superoxide release, but the responses seem to differ somewhat from those in alveolar macrophages. Stimulation of neutrophils with PMA, concanavalin A, or chemotactic factors induces either triphasic or biphasic changes in transmembrane potential; i.e., both depolarization and hyperpolarization occur (Korchak and Weissman, '78; Whitin et al., '80; Jones et al., '80). There is some evidence to indicate that the delayed hyperpolarization that occurs in neutrophils is due to the activation of an electrogenic Na^+-K^+ pump (Becker et al., '78; Jones et al., '80). In contrast, only depolarization of alveolar macrophages occurs in response to zymosan or PMA. Since in alveolar macrophages the concentration gradients for Na^+ and K^+ are smaller than in PMN (Castranova et al., '79), the activity of the pump is low and would probably have little effect on transmembrane potential. Thus, the membrane potential changes, and probably the underlying changes in permeability of the membranes to ions, are different in neutrophils and alveolar macrophages.

The membrane permeability changes that lead to the depolarization induced by zymosan in alveolar macrophages are not yet known. One possibility is that Na^+ influx is responsible for the depolarization. In fact, the data in Table 1 may be taken to indicate the involvement of Na^+ in this process; i.e., removal of extracellular Na^+ results in a decrease in superoxide release. Unfortunately, in these experiments the Na^+ was replaced with K^+, and increased amounts of extracellular K^+ lead to depolarization (Castranova et al., '79). However, in other experiments we replaced the extracellular Na^+ with choline and found a 30% decrease in the level of depolarization induced by zymosan. This result suggests that Na^+ influx is at least partially responsible for the depolarization. In addition, we have found that verapamil, an inhibitor of Ca^{++} fluxes, produces a decrease in superoxide anion release from rat alveolar macrophages. Therefore, we tested the effects of verapamil on the membrane depolarization induced by zymosan. We found that small doses of verapamil produce a decrease in the magnitude of the depolarization. However, at higher concentrations the verapamil reacts with the Di-S-C_3(5). Thus, definitive conclusions cannot be drawn from these experiments. Nevertheless, these findings suggest that Na^+, and possibly Ca^{++}, influx may occur during the depolarization.

In summary, these results indicate that the release of superoxide anion from rat alveolar macrophages in response to foreign particles is related to depolarization of the cell membranes. The change in transmembrane potential precedes the release of superoxide and, thus, may act as a signal to initiate the secretory and phagocytotic responses of the cells. It is possible that increases in the permeability of the membrane to sodium, and perhaps to calcium, are involved in the depolarization.

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