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# Mg.ATP PRIMES SUPEROXIDE-GENERATING RESPONSES IN ELECTROPERMEABILIZED NEUTROPHILS

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The present study utilizes an electropermeabilized cell system to determine the effect of Mg.ATP on neutrophil superoxide ($O_2^-$)-generating responses stimulated by suboptimal concentrations of fMLP, GTPγS and PMA. Permeabilization in the presence of exogenously added Mg.ATP was neither sufficient to initiate $O_2^-$ release nor necessary for stimulated $O_2^-$ production. However, the inclusion of Mg.ATP in the permeabilization medium primed the $O_2^-$-generating responses mediated by suboptimal concentrations of these stimuli. The site of action of Mg.ATP is intracellular. Moreover, the fact that Mg.ATP primes responses stimulated by fMLP, GTPγS and PMA suggests that the modulatory effect is at the level of protein kinase C. 

Receptor-mediated activation of neutrophils results in the production of substantial amounts of superoxide ($O_2^-$). In intact neutrophils this stimulatory pathway involves the activation of a phosphoinositide-specific phospholipase *c* to yield inositol 1,4,5 trisphosphate and 1,2-diacylglycerol; the former of these second messengers mobilizes intracellular $Ca^{2+}$ (1) while the latter activates protein kinase C (2). Although it is generally accepted that the activation of protein kinases (e.g., protein kinase C) requires the presence of ATP it has proved difficult to evaluate the role and requirement for ATP in signal transduction pathways of intact cells. Techniques that render the plasma membrane permeable have therefore been instrumental in the study of cellular signalling mechanisms (3,4). Electropermeabilization, as a means of introducing effector molecules into the cytosol, has distinct advantages over other permeabilization techniques (e.g., detergents and streptolysin O) in that the procedure apparently produces small pores in the plasma membrane yet signal transduction pathways remain relatively unimpaired (5). More recently,

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Abbreviations: $O_2^-$, superoxide; SOD, superoxide dismutase; ATP, adenosine 5'-triphosphate; LDH, lactate dehydrogenase ; EGTA, ethylene glycol-bis(ß-aminoethyl ether) N, N, N', N'-tetraacetic acid; fMLP, N-formyl-met-leu-phe; GTPγS, guanosine-5'-O-(3-thiotriphosphate); PMA, Phorbol 12-myristate 13-acetate.

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electropermeabilization has proved to be a useful technique in the study of the signal transduction pathway that activates the respiratory burst (6-9).

In the present study, electropermeabilization has been used to determine the effect of Mg.ATP on neutrophil $O_2^-$-generating responses stimulated by suboptimal amounts of chemotactic peptide (i.e., fMLP), the nonhydrolysable guanine nucleotide analog, GTPγS, and a phorbol ester (i.e., PMA). The results indicate that the electropermeabilization of neutrophils in the presence of exogenously added Mg.ATP primes the activation of NADPH oxidase mediated by all three of these stimuli. Moreover, these results suggest an intracellular site of action of Mg.ATP, probably at the level of protein kinases.

## MATERIALS AND METHODS

### Materials

ATP ($Mg^{2+}$ salt), EGTA, fMLP and PMA were purchased from Sigma. GTPγS was acquired from Boehringer Mannheim Biochemicals. Sources of all other materials have been described elsewhere (10).

### Preparation of Neutrophils

Casein-elicited guinea pig peritoneal neutrophils were isolated as described elsewhere (11). Cell viability was ≥ 95% for each preparation as measured by LDH release.

### Electropermeabilization of Neutrophils

Neutrophils ($8 \times 10^6$) were suspended in 0.8 ml of ice-cold permeabilization medium (138 mM KCl, 2.7 mM NaCl, 16.2 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, 0.5 mM $MgCl_2$, 7.5 mM D-glucose, 1 mM EGTA and 0.193 mM $CaCl_2$; pH 7.1) in a BioRad pulser cuvette. Where indicated Mg.ATP (3.5 mM) was added to the permeabilization medium. The mediums' $[Ca^{2+}]_{free}$, calculated from stability constants using the EQCAL program (Biosoft), was 50 nM. Permeabilization was achieved by two discharges of 4.5 kV/cm from a 25 μF capacitor (BioRad Gene Pulser). Electropermeabilized cells were used immediately, except where indicated. Upon electropermeabilization, cell viability, determined by the release of LDH, was 83.7 ± 4.9 % (n=4).

### Superoxide Release

Superoxide ($O_2^-$) release by the NADPH oxidase of electropermeabilized neutrophils was monitored at 37 °C by the continuous spectrophotometric measurement of the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* at 550 nm [e.g., (10)]. The assay mixture consisted of a modified Dulbecco's phosphate buffered saline medium, PBS (138 mM NaCl, 2.7 mM KCl, 16.2 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, 0.5 mM $MgCl_2$, 1 mM EGTA and a final $[Ca^{2+}]_{free}$ of 50 nM, 7.5 mM D-glucose; pH 7.1) containing 0.105 mM ferricytochrome *c* and $10^6$ cells/ml. The reference cuvette contained 30 μg/ml SOD in addition to the above constituents. The final [Mg.ATP] in all assays utilizing electropermeabilized neutrophils was 437.5 μM.

## RESULTS AND DISCUSSION

The superoxide generating NADPH oxidase retains the ability to be activated by both fMLP and PMA in electropermeabilized neutrophils. The rate of $O_2^-$ production by PMA-stimulated electropermeabilized neutrophils did not differ from that produced by intact (i.e., non-electropermeabilized) cells (Table 1). However, the response towards fMLP was slightly

**Table 1**

| | Max. rate of superoxide ($O_2^-$) production (nmoles $O_2^-$/min/$10^6$ cells) | |
|---|---|---|
| | fMLP (1.0 $\mu$M) | PMA (50 ng/ml) |
| Intact neutrophils | 5.59 ± 1.11 (n=16) | 6.43 ± 0.96 (n=16) |
| Freshly electropermeabilized | 4.23 ± 0.84 (n=7) | 6.39 ± 0.41 (n=8) |
| Electropermeabilized, resuspended | 3.88 ± 0.19 (n=3) | 7.10 ± 0.86 (n=3) |

Comparison of $O_2^-$ generating responses in intact and electropermeabilized neutrophils. Intact neutrophils (1 x $10^6$) were suspended in assay medium and incubated for 3 min at 37 °C prior to the addition of the stimulus. Electropermeabilized neutrophils were either added directly to the assay medium and treated as above or pelleted (1500 g.min, 4 °C) and resuspended in fresh permeabilization medium prior to transfer to the assay mixture. Rates of $O_2^-$ production are expressed as means ± S.D. of the number of experiments indicated in parentheses.

diminished (approx. 30 %) in electropermeabilized neutrophils, indicating that a degree of damage to either the fMLP receptor or to the membrane itself may occur upon permeabilization. In sharp contrast to the work of Grinstein and his collaborators (6-9) activation of the respiratory burst in electropermeabilized neutrophils did not require the presence of exogenously added ATP and/or NADPH in either the permeabilization or the assay media, suggesting that upon electropermeabilization minimal loss of these intermediates occurs (12,13). Indeed, when electropermeabilized neutrophils were pelleted (1500 g.min, 4 °C) and resuspended in fresh medium, thus removing any ATP and NADPH which may have been released, the rates of fMLP- and PMA-mediated $O_2^-$ production were much the same as those observed in freshly permeabilized neutrophils (Table 1). The fact that exogenously added ATP (and NADPH) is not necessary for the stimulation of $O_2^-$ production by electropermeabilized neutrophils represents a fundamental methodological difference between the studies described herein and those of Grinstein and his fellow workers (6-9).

Recent studies (14-17) have described the presence of a receptor for ATP at the surface of human neutrophils and HL60 cells; the binding of ATP to these receptors appears to activate NADPH oxidase via guanine nucleotide-binding (G) proteins. In the present study, the incubation of intact neutrophils in the presence of exogenously added Mg.ATP (100 $\mu$M - 2 mM) neither activated NADPH oxidase *per se* nor primed neutrophils that were subsequently stimulated by suboptimal levels of fMLP and PMA; higher concentrations of Mg.ATP ($\geq$ 2 mM) slightly inhibited these stimulated responses in intact cells (results not shown). Although it is not known if elicited guinea-pig neutrophils possess

similar ATP receptors, it is well established that these cells possess an ecto-ATPase activity (18,19), which may serve to limit the quantity of extracellular ATP available.

Electropermeabilization of neutrophils in the presence of exogenously added Mg.ATP was neither sufficient to initiate $O_2^-$ release nor necessary for the stimulation of $O_2^-$ production by fMLP, PMA and GTPγS. However, the inclusion of Mg.ATP in the permeabilization medium modulated the $O_2^-$-generating responses mediated by suboptimal concentrations of these stimuli (Figs. 1 and 2). The maximal rates of $O_2^-$ production in

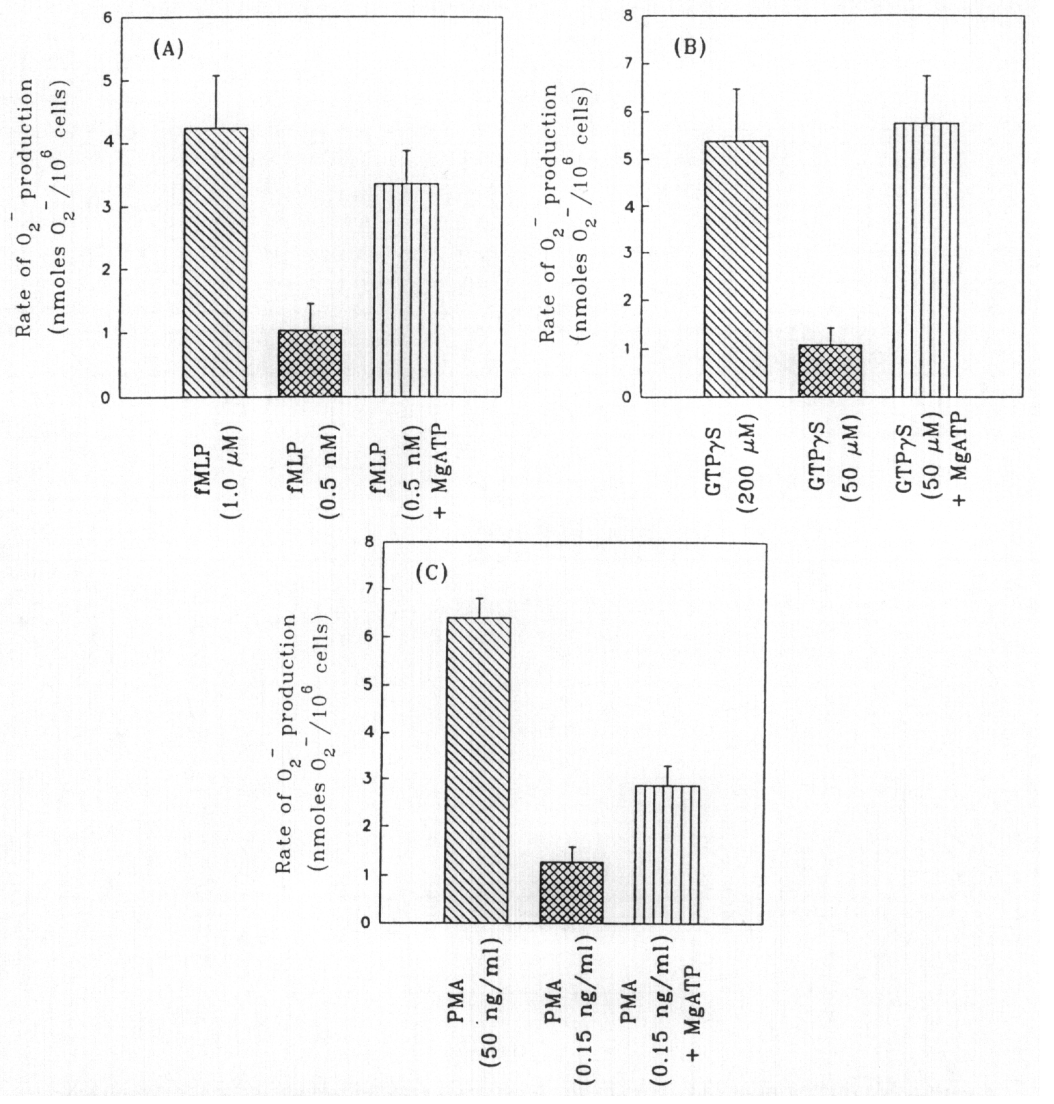


**Figure 1.** The effect of Mg.ATP on $O_2^-$ production stimulated by suboptimal amounts of (A) fMLP, (B) GTPγS, and (C) PMA. Each panel shows the mean (± S.D.) rates of $O_2^-$ production from, i) electropermeabilized cells stimulated by optimal amounts of fMLP (1 μM), GTPγS (200 μM) and PMA (50 ng/ml); *diagonal lines*, ii) electropermeabilized cells stimulated by suboptimal amounts of fMLP (5 nM), GTPγS (50 μM) and PMA (0.15 ng/ml); *crosshatch*, and iii) neutrophils permeabilized in the presence of Mg.ATP and stimulated by suboptimal amounts of these stimuli; *vertical lines*, respectively.

electropermeabilized neutrophils primed by Mg.ATP and stimulated by fMLP (5 nM), GTPγS (50 μM) and PMA (0.15 ng/ml) were enhanced 3-fold, 5-fold and 2.25-fold, respectively (Fig. 1 A, B and C). Furthermore, ATP priming reduced the onset time of stimulated $O_2^-$ production (Fig. 2 A, B and C). The ability of the nonhydrolysable guanine nucleotide analog, GTPγS, to stimulate $O_2^-$ production by electropermeabilized neutrophils (Figs. 1 B and 2 B) probably results from the activation of a G protein, termed $G_p$, which regulates the receptor-coupled activation of phospholipase *c* (20,21). It is noteworthy that GTPγS only activates the respiratory burst in permeabilized neutrophils (7, Hartfield and Robinson, in preparation) confirming the postulate that the plasma membrane is rendered

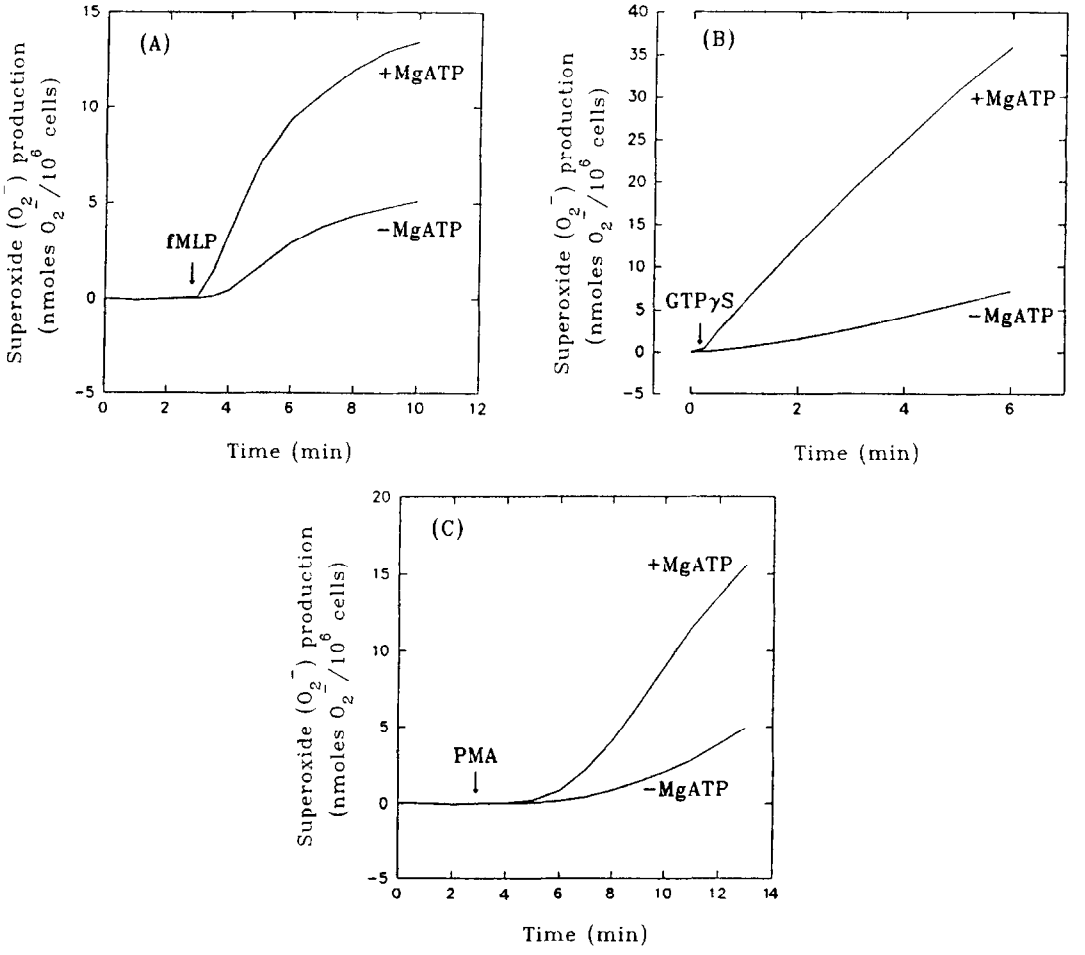


**Figure 2.** Mg.ATP priming of $O_2^-$ production in electropermeabilized neutrophils stimulated by suboptimal amounts of (A) fMLP (5 nM), (B) GTPγS (50 μM), and (C) PMA (0.15 ng/ml). Each panel shows the time course of $O_2^-$ production by neutrophils electropermeabilized either in the absence or the presence of Mg.ATP and stimulated by suboptimal amounts of fMLP, GTPγS and PMA, respectively. In panels (A) and (C) electropermeabilized cells (1 x $10^6$) were preincubated at 37 °C for 3 min prior to the addition of the stimulus (↓); in panel (B) GTPγS was included in the permeabilization medium. The results are representative of 4 experiments.

permeable to small molecules ( $\leq$ 1000 M.W. units). Accordingly, Mg.ATP (M.W. 576.2) can gain access to the cytosolic milieu of electropermeabilized neutrophils, indicating that the site of action of Mg.ATP is intracellular.

It is usually assumed that the presence of cytosolic ATP is required for the induction of receptor-mediated $O_2^-$ production. Additionally phosphorylation appears to be required for the respiratory burst (22), suggesting that ATP may donate phosphate for substrate phosphorylation by protein kinases. Several lines of evidence lend support to this assumption. Permeabilized cells phosphorylate endogenous proteins from exogenous [$\gamma$-$^{32}$P]-ATP (23), and respond to both protein kinase C agonists (13,24) and antagonists (6,7,13,25); the depletion of ATP, either before or after permeabilization, inhibits oxygen consumption by electropermeabilized human neutrophils (8,9). Furthermore, the close correlation between activation of protein kinase C and neutrophil functions, particularly the activation of the respiratory burst (26,27), has suggested an integral role for this enzyme in stimulus-response coupling. In conclusion, the present results suggest that Mg.ATP primes the activation of NADPH oxidase in electropermeabilized neutrophils by regulating the effective affinity of fMLP, GTP$\gamma$S and PMA as stimuli of the respiratory burst. Moreover, Mg.ATP probably modulates these responses by means of phosphorylation reactions mediated by protein kinase C.

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