Exposure of Human Neutrophils to Exogenous Nucleotides Causes Elevation in Intracellular Calcium, Transmembrane Calcium Fluxes, and an Alteration of a Cytosolic Factor Resulting in Enhanced Superoxide Production in Response to FMLP and Arachidonic Acid

By Richard A. Axtell, Rebecca R. Sandborg, James E. Smolen, Peter A. Ward, and Laurence A. Boxer

Exposure of human neutrophils to micromolar concentrations of both hydrolyzable and nonhydrolyzable purine nucleotides caused the generation of transient rises in intracellular calcium (Ca²⁺), Ca²⁺ fluxes across the membrane, and primed the cells for enhanced production of superoxide (O₂⁻) when subsequently exposed to agonists such as FMLP and arachidonic acid. The neutrophils were most sensitive to adenosine triphosphate (ATP) and ATP-γ-S, which produced Ca²⁺ transients and enhanced O₂⁻ production at concentrations as low as 1 to 5 μmol/L, with a doubling of O₂⁻ generation at 25 to 50 μmol/L. Adenosine diphosphate (ADP), guanosine triphosphate (GTP), and 5'-adenylylimidodiphosphate (AMP-PNP) required approximately 10-fold higher concentrations to cause similar effects. Adenosine did not cause Ca²⁺ fluxes or a Ca²⁺ transient and was inhibitory of O₂⁻ production. There was a strong correlation between a nucleotide's ability to generate a Ca²⁺ response and its ability to enhance O₂⁻ generation. Nitrogen cavitation and subcellular fractionation of the neutrophils after a brief exposure to ATP, ATP-γ-S, and AMP-PNP revealed that the enhanced O₂⁻ generating capacity was stable and detectable in a cell-free assay system. By combining variously treated cytosolic and membrane fractions, it was found that the enhanced O₂⁻ production was attributable to a modification of a component(s) of the cytosol.

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Neutrophils respond to numerous extracellular stimuli including bacterial components (formylated peptides), products of complement activation (C5), opsonized particles, and immune complexes. These factors act through cell surface receptors to mediate chemotaxis, phagocytosis, degranulation, and oxygen burst activity. Recent work in this and other laboratories has suggested the existence of a new class of exogenous mediators of neutrophil activation. In vivo experiments have shown that there is increased tissue damage and oxygen radical production when neutrophils are stimulated in the presence of platelets. Subsequent in vitro experiments have confirmed that platelets will enhance superoxide (O₂⁻) production by neutrophils in response to several stimuli, including immune complexes, opsonized zymosan, C5 and N-formyl-methionyl-leucyl-phenylalanine (FMLP).

It has recently been shown that the platelet effect can be mediated by either platelet lysates or secretory products from thrombin-stimulated platelets, the apparent mediators being adenosine triphosphate (ATP) and adenosine diphosphate (ADP) contained in the dense granules. Subsequent investigations have identified several effects of ADP, ATP, and other purine nucleotides (and their non-hydrolyzable analogs) on human neutrophils. Freyer et al. have reported that exposure to ATP and ATP-γ-S results in recruitment of CD11b/CD18 to the cell membrane with a concomitant increase in neutrophil aggregation and adhesion. Balazovich and Boxer have reported that exogenous ATP is capable of causing both the release of specific granule contents and the activation and translocation of protein kinase C. Kuhns et al. found that ATP, uridine triphosphate (UTP), and inosine triphosphate (ITP), but not ADP (in concentrations less than 200 μmol/L) were capable of generating a Ca²⁺ transient and enhancing FMLP-induced O₂⁻ production. In addition, Ward et al. reported that with both rat and human neutrophils, the non-hydrolyzable analogs 5'-adenylylimidodiphosphate (AMP-PNP) and β,γ-methyladenosine 5'-triphosphate (AMP-PCP) were capable of enhancing O₂⁻ production in response to immune complexes. The non-hydrolyzable nucleotides were not tested with FMLP as the stimulus. Further work by Ward et al. has shown that both ATP and ADP are capable of causing Ca²⁺ transients and enhanced O₂⁻ production in response to FMLP, but interpretation of these latter observations is somewhat confounded by the inclusion of cytochalasin B in these assays.

In this report, we present data indicating that with several purine nucleotides, both native and hydrolytically resistant, there is a consistent correlation between a nucleotide's ability to raise intracellular Ca²⁺ and its ability to enhance O₂⁻ production in response to the agonists FMLP and arachidonic acid. We will also present data indicating that this "priming" effect of exogenous nucleotides is mediated through a modification of the cytosolic factor(s).

Materials and Methods
Materials. Anhydrous dextrose was obtained from Columbus Chemical Industries (Columbus, WI), fura-2 acetoxyethyl ester was purchased from Molecular Probes (Junction City, OR), and 6% dextran 75 in 0.9% sodium chloride for injection was obtained from Abbott Laboratories (North Chicago, IL). Horse heart ferricytochrome C (grade VI), superoxide dismutase (SOD, from bovine erythrocytes), FMLP, ethylene glycol tetra-acetic acid (EGTA), Trizma base, Triton-X-100, adenosine 5'-triphosphate (disodium salt), guanosine 5'-triphosphate (GTP; sodium salt type III), adeno-
sine 5'-diphosphate (sodium salt), adenosine, citric acid trisodium salt (dihydrate), citric acid (monohydrate), HEPES, PIPES, and 5'-adenyllylimido-diphosphate (AMP-PNP, tetralithium salt), were purchased from Sigma Chemical Company (St Louis, MO). Adenosine-5'-O-(3-thiotriphosphate) tetralithium salt (ATP-γ-S) was obtained from Boehringer Manheim Biochemicals (Indianapolis, IN), and Ficoll-Paque and Percoll were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Arachidonic acid was obtained from Nu-Chek-Prep, Inc (Elysian, MN), and sodium dodecylsulfate from Bio-Rad Laboratories (Richmond, CA). MAPTAM (bis- (2-acetoxymethyl ester) was purchased from Calbiochem (San Diego, CA). All other reagents were laboratory grade.

Preparation of human neutrophils. Blood was collected from healthy volunteers and anticoagulated with acid-citrate-dextrose (ACD). The neutrophils were purified by dextran sedimentation followed by hypotonic lysis to remove red blood cells and centrifugation through Ficoll-Paque to remove mononuclear cells. These preparations contained greater than 95% neutrophils. For the whole cell assays, the neutrophils were used immediately.

For the cell-free assays, the neutrophils were subcellularly fractionated following the Borregaard technique with certain modifications. The cells were suspended in ice-cold relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 3.5 mmol/L MgCl₂, and 10 mmol/L PIPES, pH 7.3) without 1 mmol/L ATP. For those experiments involving exposure to nucleotides, the cells were suspended in ice-cold relaxation buffer with the desired nucleotide concentration, incubated for 5 minutes at 4°C, and then washed twice with nucleotide-free relaxation buffer. The cells were adjusted to 7.5 x 10⁸ cells/mL and disrupted by nitrogen cavitation after pressurization at 420 psi for 20 minutes. The cavitate was collected into EGTA (pH 7.4) sufficient for a final concentration of 1.25 mmol/L and then centrifuged at 500g for 10 minutes at 4°C. The supernatant was then loaded onto precooled discontinuous Percoll gradients (also lacking in nucleotides) of densities 1.07 and 1.110 g/mL, and the gradients were centrifuged at 48,000g for 16 minutes at 4°C. After centrifugation, the cytosolic and membrane fractions were harvested with a Pasteur pipette. The cytosolic fraction (the aqueous layer extending down to just above the membranes at the aqueous-light Percoll interface) was then spun at 320,000g for 68 minutes to remove contaminating membranes. The membrane fraction (extending from the aqueous-light Percoll interface down to just above the specific granule bands) was centrifuged at 180,000g for 95 minutes at 4°C to remove the Percoll. The membranes were then collected from just above the Percoll pad and either used immediately or stored at −70°C at a concentration of 5 x 10⁷ cell equivalents/mL. Cytosol was also stored at −70°C, and activity was stable for at least 2 weeks.

For experiments using the intracellular Ca²⁺ chelator MAPTAM, the purified neutrophils (10³ cells/mL) were suspended in a Ca²⁺-free HEPES buffer (150 mmol/L NaCl, 5.6 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L glucose, and 10 mmol/L PIPES, pH 7.3) to which either MAPTAM (final concentration 100 µmol/L) or DMSO (the diluent for the MAPTAM, final concentration of 0.5% bovine serum albumin (BSA) and incubated another 15 minutes. After incubation, the neutrophils were washed twice with nucleotide-free relaxation buffer and stored at room temperature at a concentration of 2 x 10⁸ cells/mL.

Assays were performed at 37°C on a Perkin-Elmer 650-108 fluorescence spectrophotometer. For each assay, 100 µL of the cell suspension was added to 890 µL of prewarmed HEPES buffer in a cuvette with a magnetic stir bar, and the cells were allowed to equilibrate for 3 minutes. The stimuli were added at 10 µL aliquots, and the change in furu-2 fluorescence was monitored continuously at an emission wavelength of 500 nm (10 nm slit width) and a single excitation wavelength of 342 nm (5 nm slit width). The intracellular Ca²⁺ levels were calculated using the formula: [Ca²⁺] = k_d (F - F_min)/(F_max - F). The dissociation constant (k_d) was taken as 224 nm. F_max was determined by lysing the cells with 0.2% Triton, and F_min was determined by first adding 5 µL of 1 mol/L Tris base to the cuvette to raise the pH to 8.5 and then adding 7.5 mol/L EGTA (pH 8.5).

Ca²⁺ uptake and effect. Assays of Ca²⁺ fluxes were performed in a manner similar to those methods described by Korchak et al. For uptake assays, purified neutrophils were resuspended in a buffer consisting of 150 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgCl₂, 0.4 mmol/L CaCl₂, and 10 mmol/L HEPES, pH 7.45 and incubated at 37°C for 5 minutes. Ca²⁺ uptake was initiated by the addition of stimulus or vehicle containing 150 µCi/mL ⁴⁵CaCl₂. Assays were carried out for 2 minutes and then terminated by the addition of 10 mol/L EGTA, pH 7.4 to the incubation medium. Aliquots of cell suspensions were immediately layered over silicon oil
and centrifuged in a microfuge for 20 seconds. The amount of radioactivity in each cell pellet was measured using liquid scintillation counting. The amount of radioactivity taken up by cells over two minutes was calculated as the difference between that measured at 2 minutes and at a zero time control.

For efflux assays, neutrophil suspensions were incubated with 150 µCi/mL 45CaCl2 for 45 minutes. Preliminary experiments indicated that isotopic equilibrium was achieved within 45 minutes. After the loading procedure, cells were washed three times at room temperature in unlabeled buffer, resuspended, and then incubated at 37°C for 5 minutes. Efflux was initiated by the addition of an aliquot of the cell suspension to the stimulus or vehicle and terminated by centrifugation through silicon oil. Aliquots of cell supernatants were analyzed for radioactivity.

All results are expressed as counts per minute per 106 cells and were analyzed for statistical significance using a Student's t test.

RESULTS

Nucleotide-induced Ca2+ transients in neutrophils. Exposure of human neutrophils to 25 µmol/L ATP, ATP-γ-S, AMP-PNP, or GTP in the absence of FMLP produced changes in intracellular Ca2+, as shown in Fig 1. ATP and ATP-γ-S caused transient rises in intracellular Ca2+ that were initiated within seconds of the addition of nucleotide and rose to a maximum within 20 seconds. The intracellular Ca2+ concentration then diminished to the "resting" level over the subsequent several minutes. At similar concentrations (25 µmol/L), AMP-PNP caused somewhat lower elevations, and GTP caused markedly smaller Ca2+ transients. As can be seen in Fig 1, the patterns of Ca2+ transients induced by ATP and ATP-γ-S were generally similar to that produced by 0.1 µmol/L FMLP, although the duration of the elevation of the intracellular Ca2+ concentration was shorter after the nucleotide stimulation than after exposure to the chemotactic peptide.

The magnitude of the induced rise in the intracellular Ca2+ concentration was found to be dependent on both the identity of the nucleotide and its concentration. In general, there was an increase in the magnitude of the Ca2+ transient with increasing nucleotide concentration (Fig 2). There was a consistent hierarchy of sensitivity to the various purine nucleotides. ATP produced detectable rises in intracellular Ca2+ at 0.5 µmol/L and near maximal rises (130 nmol/L) at 10 µmol/L concentrations. The neutrophils were slightly less sensitive to ATP-γ-S with detectable Ca2+ transients occurring at 1 µmol/L concentrations and a maximal response occurring at 50 µmol/L.

The neutrophils were approximately an order of magnitude less sensitive to GTP and ADP, exhibiting a threshold of 5 to 10 µmol/L, with a maximal Ca2+ rise occurring with exposure to nucleotide concentrations in the 100 µmol/L range. As reported by other investigators using fluorescent probes such as fura-2/AM and adenosine, ATP (0.1-100 µmol/L) caused a wide variation in the maximal amplitude of the Ca2+ transient seen from experiment to experiment. In these experiments, the maximal rises in Ca2+ concentration ranged from 120 to 750 nmol/L, although mean values were in the range of 150 to 185 nmol/L. The near equivalent efficacy of ATP and ATP-γ-S in producing a Ca2+ transient suggests that hydrolysis of the nucleotide is not required for this effect.

Nucleotide-induced Ca2+ fluxes in neutrophils in the presence and absence of FMLP. FMLP-induced changes in the intracellular Ca2+ concentrations of neutrophils represent the net effect of three sequential events. Within 1 second of exposure to the chemotactic peptide, there is a release of Ca2+ from internal stores, which is followed within 3 seconds by the initiation of an influx of Ca2+ from extracellular fluid. After 10 to 12 seconds, an efflux of Ca2+ begins, which eventually returns the intracellular Ca2+ concentration to resting levels. To determine whether the changes in intracellular Ca2+ observed in response to exposure to the nucleotides were related to changes in Ca2+ movement across the cell membrane, we investigated the flux of 45Ca2+ in intact human neutrophils in response to ATP, ATP-γ-S, and...
FMLP. All measurements were performed after 2 minutes of exposure to each stimulus. As seen in Table 1, the influx of 
$^{45}$Ca$^{2+}$ from the medium was markedly increased over control by the presence of 0.1 μmol/L FMLP. Exposure of the neutrophils to 100 μmol/L concentrations of ATP and ATP-$\gamma$S produced similar increases in $^{45}$Ca$^{2+}$ uptake. In addition, treatment of the cells with ATP and FMLP simultaneously resulted in a greater $^{45}$Ca$^{2+}$ uptake than that produced in response to either stimulus alone, the net effect of which was almost additive in nature.

We then examined Ca$^{2+}$ efflux from cells that had been preloaded with $^{45}$Ca$^{2+}$. As seen in Table 2, exposure of the neutrophils to 0.1 μmol/L FMLP resulted in the release of 34% of the $^{45}$Ca$^{2+}$. Both ATP and ATP-$\gamma$S (at 100 μmol/L concentrations) also produced a significant $^{45}$Ca$^{2+}$ efflux, although the magnitude was less than that seen with the chemotactic peptide alone. Once again, the greatest $^{45}$Ca$^{2+}$ flux (44% of the preloaded total) occurred after simultaneous exposure of neutrophils to ATP and FMLP. In marked contrast, exposure to adenosine did not produce significant $^{45}$Ca$^{2+}$ influx or efflux in neutrophils. This is consistent with our observation that adenosine fails to produce a Ca$^{2+}$ transient (Figs 1 and 2).

**Effects of nucleotides and adenosine on FMLP-induced O$_2^-$ production.** It is commonly assumed that the FMLP-induced changes in intracellular Ca$^{2+}$ concentration are a prerequisite for several neutrophil functional responses, including adhesion, degranulation, phagocytosis and O$_2^-$ production. As previously illustrated, exposure of neutrophils to exogenous purine nucleotides causes similar elevations in intracellular Ca$^{2+}$ and generates Ca$^{2+}$ fluxes across the membrane. These nucleotides do not, however, by themselves cause the generation of any O$_2^-$. They are, however, capable of enhancing O$_2^-$ production in response to agonists such as FMLP and arachidonic acid. Pre-incubation of neutrophils with these nucleotides caused an increase in both the maximal rate of superoxide production and the total amount of superoxide generated. As can be seen in Fig 3, 50 μmol/L concentrations of ATP, ATP-$\gamma$S, and GTP enhanced O$_2^-$ production over the full range of effective chemotactic peptide concentrations. The most pronounced enhancement induced by the nucleotides was seen at 5 x 10$^{-3}$ to 5 x 10$^{-7}$ mol/L FMLP, where the rate of O$_2^-$ production was more than doubled. In contrast, 50 μmol/L concentrations of adenosine profoundly inhibited FMLP-induced O$_2^-$ production.

Despite the similar responses of neutrophils to 50 μmol/L concentrations of ATP, ATP-$\gamma$S, and GTP, there were substantial differences in the sensitivity of FMLP-stimulated neutrophils to lower concentrations of the nucleotides. In the experiments shown in Fig 4, the concentration of the chemotactic peptide was held constant at 10$^{-7}$ mol/L and the nucleotide concentrations were varied between 0.1 μmol/L and 100 μmol/L. The neutrophils were most sensitive to ATP, with a doubling of the rate of O$_2^-$ production occurring

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**Table 1. $^{45}$Ca$^{2+}$ Uptake in Response to FMLP and Various Adenine Compounds**

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Calcium Uptake</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>1,267 ± 176</td>
<td>100</td>
</tr>
<tr>
<td>FMLP (n = 8)</td>
<td>4,210 ± 348*</td>
<td>375 ± 50*</td>
</tr>
<tr>
<td>ATP (n = 6)</td>
<td>2,980 ± 645*</td>
<td>205 ± 25*</td>
</tr>
<tr>
<td>ATP-$\gamma$S (n = 6)</td>
<td>4,062 ± 543*</td>
<td>387 ± 86*</td>
</tr>
<tr>
<td>ATP + FMLP (n = 6)</td>
<td>5,470 ± 691*</td>
<td>424 ± 68*</td>
</tr>
<tr>
<td>Adenosine (n = 3)</td>
<td>1,114 ± 208</td>
<td>102 ± 6</td>
</tr>
</tbody>
</table>

Neutrophils were exposed to 0.1 pmol/L FMLP and/or 100 pmol/L concentrations of ATP, ATP-$\gamma$S, or adenosine for 2 minutes, and the influx of $^{45}$Ca$^{2+}$ from the medium was measured as described in "Materials and Methods."

*Differs significantly from control group (P < .05).

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**Table 2. $^{45}$Ca$^{2+}$ Efflux from Neutrophils in Response to FMLP and Various Adenine Compounds**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium Efflux</th>
<th>% Control</th>
<th>% Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>284 ± 47</td>
<td>100</td>
<td>4 ± 0.3</td>
</tr>
<tr>
<td>FMLP (n = 7)</td>
<td>2,434 ± 330</td>
<td>884 ± 67*</td>
<td>34 ± 2*</td>
</tr>
<tr>
<td>ATP (n = 6)</td>
<td>991 ± 164*</td>
<td>331 ± 15*</td>
<td>14 ± 1*</td>
</tr>
<tr>
<td>ATP-$\gamma$S (n = 4)</td>
<td>1,013 ± 217*</td>
<td>318 ± 42*</td>
<td>13 ± 1*</td>
</tr>
<tr>
<td>ATP + FMLP (n = 4)</td>
<td>2,808 ± 299*</td>
<td>1,136 ± 97*</td>
<td>44 ± 3*</td>
</tr>
<tr>
<td>Adenosine (n = 3)</td>
<td>265 ± 93</td>
<td>83 ± 4</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

Results are expressed as the percent of internalized $^{45}$Ca$^{2+}$ released after 2 minutes of stimulation with the indicated agonists. Final concentrations were 0.1 μmol/L for FMLP and 100 μmol/L for ATP, ATP-$\gamma$S, and adenosine.

*Differs significantly from control group (P < .05).
at a concentration of 5 μmol/L. They were slightly less sensitive to ATP-γ-S (the rate of O2− production doubling at 10 μmol/L), and approximately an order of magnitude less sensitive to GTP and ADP. As before, adenosine profoundly inhibited FMLP-induced O2− production, with 76% inhibition at 0.1 μmol/L and 93% inhibition at 100 μmol/L.

When the ability of these four nucleotides to generate Ca2+ transients and to augment FMLP-induced O2− production in human neutrophils were compared, a close correlation between the two phenomena was evident (Figs 2 and 4). The lowest concentration of each nucleotide at which a Ca2+ transient was detected was essentially the same concentration at which enhancement of O2− production by that nucleotide was first seen. The subsequent dose-response curves for the two phenomena were then nearly superimposable for each nucleotide. Thus, the nucleotide concentration that produced the maximal Ca2+ transient was the concentration that produced maximal enhancement of O2− production.

Effect of EGTA on superoxide production and Ca2+ transients. The addition of EGTA to the extracellular medium (to chelate-free Ca2+) immediately before stimulation of neutrophils by FMLP has been shown to decrease the magnitude of the rise in intracellular Ca2+ and to hasten the return of the intracellular Ca2+ concentration to resting levels. A similar effect of EGTA on human neutrophils occurred with the Ca2+ transient evoked by ATP. As can be seen in Fig 5, when 10 mmol/L EGTA (adequate to reduce extracellular Ca2+ to less than 0.1 μmol/L) was added 30 seconds before the stimulus, the usual initial rapid rise (0 to 5 seconds) was essentially intact. However, later sustained elevations (5 to 20 seconds) were reduced, as was the maximal increment in Ca2+ concentration. The intracellular Ca2+ concentration subsequently returned more quickly to the baseline value. Presumably, these changes reflect an abrogation of the influx of extracellular Ca2+ and are consistent with the data (Table 1) that indicated that both ATP and ATP-γ-S induced uptake of Ca2+ from the extracellular medium.

Extracellular EGTA also affected FMLP-induced O2− production. As seen in Table 3, the initial rate of O2− production was not significantly diminished by the addition

![Graph](image)

**Fig 4.** Enhancement of FMLP-induced O2− production is a function of nucleotide/nucleoside identity and concentration. Human neutrophils were incubated with increasing concentrations of ATP (O--O), ATP-γ-S (X--X), GTP (△--△), ADP (□--□), and adenosine (□--□), and then stimulated with 0.1 μmol/L FMLP. The effect on the maximal O2− production rate was then compared for the different nucleotides and adenosine. The nucleotide or adenosine were added after 210 seconds of incubation and the FMLP at 300 seconds. Values are means ± SD for three experiments.

**Table 3. Effect of EGTA on FMLP-Induced O2− Production by Human Neutrophils in the Presence and Absence of ATP**

<table>
<thead>
<tr>
<th>Parameter Analyzed</th>
<th>FMLP Alone</th>
<th>ATP + FMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial O2− Rate</td>
<td>95 ± 5.2</td>
<td>79.3 ± 5.5*</td>
</tr>
<tr>
<td>Total O2− Produced</td>
<td>71.6 ± 11*</td>
<td>45.7 ± 1.5*</td>
</tr>
<tr>
<td>Burst Duration</td>
<td>70.3 ± 7.2*</td>
<td>60 ± 5.0*</td>
</tr>
</tbody>
</table>

Results are expressed as the percent of values obtained in the absence of EGTA ± SD. Baseline O2− rates were 51 nmol O2−/min/10⁶ cells for 0.1 μmol/L FMLP alone and 92 nmol O2−/min/10⁶ for FMLP + 10 μmol/L ATP. Baseline total O2− production was 122 nmol/10⁶ cells for 0.1 μmol/L FMLP alone and 242 nmol/10⁶ cells for FMLP + 10 μmol/L ATP. Baseline burst duration was 4.85 minutes for FMLP alone and 5.0 minutes for ATP plus FMLP.

*Differs significantly from non-chelated results (P < .05).
of 3 mmol/L EGTA (adequate to reduce extracellular Ca\(^{2+}\) to less than 0.1 \(\mu\)mol/L). However, the burst duration and, hence, the total \(O_2^-\) produced was significantly decreased, consistent with the hypothesis that a \(Ca^{2+}\) influx was necessary for the maintenance but not the initiation of the respiratory burst.

The chelation of extracellular \(Ca^{2+}\) had a more pronounced effect on the ability of ATP to augment FMLP-induced superoxide production. There was a greater than 20% reduction in the nucleotide's enhancement of the initial rate of \(O_2^-\) production, although the rate was still higher than with FMLP alone. The burst duration was also shortened with the result that the total \(O_2^-\) generated was decreased by more than 50%. It appears that the mechanism by which ATP enhances \(O_2^-\) production is substantially dependent on the presence of extracellular \(Ca^{2+}\).

**Effect of exogenous nucleotides on \(O_2^-\) production in a cell-free assay.** To attempt to elucidate the mechanism by which nucleotides are able to enhance \(O_2^-\) production, we examined the effect of a brief exposure to exogenous nucleotides on NADPH oxidase activity when the cells were subsequently fractionated and then reconstituted in a cell-free assay. This approach allows one to assess the stability of any modification and to identify the site of that modification (ie, cytosol, membrane, or both). As elaborated in "Materials and Methods," purified neutrophils were exposed to various concentrations (0, 1, 5, or 50 \(\mu\)mol/L) of ATP, ATP-\(\gamma\)-S, or AMP-PNP for 5 minutes at 4°C, washed, cavitaded, and fractionated. The cytosolic and membrane fractions from the variously exposed cells were then combined in the indicated combinations, and \(O_2^-\) production was assayed. Both SDS and arachidonic acid produced qualitatively identical responses, but SDS was used in the majority of the assays because it was more stable than arachidonic acid and gave more consistent results.

In the experiments shown in Fig 6, the cytosol and membranes from cells exposed to 0, 1, 5, or 50 \(\mu\)mol/L concentrations of either ATP, ATP-\(\gamma\)-S, or AMP-PNP were recombined, and \(O_2^-\) generation in response to 108 \(\mu\)mol/L SDS was measured. As can be seen, all three nucleotides caused significant enhancement of \(O_2^-\) generation when compared with cells not exposed to nucleotide. The effect was evident at 1 \(\mu\)mol/L concentrations of the nucleotides and increased with increasing nucleotide concentration up to 50 \(\mu\)mol/L. Nucleotide concentrations greater than 50 \(\mu\)mol/L did not consistently produce further enhancement of cell-free \(O_2^-\) generation (data not shown). Brief exposure to 5 to 50 \(\mu\)mol/L concentration of ATP or AMP-PNP resulted in more than a doubling of the rate of \(O_2^-\) production, while 50 \(\mu\)mol/L ATP-\(\gamma\)-S was somewhat less effective at enhancing \(O_2^-\) generation. Comparison of Figs 4 and 6 reveals that the degree of \(O_2^-\) enhancement, the range of effective concentrations, and the efficacy of both native and non-hydrolyzable nucleotides are quite similar for the whole cell and the cell-free systems.

Direct comparison of the nucleotide effects on \(O_2^-\) production are possible only for amphiphiles such as arachidonic acid, which are capable of eliciting \(O_2^-\) generation in both systems. Figure 7 shows the effect of 25 \(\mu\)mol/L concentrations of three nucleotides (ATP, ATP-\(\gamma\)-S, and AMP-PNP) on arachidonic acid-induced \(O_2^-\) production in intact neutrophils. As with FMLP, the nucleotide was added 90 seconds before the agonist, and as with FMLP, micromolar concentrations of all three nucleotides enhanced \(O_2^-\) production. At intermediate concentrations of the fatty acid (30 to 60 \(\mu\)mol/L), these nucleotides produced a 60% to 160% increase in \(O_2^-\) production, although at maximally stimulatory concentrations (80 \(\mu\)mol/L) there was little or no enhancement. Once again, the effects of the nucleotides were first seen at submicromolar (0.5 \(\mu\)mol/L) concentrations and reached a maxima at approximately 100 \(\mu\)mol/L (data not shown).

There was a significant consistency in the effects of these nucleotides on \(O_2^-\) production, whether the cells are stimulated with amphiphile immediately as intact cells (Fig 7) or if they are disrupted, reassembled, and then stimulated with arachidonic acid or SDS (Fig 6). Although the mechanisms
by which amphiphiles elicit O$_2^-$ generation in the whole cell and cell-free systems may be different, the similarities with respect to the range of effective nucleotide concentrations (1 to 50 μmol/L), the degree of enhancement (50% to 200%) and the efficacy of both hydrolyzable and non-hydrolyzable analogs suggest that the mechanisms by which the resultant O$_2^-$ production is enhanced may be the same in both systems.

In the preceding cell-free experiments, isolated membranes were recombined with their corresponding cytosols, and the O$_2^-$ generated was quantitated. With the cell-free system, one can independently assay the membranes and cytosol from neutrophils treated with varying concentrations of various nucleotides. In the experiments shown in Table 4, neutrophils were exposed to 50 μmol/L ATP-γ-S, 50 μmol/L AMP-PNP, or no nucleotide, washed, fractionated and then assayed for superoxide production in response to SDS. As expected, reconstituting the cytosol and membranes from neutrophils exposed to ATP-γ-S resulted in O$_2^-$ production at nearly twice the rate of components from nonexposed neutrophils, and recombination of membrane and cytosol from neutrophils treated with AMP-PNP resulted in O$_2^-$ production that was almost three times the basal rate. By combining the three different membrane preparations with standard cytosol (not exposed to any nucleotide) it could be seen that the different membrane preparations were nearly identical with respect to their ability to support O$_2^-$ production. The converse combination of standard (non-nucleotide exposed) membrane with the three cytosols, revealed that the cytosol accounted for virtually all the observed enhancement of O$_2^-$ production. ATP-γ-S cytosol doubled the rate and AMP-PNP cytosol tripled the rate of O$_2^-$ production, as compared with cytosol from cells not exposed to exogenous nucleotide.

The cell-free system can also be used to further define the role of Ca$^{2+}$ in nucleotide-induced priming. Extracellular Ca$^{2+}$ is required by intact cells for maximal O$_2^-$ production in response to agonists such as FMLP, arachidonic acid, immune complexes, and Cs, which complicates the assessment of the requirement for Ca$^{2+}$ as an intermediary in nucleotide-induced priming. The cell-free O$_2^-$ generating system does not require Ca$^{2+}$ for optimal O$_2^-$ production, and excess Ca$^{2+}$ is actually inhibitory. Thus, this is an ideal system for evaluating the role of Ca$^{2+}$. Intact neutrophils at 4°C in Ca$^{2+}$-free relaxation buffer are capable of generating calcium transients in response to FMLP and micromolar concentrations of ATP, ATP-γ-S, and AMP-PNP, although the amplitude and rate of rise are less than those generated at 37°C in the presence of extracellular Ca$^{2+}$ (Fig 8). When the cells are loaded with 100 μmol/L MAPTAM (an intracellular Ca$^{2+}$ chelator) this transient is no longer detectable with fura-2/AM (data not shown), consistent with results reported by Korchak et al. We therefore evaluated the effect that MAPTAM loading of neutrophils had upon the ability of exogenous nucleotides to enhance O$_2^-$ production when the cells were subsequently fractionated and assayed in a cell-free system. As can be seen in Fig 9, MAPTAM alone had no effect on O$_2^-$ generation in the cell-free system, but its presence completely abrogated the usual twofold enhance-

**Table 4. Cell-Free O$_2^-$ Production by Subcellular Fractions of Neutrophils Exposed to ATP-γ-S, AMP-PNP, or No Nucleotide**

<table>
<thead>
<tr>
<th>Membranes</th>
<th>No Nucleotide</th>
<th>ATP-γ-S</th>
<th>AMP-PNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No nucleotide</td>
<td>100 ± 9.5</td>
<td>209 ± 9.5</td>
<td>306 ± 9.2</td>
</tr>
<tr>
<td>ATP-γ-S</td>
<td>98 ± 6</td>
<td>190 ± 4.6</td>
<td>—</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>99 ± 6</td>
<td>—</td>
<td>298 ± 20</td>
</tr>
</tbody>
</table>

The rate of O$_2^-$ production by cytosol and membrane from neutrophils not exposed to any exogenous nucleotide was arbitrarily assigned a value of 100%, and the other combinations' O$_2^-$ production are expressed as a percentage of this standard mixture. Actual rate of O$_2^-$ production by non-nucleotide cytosol and membrane was 15.6 nmol/min/10$^7$ cell equivalents of membrane. Stimulus was 108 μmol/L SDS. Values are means ± SD for at least three experiments (cavitations). ATP-γ-S: 50 μmol/L; AMP-PNP, 50 μmol/L.
ment that is normally seen after exposure to 50 \(\mu\text{mol/L}\) ATP-\(\gamma\)-S before cavitation (Fig 6). This confirms the requirement for a transient elevation of intracellular Ca\(^{2+}\) for nucleotide-mediated priming of neutrophils to occur.

**DISCUSSION**

Exogenous nucleotides are capable of exerting numerous effects on human neutrophils. In contrast to many other cell types, such as J774 murine macrophages, thymocytes, and Friend erythroleukemia cells that require high (0.3 to 5 \(\mu\text{mol/L}\)) concentrations of the nucleotides for an effect to be seen, neutrophils respond to more physiologic (1 to 50 \(\mu\text{mol/L}\)) concentrations. Meyer et al\(^2\) calculated that platelets contain 1.9 \(\mu\text{mol}\) of secretable ATP and 2.6 \(\mu\text{mol}\) of secretable ADP per 10\(^{11}\) platelets. Thus, assuming a normal platelet count of 300,000/\(\mu\text{L}\), a serum concentration of 22 \(\mu\text{mol/L}\) ATP and 32 \(\mu\text{mol/L}\) ADP could be achieved. Although such a massive systemic release of platelet granules would never occur, it is reasonable to assume that nucleotide concentrations in the 5 to 30 \(\mu\text{mol/L}\) range are achieved in the vicinity of a fibrin clot or wherever platelet aggregation and degranulation have occurred. This makes it highly likely that the effects discussed in this report are physiologically relevant.

These nucleotides appear to be exerting their effects through a surface receptor, a conclusion based on the micromolar sensitivity and the near-equivalent efficacy of the non-hydrolyzable analogs (indicating that the nucleotides are not serving as a source of high energy phosphate). In addition, our cell-free experiments demonstrated that the priming effects of these nucleotides persisted after brief exposure and subsequent washing (less than 0.05% remaining after two washes) (data not shown), indicating that their continued presence is not required for the persistence of the priming phenomenon. All of these observations support the hypothesis that these purine nucleotides are modifying neutrophil function by acting upon a “purinergic” receptor mechanism such as that described by Burnstock\(^3\) and Su\(^11\) in neural tissue. Agonists such as ATP, ADP, ATP-\(\gamma\)-S, and AMP-PNP could be binding to the “P2” receptor, while the inhibitory adenine compounds AMP and adenosine act by binding to the “P1” receptor. The greater efficacy of the nucleotide triphosphates ATP and ATP-\(\gamma\)-S as compared with ADP is also consistent with such a hypothesis.

One of the first events that occur after ligand binding in several cell types is a rise in intracellular Ca\(^{2+}\). This cation then acts as a second messenger producing alterations in cell function that are distinct for each cell type. This signal transduction mechanism has been well described in neutrophils for ligands such as FMLP. Based on the present data, it would appear that Ca\(^{2+}\) performs a similar role for neutrophil responses to the nucleotides. In intact cells, the observation that exogenous EGTA blunts both the Ca\(^{2+}\) response and the ability of the nucleotides to enhance \(O_2^-\) production, as well as the tight correlation between a nucleotide’s ability to generate a Ca\(^{2+}\) transient and its ability to enhance \(O_2^-\) production, strongly support such a hypothesis. In addition, the observations in the cell-free system that the abolition of the nucleotide-induced Ca\(^{2+}\) transient with MAPTAM resulted in the absence of any enhancement of \(O_2^-\) production further strengthens this conclusion. However necessary an elevation in intracellular Ca\(^{2+}\) may be for the manifestation of the nucleotide response, it is only an intermediary since the nucleotide effect on \(O_2^-\) production persists long after intracellular Ca\(^{2+}\) levels have returned to baseline. In addition, in the cell-free \(O_2^-\) assay system the enhanced activity of the cytosol persists, despite the presence of EGTA.

Recently published work with transformed myeloid and monocytic cell lines has provided further insight into the effects of purine nucleotides on hematopoietic cells. Dubyak et al\(^9\) and Cowen et al, working with “granulocytes” differentiated from HL-60 cells, have shown that exposure to micromolar concentrations of ATP, ATP-\(\gamma\)-S, and ADP resulted in the generation of Ca\(^{2+}\) transients and phospholipid remodeling as evidenced by inositol triphosphate accumulation. These investigators found the same hierarchy of nucleotide sensitivity—ATP > ATP-\(\gamma\)-S > ADP > AMP—adenosine—as has been reported for P2 purinergic receptors in other systems. Greenberg et al, working with J774 macrophages, found a similar hierarchy of response to the various nucleotides and determined that the rises in intracellular Ca\(^{2+}\) occurring in response to micromolar concentrations (less than 50 \(\mu\text{mol/L}\)) of purine nucleotides represented both a release from internal stores and a influx across the cell membrane. Finally, Seifert et al\(^8\) have reported that purine nucleotides are capable of enhancing the \(O_2^-\) production by DMSO and dibutyryl cyclic AMP (cAMP) differentiated HL-60 cells occurring in response to FMLP, as is seen in normal mature neutrophils. The dibutyryl-cAMP differentiated cells, however, were capable of generating \(O_2^-\) in response to treatment with cytochalasin B (1 \(\mu\text{g/mL}\)) and ATP (1 to 100 \(\mu\text{mol/L}\)) alone (without FMLP), which is at variance with what is seen in normal mature neutrophils.

The actual mechanism by which the neutrophil is modified to respond more vigorously to stimuli such as FMLP, arachidonic acid, and immune complexes is yet to be determined. From the information obtained from our cell-free studies, one can exclude membrane changes such as increased receptor number, altered receptor affinity, or a modification of the membrane bound portion of the oxidase from consideration. Likewise, although granule fusion occurs in response to these nucleotides, it does not appear to have a role in this priming process. It is clear that the nucleotides are able to effect a stable (for several hours at 4\(^\circ\)C) modification of a cytosolic constituent(s), but how this interacts with the components of the NADPH oxidase remains to be discovered. Potential mechanisms include alteration of cytosolic components by the action of proteases, phosphatases, or kinases.

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Exposure of human neutrophils to exogenous nucleotides causes elevation in intracellular calcium, transmembrane calcium fluxes, and an alteration of a cytosolic factor resulting in enhanced superoxide production in response to FMLP and arachidonic acid

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