Functional Consequences of Interactions between Human Neutrophils and ATP, ATPγS, and Adenosine[a]

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INTRODUCTION

There is an increasing body of evidence suggesting that nucleotides such as ATP can serve as important extracellular factors to bring about significant functional changes in phagocytic cells. For instance, ATP and its analogues (for example, adenosine-5′-*O*-(3-thiotriphosphate), or ATPγS) can interact with neutrophils to cause increases in intracellular calcium.[1-4] This appears to be associated with "priming" of these cells for enhanced superoxide (O_2^-) responses to agonists such as chemotactic peptides.[1,2,5,6] Because platelets contain and, when activated, release substantial amounts of ATP and ADP, mammalian systems have the potential for delivering significant amounts of adenine nucleotides to sites of inflammation. It may well be that *in vivo* secretion products of platelets will bring about an amplification of oxygen radical formation by phagocytic cells, and so intensify tissue injury at the inflammatory site. This concept is supported both by *in vitro* and *in vivo* observations.[6-8] In contrast to ATP with respect to phagocytic cells, adenosine has an opposing effect: suppression of O_2^- responses to chemotactic peptides.[5,9] In this study, we have assessed the effects of ATP (and/or ATPγS) and adenosine on chemotactic peptide receptor numbers and affinities, the cell membrane content of CR3 (complement receptor III, or, as defined by monoclonal antibodies, Mol antigen (CD11b)), and the intracellular changes in calcium induced by exposure of neutrophils to chemotactic peptide.

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MATERIALS AND METHODS

Reagents

Except as noted, reagents were purchased from Sigma Chemical Company (St. Louis, MO). ATPγS was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). *N'*-formyl-Met-Leu-[^{3}H]Phe ([^{3}H]fMLP) (56.9 Ci/mmol) was purchased from DuPont-New England Nuclear Research Products (Boston, MA).

Preparation of Neutrophils

For most studies, neutrophils were prepared as previously described.[10] Briefly, blood was anticoagulated with $\frac{1}{10}$ volume of acid citrate dextrose and subjected to centrifugation (400 × g, 15 min, room temperature). The upper layer of platelet-rich plasma was then removed. The remaining pellet containing red blood cells and leukocytes was diluted 1:1 with phosphate-buffered saline (140 mM NaCl, 1 mM KH_2PO_4, 5 mM Na_2HPO_4, pH 7.3), and 25-ml aliquots were layered over 15 ml of Ficoll-Hypaque (specific gravity of 1.077). Centrifugation was carried out (400 × g, 30 min), and the upper layers were aspirated, leaving the red cell pellet containing neutrophils. Red cells in the cell pellets were then lysed with equal volumes of 150 mM NH_4Cl containing 1 mM EDTA. The remaining neutrophils were washed with 50 ml of phosphate-buffered saline and resuspended in Tris-buffered Hanks' Balanced Saline Solution (HBSS) and used immediately.

The second method of neutrophil preparation was similar to that described previously,[1] with the exception that lysis of the red cell pellet was omitted. Briefly, heparinized whole blood was diluted 1:1 with 6% (w/v) T-500 dextran (Pharmacia, Piscataway, NJ) and sedimented a 1 × g for 40 min. Twenty-five aliquots of the upper leukocyte-rich layer were layered over 15 ml of Ficoll-Hypaque (specific gravity of 1.077) and centrifuged (400 × g, 30 min). The upper layers were removed by aspiration and the neutrophil pellet was washed once with phosphate-buffered saline and used immediately.

N'-*Formyl-Met-Leu-[^{3}H]-Phe Binding*

Neutrophils suspended in binding buffer (140 mM NaCl, 1 mM KH_2PO_4, 5mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.3) were prewarmed for 3 min and then exposed to either 10 μM ATPγS, 10 μM adenosine, or buffer for 5 min. Incubations were terminated by placing the cells on ice followed by addition of phenylmethylsulfonyl fluoride (1 mM). [^{3}H]fMLP binding was carried out at 4 °C by incubating 100 μl of cells (8×10^5 cells) with 25 μl of [^{3}H]fMLP (3-300 nM) and 25 μl of either buffer or cold fMLP (360 μM). After 1-1.5 hr, cells were harvested by vacuum filtration over glass fiber filters (Whatman GF/C, Whatman International, Maidstone, United Kingdom) and washed with binding buffer (4 × 4 ml). Filters were dried overnight, scintillation fluid (Safety Count, Research Products Interna-

tional, Mount Pleasant, IL) was added, and radioactivity was assessed in a Beckman LS 5801 scintillation counter (Fullerton, CA). Nonspecific binding (uptake of [^{3}H]fMLP in the presence of unlabeled 60 μM fMLP) was usually less than 13% of the total binding. Scatchard plots were obtained with the aid of the program EBDA/ligand (Elsevier-Biosoft, Cambridge, United Kingdom). Analysis of [^{3}H]fMLP binding using concentrations of 2.5-200 nM showed no significant improvement of fit for the Scatchard plot using a two receptor site model over a single site (F test, $p > .05$).

O_2^- Response of Neutrophils

Generation of O_2^- was determined by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* as described previously.[2] Enhancement studies were performed by the addition of prewarmed cells to reaction mixtures containing either ATP, ATPγS, or adenosine. After 2 min, fMLP was added. For experiments involving cytoplasts, a preliminary assay of O_2^- production was performed to determine the number of cells necessary to generate O_2^- in amounts that would be the same for cytoplasts and intact neutrophils. This adjustment of cell number allowed for comparison of the relative enhancement of these two groups. The O_2^- responses were comparable between intact neutrophils kept on ice and intact neutrophils exposed to Ficoll and cytochalasin B under conditions similar to those involved in cytoplast preparation (data not shown).

Neutroplast Preparation

Neutrophils (10^8) were suspended in 10 ml of 12.5% (w/v) Ficoll solution containing 17.4 μM cytochalasin B at 37 °C. This was layered onto a discontinuous gradient (10 ml of 17% Ficoll and 10 ml of 25% Ficoll, each containing 17.4 μM cytochalasin B), and was subjected to ultracentrifugation at 34 °C at 79,500 $\times$ g for 30 min. The band of neutroplasts at the interface of the 12.5% and 17% Ficoll layers was aspirated, diluted to 50 ml with phosphate-buffered saline, and centrifuged at 725 $\times$ g for 6 min. The pellet was resuspended in HBSS and used. Enzyme content of neutroplasts produced in this manner showed less than 3.5% of β-glucuronidase or vitamin B_{12} binding protein by standard assay in conformity with other reports.[11] Transmission electron microscopy also confirmed granule depletion (data not shown).

Statistical Analysis

The data was expressed as the mean ($\bar{x}$) and standard error of the mean (SEM). A paired t test was used to compare the response between two treatments. Because of the variability of specific fluorescence for control samples in different experiments, CR3 (Mo1) fluorescence data was compared using a t test of the mean log values of specific fluorescence. Statistical significance was defined at $p < .05$.

Immunofluorescence Analysis

The generation and characterization of murine monoclonal antibodies anti-Mo1 (anti-CD11b, IgG2a; clone 44) and anti-I3 (IgG2a; clone 9-4) have been described.[10] Neutrophils were subjected to indirect immunofluorescence staining for the expression of the Mo1 determinant relative to background staining by an isotype-identical negative control antibody (anti-I3) and subjected to flow cytometric analysis. For each experiment, specific fluorescence intensity represents the computed mean channel number (0-230 channels, linear scale) of cells stained with the anti-Mo1 antibody minus the mean channel number of cells stained with the anti-I3 reagent. The photomultiplier tube setting for all experiments was 1000.

Changes in the Concentration of Intracellular Calcium

Measurements of $[Ca^{2+}]_i$ using the fluorescent probe fura-2 were described previously.[2] Briefly, neutrophils were suspended in Hepes buffered saline (2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose), loaded for 15 min at 37 °C with 20 μM fura-2-AM (the acetoxymethyl ester of fura-2, Calbiochem, La Jolla, CA), and then loaded for an additional 15 min after a 10-fold dilution. The cells were centrifuged and resuspended in buffer and stored on ice until use. Changes in $[Ca^{2+}]_i$-dependent fluorescence were measured in a Perkin-Elmer LS-5B luminescence spectrometer using a thermally equilibrated cuvette holder at 37 °C. Changes in $[Ca^{2+}]_i$ were determined as previously described.[2]

RESULTS

Effects of ATP and ATPγS on Superoxide Response of Neutrophils Prepared by Two Different Methods

Because the usual procedure of isolating neutrophils from human blood includes the lysis of red cells by addition of NH_4Cl, the ability of ATPγS to enhance O_2^- responses in fMLP-stimulated cells was evaluated in neutrophils prepared by addition or omission of the fluid used to lyse red cells (150 mM NH_4Cl). Neutrophils (1×10^6/ml) were then stimulated with 100 nM fMLP in the presence of increasing concentrations of ATPγS, and the O_2^- responses were measured (FIG. 1). As is evident from the data, the addition of ATPγS caused the production of O_2^- to be enhanced in a dose-dependent manner, but there was a remarkably amplified effect on O_2^- production in cells that had not been exposed to the lysing solution. In this case, there was a > 100% increase in the production of O_2^- as compared to a 27% increase in cells that had been in contact with the lysing solution. Reasons for the differences are not understood. In all subsequent experiments to be reported, values were obtained using neutrophils that had been isolated in the conventional manner

(using the lysing procedure), because contamination of neutrophils with red cells was incompatible with some of the analytical procedures.

As shown in FIGURE 2, the ability of ATP and ATPγS to prime neutrophils for subsequently enhanced O_2^- responses was proportional to the concentration of nucleotide employed. Enhancement in the O_2^- responses of neutrophils subsequently stimulated with 100 nM fMLP was found in the low μM dose range of nucleotide. Although the ATP effect tended to reach a plateau concentration of 10 μM, the effect of ATPγS was maintained and was proportional to the concentration of nucleotide, even at 100 μM (FIG. 1).

The well-known ability of adenosine to inhibit O_2^- responses of human neutrophils is shown in FIGURE 3 as a dose-response relationship. Neutrophils were exposed to varying concentrations of adenosine at 37 °C for 2 min, and these exposures were followed by the addition of 100 nM fMLP. Production of O_2^- over a 30-min period was then determined. As is apparent from the data, the inhibitory effects of adenosine were dose related, with maximal inhibition found at a concentration of 1 μM.

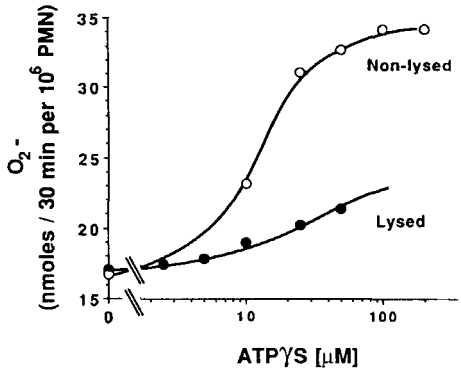

FIGURE 1. Enhancement by ATPγS of O_2^- responses in fMLP-stimulated neutrophils as a function of whether cells had been previously exposed to 0.15 M NH_4Cl, the lysing solution for RBCs.

Effects of ATPγS on Calcium Changes Induced by fMLP

In order to determine if the priming effects of ATPγS might be related to a greater increase in intracellular calcium after cell stimulation with fMLP, human neutrophils (5×10^6) that had been preloaded with fura-2 were first exposed to 25 μM ATPγS for 5 min at 37 °C, and then 1 μM fMLP was added. The intracellular calcium levels were continuously monitored in a spectrofluorometer. For convenience, computed concentrations of intracellular calcium at selected time points were used for graphic expression. As is apparent from the data in FIGURE 4, the intracellular changes in calcium in response to cell contact with fMLP were indistinguishable from the calcium changes induced in fMLP-stimulated cells that had been previously exposed to ATPγS. When the two sets of data (from ATPγS- or buffer-pretreated cells) were compared, there was no statistically significant differences at any time point. Thus, cells that are primed with ATPγS do not demonstrate a different pattern of increase in intracellular calcium when the cells are subsequently stimulated with fMLP.

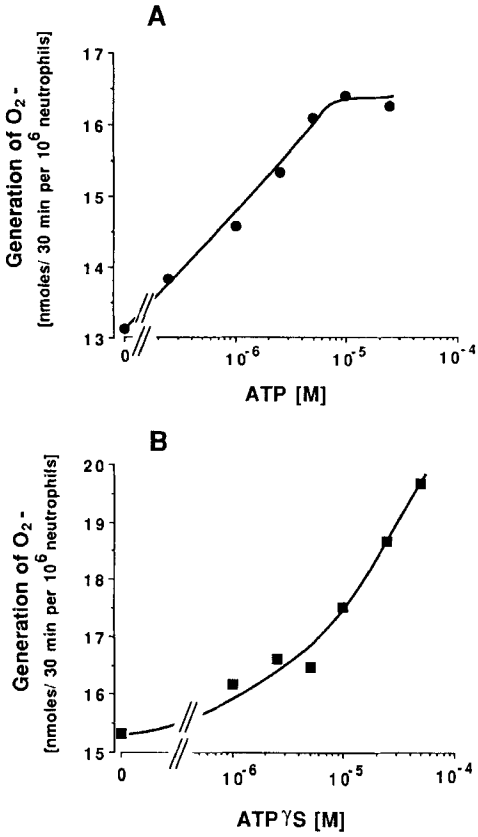

FIGURE 2. Dose-response relationships between O_2^- production in fMLP-stimulated neutrophils and the concentration of (**A**) ATP or (**B**) ATPγS.

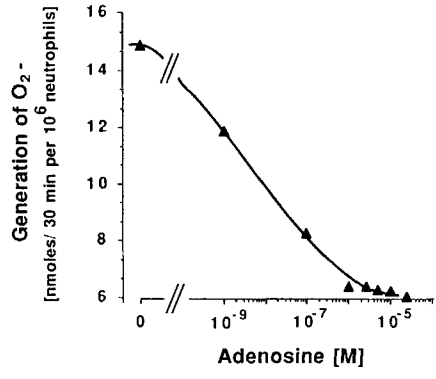

FIGURE 3. Dose-response relationship of inhibition by adenosine of O_2^- in fMLP-stimulated neutrophils.

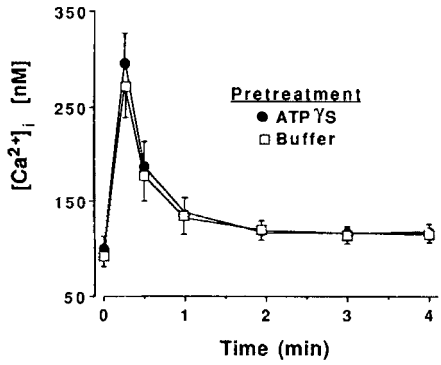

FIGURE 4. Increases in intracellular calcium in fMLP-stimulated neutrophils that had been pretreated with ATPγS (25 μM) or buffered salt solution.

In order to determine if, under the experimental conditions employed, calcium changes might be at a plateau and, therefore, any ATPγS effects on calcium levels might be masked, the experiment described by the data in FIGURE 5 was carried out. Cells were exposed to either 25 μM ATPγS or buffered salt solution for 5 min at 37 °C, and then fMLP over a wide range of concentrations was added. Changes in intracellular calcium were measured. In cells incubated 5 min at 37 °C, the "basal" concentrations of intracellular calcium were 61.5 ± 3.3 and 62.3 ± 2.6 nM in primed (ATPγS-treated) and unprimed cells, respectively. Thus, the levels of intracellular calcium in these two preparations of cells prior to stimulation with fMLP were indistinguishable. When cells were exposed to a range of fMLP concentrations (10^{-11}-10^{-6} M), the increases in intracellular calcium were the same in the two sets of cells whether they had been previously (5 min earlier) exposed to 25 μM ATPγS or to buffered salt solution. Thus, at no concentration of fMLP does prior cell contact with ATPγS alter the maximal increase in intracellular calcium induced by fMLP. In whatever manner ATPγS brings about enhancement of O_2^- in fMLP-stimulated neutrophils, this does not appear to be related to exaggerated increases in intracellular levels of calcium induced by cell contact with fMLP.

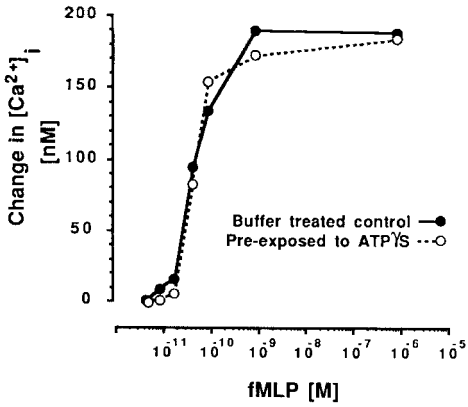

FIGURE 5. Peak changes in intracellular calcium in neutrophils exposed to a range of concentrations of fMLP, as a function of prior exposure of cells (5 min earlier) to buffered salt solution or 25 μM ATPγS.

Effects of ATPγS and Adenosine on fMLP Receptors and CR3 Content

Consideration was given to the possibility that ATPγS and adenosine may alter neutrophil responses to fMLP by causing changes in fMLP receptor content on the surfaces of intact neutrophils. Accordingly, after the neutrophils were pretreated with 10 μM adenosine or ATPγS, the cells were allowed to equilibrate with [^{3}H]fMLP, and the specific binding of the radioactive ligand was determined. From these measurements, the number of receptors/cell and the binding affinities (K_d) for [^{3}H]fMLP were calculated. The results of these studies are described in TABLE 1, where it is evident that cell contact with either adenosine or ATPγS resulted in a small (10-17%) drop in the mean number of receptors/cell without a change in the affinity of the binding of [^{3}H]fMLP to the neutrophils. Because it is known that agonists (including fMLP) for neutrophils will cause changes in the content of the cell membrane resulting

TABLE 1. Effects of ATPγS and Adenosine on [^{3}H]fMLP Binding to Neutrophils[a]

Experiment	Material Added to Neutrophils	[^{3}H]fMLP Receptor Number/Cell (mean ± SEM)	K_d (nM, mean ± SEM)
1	Buffer	13,183 ± 1790	4.64 ± 0.65
	Adenosine (10 μM)	11,974 ± 1559[b]	4.14 ± 0.05[c]
2	Buffer	16,530 ± 1438	4.46 ± 0.49
	ATPγS (10 μM)	13,717 ± 1193[d]	4.13 ± 0.49[c]

[a] Derived from reference 10.
[b] As compared to the data line above, $p < .007$, $N = 7$.
[c] As compared to the data line above, p is not significant.
[d] As compared to the data line above, $p < .02$, $N = 9$.

from fusion of secretory granules, cells were exposed to ATPγS and both the content of CR3 and the fMLP receptor number were measured in cells from the same donor pool. The results are described in TABLE 2. CR3 cell content (Mol antigen, CD11b), as assessed by the use of fluorescent antibody and flow cytometry, was greatly increased. Consistent with the data in TABLE 1, the number of fMLP receptors fell by 27% when cells were exposed to ATPγS (TABLE 2). These data indicate that ATPγS has paradoxical effects on CR3 and fMLP receptors of human neutrophils. Because it is known that secretory granules contain both fMLP and CR3 receptors and that granule fusion to the cell membrane can result in adduction of these receptors to the surface of the neutrophil,[12,13] the data in TABLE 2 suggest that adduction of these two different receptors to the cell membrane may be separately regulated. The findings described in TABLES 1 and 2 do not point to a consistent pattern of change in fMLP receptor content as an explanation for why ATPγS and adenosine alter O_2^- responses of neutrophils stimulated with fMLP.

Effects of ATPγS and Adenosine on O_2^- Production: Independence of a Requirement for Cytoplasmic Granules

Because, as discussed above, fusion of cytoplasmic granules to the cell membrane of the neutrophil can change the composition of that membrane, the question was explored as to whether the availability of cytoplasmic granules was required for the enhancing effects of ATPγS and the inhibitory effects of adenosine on O_2^- responses of fMLP-stimulated cells. Accordingly, neutroplasts, which lack cytoplasmic granules but still respond to fMLP or to phorbol ester with O_2^- responses, were prepared and used in amounts that produced O_2^- responses similar to those of 500,000/ml neutrophils stimulated with 100 nM fMLP. It should be emphasized that these studies employed neutrophils and neutroplasts that had been prepared from the same pool of cells, allowing simultaneous comparison of O_2^- responses of neutroplasts and neutrophils. As is evident from the data in TABLE 3, the O_2^- responses of fMLP-stimulated neutrophils and neutroplasts in the absence of ATPγS or adenosine were similar. The enhancing effects of 10 μM ATPγS and the inhibitory effects of 10 μM adenosine were quantitatively similar in the two cell preparations. These data prove beyond reasonable doubt that however ATPγS and adenosine alter O_2^- responses in fMLP-stimulated cells, the presence of cytoplasmic granules is not required.

DISCUSSION

There is an increasing body of evidence suggesting that adenine nucleotides and adenosine may be important modulators of cellular responses. ATP (and its analogues) and to a lesser extent ADP will cause transient increases in levels of intracellular calcium immediately after cell exposure. In some systems it is known that this phenomenon is related to synthesis of inositol phosphates. The functional significance of these changes has been defined in neutrophils where it has been demonstrated that for cells first treated with ATP, and then exposed to the chemotactic peptide fMLP, the production of O_2^- and the extracellular release of lysosomal enzymes are increased. The biological relevance of these observations may be linked to the finding that for human neutrophils stimulated with fMLP or with immune complexes, in the presence of intact platelets, platelet lysates, or platelet secretion products, the generation of

TABLE 2. Effects of ATPγS on CR3 Content and fMLP Receptors of Neutrophils[a]

Material Added	CR3 Content[b] (mean ± SEM)	[^{3}H]fMLP Receptor Number/Cell (mean ± SEM)
Buffer	3.20 ± 0.60	11,068 ± 2745
ATPγS (10 μM)	9.66 ± 3.54[c]	8,111 ± 1828[d]

[a] Summarized from reference 10 ($N = 8$).
[b] Specific fluorescence intensity measured by flow cytometry, expressed on linear scale.
[c] As compared to the data line above, $p < .03$.
[d] As compared to the data line above, $p < .05$.

TABLE 3. O_2^- Responses of Neutrophils and Neutroplasts Stimulated with fMLP[a]

Preparation Employed	Addition	O_2^- Response (nmol/30 min, mean ± SEM)	Significance[b]
Neutrophils	None	7.1 ± 1.7	
	ATPγS (10 μM)	9.1 ± 2.2	$p < .04$
	Adenosine (10 μM)	3.7 ± 1.1	$p < .03$
Neutroplasts	None	7.4 ± 1.0	
	ATPγS (10 μM)	10.1 ± 1.4	$p < .002$
	Adenosine (10 μM)	2.7 ± 0.6	$p < .001$

[a] Summarized from reference 10.

[b] All tests for significance were by comparison to the cell preparation to which there were no additions.

O_2^- is significantly amplified.[6] Also, when complement activation is brought about *in vivo* by infusion of cobra venom factor, the resulting injury of pulmonary microvascular endothelial cells is greatly attenuated by prior platelet depletion.[7] Because the vascular damage in this model is known to be directly attributable to toxic oxygen products of activated neutrophils, it may well be that the role of platelets in this inflammatory model is in part linked to the release of adenine nucleotides, which enhance oxygen radical production by neutrophils.

Although ATPγS causes increased CR3 expression on the plasma membrane of neutrophils, it has the opposite effect on fMLP receptor number (with no change in receptor affinity). It seems likely that the effects of ATP, ATPγS, and adenosine on fMLP-induced O_2^- responses in neutrophils cannot be attributed to changes in fMLP receptor content. This is further underscored by the fact that fMLP-induced O_2^- responses in granule-deficient neutroplasts are fully responsive to ATPγS and adenosine, even though granule fusion events are precluded.

It is possible that ATP, ATPγS, and adenosine may be altering a late step in the signal transduction pathway, such as the activity, translocation, or proteolysis of protein kinase C, or perhaps a direct effect on NADPH oxidase. Alternatively, one or more of these adenine compounds could be causing selective enhancement (by ATP or ATPγS) or inhibition (by adenosine) of diacylglycerol production from a phosphatidylglyceride other than phosphatidylinositol, such as phosphatidylcholine. In this way, there would be no change in the amount of inositol trisphosphate formed, but there could be an asymmetrical generation of diacylglycerol, higher in the case of ATP (or ATPγS) followed by fMLP, and lower in the case of adenosine followed by fMLP. The evidence assembled to date does not allow a distinction to be made between the effects of the adenine compounds on early or late steps in signal transduction.

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DISCUSSION OF THE PAPER

L. L. SLAKEY (*University of Massachusetts, Amherst, MA*): I am struck by the similarity between the relationship of nucleotides and adenosine to platelet responsiveness, and the results that you report. That is, in the platelet, ADP is proaggregatory, and adenosine is antiaggregatory, and you find that ATP and ADP enhance the neutrophil O_2^- response and that adenosine inhibits it. My comment springs from the hypothesis that the pathway of nucleotide hydrolysis at cell surfaces can serve as a timer. Early after ATP/ADP release, both platelet and neutrophil responses are enhanced. As adenosine is produced, the response is inhibited. Both the platelet and the neutrophil would be in the neighborhood of endothelial cells, and the endothelial ectonucleotidases, unlike those on some other cell types, do tend to create a distinct

time gap between the appearance of a bolus of ATP or ADP, and the generation of an adenosine-rich milieu.

WARD: The brief appearance and subsequent disappearance of intravascular aggregates of platelets may well be due to the release by the platelets of ATP and ADP. ATP and ADP are metabolized very rapidly to adenosine, which then results in the disaggregation and disappearance of the platelets.

Y. H. EHRLICH (*College of Staten Island, New York, NY*): My questions regard the mechanism of action. What is the effect of ATP analogues other than ATP[S]? Could the measured binding of ATP[^{35}S] be actually or partly this phosphorylation of proteins rather than binding?

WARD: Human neutrophils respond with 1) calcium transients and 2) enhanced O_2^- response to other nucleotide triphosphates with the following rank orders of potency: 1) ATP > > 2-Me-S-ATP > AMP-PCP>; 2) UTP $\geq$ ATP > ITP > GTP > CTP. Our studies with ATP[^{35}S] suggest that the binding is reversible (in an excess of cold ATP[S]) and shows no covalent thiophosphorylation (as determined by SDS-PAGE). Further, examination of label eluted from neutrophils shows that all of the label is in the form of ATP[^{35}S] without evidence of hydrolysis.

R. F. COLMAN (*University of Delaware, Newark, DE*): Is the protein kinase C system involved? Have you looked at specific inhibitors of the enzyme for effects on the potentiating effect of ATP[S]?

WARD: The endpoint is O_2^- production, which seemed to be blocked by inhibitors of protein kinase C. Thus, we have not used these inhibitors. There is still no evidence either for or against the ability of ATP[S] to modify protein kinase C.

J. S. WILEY (*Austin Hospital, Heidelberg, Australia*): The increase in CR3 expression on neutrophils due to exposure to ATP[S] is impressive. Do other agonists give increased CR3 expression of the same magnitude?

WARD: Like other neutrophil agonists (such as formyl chemostatic peptide, C5a, phorbol ester, and calcium ionophore A23187), ATP causes increased expression of CR3 by fusing secondary granules to the cell membrane. ATP carries out this action in a dose-dependent manner.