Activation of electroporabilized neutrophils by adenosine 5'-[γ -thio]triphosphate (ATP[S])

Role of phosphatases in stimulus–response coupling

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Electrically permeabilized human neutrophils were used to study the mechanism of activation of the NADPH oxidase by chemotactic factors. The respiratory burst elicited by formyl-methionyl-leucyl-phenylalanine (fMLP) was strictly dependent on the addition of ATP. The response was also supported by adenosine 5'-[γ -thio]triphosphate (ATP[S]), but not by the non-hydrolysable analogue (p[NH]ppA). In the presence of ATP, displacement of fMLP from its receptor by antagonist peptides resulted in the abrupt termination of the O₂-consumption burst. In contrast, the response persisted after displacement of fMLP when ATP[S] was present. This finding is consistent with the formation of biologically active thiophosphoproteins which are resistant to cleavage by cellular phosphatases. Accordingly, lower concentrations of ATP[S], as compared with ATP, were required to support the fMLP response. The data indicate that protein phosphatases control the extent and duration of the response in cells stimulated with chemoattractants. Unlike ATP, sub-millimolar concentrations of ATP[S] elicited a spontaneous respiratory burst in the absence of fMLP or other stimuli. This effect was inhibited by p[NH]ppA and was not observed in intact (non-permeabilized) cells, indicating interaction of ATP[S] with an intracellular adenine-nucleotide-binding site, possibly a protein kinase. These results suggest that protein kinases are active in neutrophils in the absence of exogenous stimuli, but that accumulation of the essential phosphoprotein(s) is normally prevented by the ongoing vigorous phosphatase activity. It is conceivable that control of the respiratory burst is exerted by inhibition of phosphatase activity, instead of or in addition to the more commonly postulated activation of protein kinases.

INTRODUCTION

Upon activation, phagocytic cells undergo an intense burst of O₂ consumption. This increased O₂ utilization is not associated with mitochondrial metabolism, but is instead required for the generation of bactericidal superoxide anions by the plasmalemmal NADPH oxidase [1]. The sequence of events coupling stimulation of the cells with the metabolic burst is not completely understood. In particular, the role of ATP remains ill-defined. It is well established that, in intact neutrophils, metabolic inhibition using agents such as 2-deoxy-D-glucose or iodoacetamide precludes the generation of superoxide after stimulation [2,3]. However, it is not clear from these experiments whether ATP is required for signal transduction or merely for the continued synthesis of NADPH, the substrate of the oxidase.

More recently, the requirement for ATP has been re-analysed in a cell-free preparation, under conditions where ATP and NADPH can be varied independently. Though some disagreement exists (cf. [4] with [5,6]), most laboratories have reported that addition of exogenous ATP is not necessary to elicit superoxide generation in cell-free systems stimulated by either arachidonate or SDS. This result is seemingly in disagreement with the

generally accepted notion that, in intact cells, stimulation is mediated by the activation of a protein kinase, possibly protein kinase C [7]. The apparent discrepancy could indicate that stimulation by arachidonate or detergent in the microsomal preparation occurs at a stage after the putative phosphorylation. It therefore remains to be determined whether ATP is required for phosphorylation reactions in cells stimulated by other physiological ligands, such as bacterial chemoattractants. Unfortunately, cell-free preparations do not retain responsiveness to chemotactic factors. In fact, most receptor-mediated responses are lost even in preparations of cells permeabilized by mild chemical procedures, such as addition of digitonin or saponin.

We have recently developed a method for the preparation of electrically permeabilized human neutrophils [8]. Exposure of the cells to intense electrical fields renders the plasma membrane permeable to molecules of *M_r* ≤ 750, without impairing their responsiveness to chemotactic factors. This preparation enabled us to analyse the ATP-dependence of the chemoattractant-induced response under conditions where NADPH was supplied exogenously and therefore maintained constant. The results reported here show that, in cells stimulated with fMLP, ATP is stringently required for stimulus–

Abbreviations used: ATP[S], GTP[S], adenosine (or guanosine) 5'-[γ -thio]triphosphate; fMLP, formyl-methionyl-leucyl-phenylalanine; Boc-PLPLP, *N*-t-butoxycarbonyl-L-phenylalanyl-D-leucyl-L-phenylalanyl-D-leucyl-L-phenylalanine; p[NH]ppA, adenosine 5'-

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response coupling. In addition, we found that the fMLP response can be potentiated by low concentrations of ATP[S], suggesting a role for phosphatases in controlling the activation process. Finally, we obtained evidence for the presence of active kinases in the absence of added stimuli, as revealed by the spontaneous O_2 -consumption burst recorded at higher concentrations of ATP[S].

EXPERIMENTAL

Materials

Ficoll 400 and Dextran T500 were purchased from Pharmacia LKB Biotechnology. EGTA, NADPH, fMLP, Boc-PLPLP, 2-deoxy-D-glucose, Hepes, GTP, p[NH]ppA and ATP (K^+ salt) were obtained from Sigma. The ATP[S] used for most of the experiments was from Boehringer Mannheim, but one batch was purchased from Fluka.

Solutions

Bicarbonate-free medium RPMI 1640 (Sigma) was buffered to pH 7.3 with 25 mM-Hepes/NaOH. Permeabilization medium contained 140 mM-KCl, 1 mM- $MgCl_2$, 1 mM-EGTA, 10 mM-Hepes (*N*-methyl-D-glucammonium or K salt, pH 7.0), and sufficient $CaCl_2$ to give a final free Ca^{2+} concentration of 100 nM, calculated as described in ref. [9]. Where indicated, ATP, ATP[S] or p[NH]ppA was added to this medium during the O_2 -consumption assay. All media were adjusted to 290 ± 5 mosM with the major salt.

Cell isolation and permeabilization

Neutrophils were isolated from fresh heparinized human blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation [10]. Contaminating red cells were removed by NH_4Cl lysis. Cells were washed, counted with a model ZM Coulter Counter and resuspended at 10^7 cells/ml in Hepes-buffered RPMI 1640.

Where indicated, the cells were depleted of ATP before permeabilization by incubation for 5 min at $37^\circ C$ in a medium containing 140 mM-NaCl, 5 mM-KCl, 10 mM-Hepes, 1 mM- $MgCl_2$, 1 mM- $CaCl_2$ and 5 mM-2-deoxy-D-glucose. The cells were then sedimented and resuspended in ice-cold permeabilization solution at 10^7 cells/ml. Portions (0.8 ml) of this suspension were then transferred to a Bio-Rad Pulser cuvette and permeabilized by using two discharges as described in ref. [8]. The cells were sedimented and resuspended in fresh ice-cold permeabilization solution between pulses. The electroporated cells were then suspended in permeabilization medium containing 2 mM-NADPH, 0.1 mM-GTP and the indicated concentration of adenine nucleotide, and used for O_2 -consumption measurements.

Other methods

O_2 consumption was measured polarographically with a model 53 or a model 5300 biological O_2 monitor (Yellow Springs Instrument Co.), as described in ref. [8]. O_2 consumption was calculated by using a solubility coefficient of 0.024 ml of O_2 /ml at $37^\circ C$, the temperature used for all experiments. All measurements were performed at least three times with blood from different donors, and are presented as representative traces or as means $\pm$ S.E.M. for the indicated numbers of

determinations (n). Straight lines were fitted by least squares.

RESULTS AND DISCUSSION

We studied the ATP-dependence of the respiratory burst induced by fMLP in electroporated cells. In preliminary experiments, otherwise untreated cells were permeabilized in the absence of ATP and tested for O_2 consumption in the absence of exogenous ATP. Under these conditions, the fMLP-induced O_2 consumption burst was greatly, though not completely, inhibited (results not shown). The residual response was probably due to the incomplete elution of intracellular ATP from the permeabilized cells at the time of the assay. This problem can be ameliorated by prolonging the interval between permeabilization and measurement of O_2 consumption. Alternatively, the residual response could be virtually eliminated by depletion of the endogenous ATP

Fig. 1. ATP-dependence of fMLP-induced O_2 consumption in permeabilized neutrophils

Cells were depleted of ATP with 2-deoxy-D-glucose, electroporated and used for O_2 -consumption measurements as described in the Experimental section. (a) Cells suspended in permeabilization medium containing NADPH and GTP in the absence (top trace) or the presence (lower trace) of 1 mM-ATP. Where indicated, 0.1 μM -fMLP was added. (b) Cells suspended in the presence of 200 μM -p[NH]ppA (top trace) or 20 μM -ATP[S] (lower trace). Other conditions were as in (a). The traces in (a) and (b) are representative of three or more experiments. (c) Dependence of the fMLP-induced response on the concentration of ATP (■) or ATP[S] (◆). Data are means $\pm$ S.E.M. of the maximal O_2 -consumption rate measured in at least three experiments. Where absent, the S.E.M. bars were smaller than the symbol. Temperature: $37^\circ C$.

pool before permeabilization (Fig. 1a). The latter protocol was found to be more reliable and was used hereafter. The effect of ATP depletion is specific and reversible, as demonstrated in the lower trace of Fig. 1(a). A large O_2 -consumption response was induced by fMLP when exogenous ATP (1 mM) was supplied to the depleted cells. In five experiments, the response in the absence of the nucleotide was less than 1% of that observed when 1 mM-ATP was re-introduced to the medium. The site of action of ATP is intracellular. This was demonstrated by measuring O_2 consumption in cells that were ATP-depleted but not permeabilized. Re-addition of exogenous ATP to these cells failed to restore the chemoattractant-induced respiratory burst (results not shown). Taken together, these results suggest that the presence of cytosolic ATP is required for the induction of an O_2 -consumption burst by fMLP.

As shown in Fig. 1(b), hydrolysis of the γ -phosphate group of ATP appears to be necessary to generate the response to chemotactic factor. Comparable or even higher concentrations of the non-hydrolysable ATP analogue p[NH]ppA failed to support the response. Similarly, the respiratory burst was absent in the presence of 1 mM-ADP (results not shown). In contrast, fMLP stimulated a marked respiratory burst in the presence of ATP[S] (Fig. 1b, lower trace), an ATP analogue whose γ -thiophosphate is known to be cleaved by a variety of enzymes, including several protein kinases [11,12]. Therefore, hydrolysis of ATP appears to be essential for the development of the response to the chemoattractant. It is noteworthy that the effect is specific for ATP; GTP (1 mM) did not support O_2 consumption.

When testing the effects of ATP[S], it became apparent that this nucleotide is, in fact, more potent than ATP itself in supporting the O_2 -consumption burst. The detailed nucleotide-concentration-dependence of the fMLP-induced burst is presented in Fig. 1(c). For reasons that will become apparent below, higher concentrations of ATP[S] were not tested. Clearly, considerably lower concentrations of ATP[S] are needed to elicit half-maximal fMLP-induced responses ($\sim 20 \mu\text{M}$), compared with ATP ($\sim 60 \mu\text{M}$). If it is assumed that phosphorylation is required for the respiratory burst, this effect can conceivably be attributed to the accumulation of biologically active thiophosphorylated proteins. It is well established that, whereas several kinases utilize ATP[S] as a substrate, the thiophosphate group inserted into proteins is highly resistant to cleavage by phosphatases [11,12]. Thus, if the formation of phosphoproteins is normally limited by the presence of active phosphatases, higher phosphoprotein levels will be attained when ATP[S] is used instead of ATP.

A corollary of this hypothesis predicts that, upon removal of the stimulatory input, the response is expected to persist considerably longer when the poorly hydrolysable thiophosphoproteins are formed, compared with the more readily cleaved phosphoproteins. This prediction was confirmed in the experiments shown in Fig. 2. As described for intact cells (see [13] for review), the respiratory burst induced by fMLP in permeabilized cells in the presence of ATP could be rapidly terminated by displacing the chemotactic peptide with the competitive inhibitor Boc-PLPLP, confirming that continued receptor occupancy is required for a sustained response (Fig. 2a). In five experiments, the rate of O_2 consumption averaged $2.8 \pm 0.27 \text{ nmol/min per } 10^6 \text{ cells}$ before and

Fig. 2. Kinetics of inactivation of the respiratory burst by the antagonist peptide Boc-PLPLP

Cells were depleted of ATP and suspended in permeabilization solution containing NADPH, GTP and either 100 μM -ATP (a) or 25 μM -ATP[S] (b). Where indicated, the respiratory burst was initiated by addition of 10 nM-fMLP. No further additions were made to the bottom trace in (a). In the top trace of (a) and in trace (b), the antagonist peptide Boc-PLPLP (10 μM) was added where noted by the arrow. Other conditions were as in Fig. 1. The traces are representative of at least three similar experiments.

$0.2 \pm 0.08 \text{ nmol/min per } 10^6 \text{ cells}$ after addition of Boc-PLPLP, when ATP was the phosphate donor. In contrast, displacement of fMLP with Boc-PLPLP affected the rate of O_2 consumption only marginally when ATP[S] was used as the substrate (Fig. 2b). In four determinations with ATP[S] the rate was $3.0 \pm 0.31 \text{ nmol/min per } 10^6 \text{ cells}$ before and $2.38 \pm 0.21 \text{ nmol/min per } 10^6 \text{ cells}$ after the inhibitory peptide was added. These findings are consistent with the formation of biologically active thiophosphoproteins which are resistant to cellular phosphatases. Moreover, the results imply that the rapid termination of the response upon removal of the stimulus, observed when ATP is used, is due to the fast dephosphorylation of critical phosphoproteins.

The results presented above suggest that protein phosphatases exert tight control of the extent and duration of the response in cells stimulated with chemoattractants. Because of the robust phosphatase activity evidenced by the experiments in Fig. 2, it is conceivable that protein kinases are active even in the absence of stimuli, but that the rate of phosphorylation is outstripped by the rate of dephosphorylation. If this were the case, accumulation of active thiophosphorylated proteins would occur when ATP[S] is the substrate. To investigate this possibility, electroporated cells were incubated in the absence of stimuli with progressively higher concentrations of the thiophosphorylated nucleotide. As shown in Fig. 3(a), a 'spontaneous' burst of O_2 consumption was induced by sub-millimolar concentrations of ATP[S], in permeabilized but otherwise untreated cells. The burst became evident after a lag that was inversely related to the concentration of the nucleotide, consistent with the gradual accumulation of thiophosphoproteins as a result of basal kinase activity. The double-reciprocal plot in Fig. 3(b) illustrates the relationship between the rate of O_2 consumption,

Fig. 3. Effect of ATP[S] on O₂-consumption in the absence of stimuli

Neutrophils were depleted of ATP and used for O₂-consumption measurements as described for Fig. 1. (a) Cells were suspended at the beginning of the trace in permeabilization medium containing NADPH, GTP and the indicated concentration (μM) of ATP[S]. Note that fMLP was not added in these experiments. (b) Concentration-dependence of the spontaneous stimulation of O₂ consumption induced by ATP[S]: double-reciprocal plot of the means ± S.E.M. of three or more determinations. Where absent, bars were smaller than the symbol. Straight line was fitted by least squares. (c) Cells suspended in permeabilization medium containing 200 μM-p[NH]ppA (top trace), 3 mM-ATP (middle trace) or no adenine nucleotide (control; lower trace). Where indicated, 200 μM-ATP[S] was added to all the samples. The traces in (a) and (c) are representative of three or more determinations. Other conditions were as in Fig. 1.

measured at 2 min, and the concentration of ATP[S]. Under the conditions used, half-maximal stimulation was obtained with 178 μM-ATP[S]. It is noteworthy that the effect of ATP[S] was not observed in intact cells (results not shown), indicating an intracellular site of action. Taken together, these observations suggest that kinases capable of stimulating NADPH oxidation are active in unstimulated cells, but that accumulation of biologically active phosphoprotein is prevented by ongoing phosphatase activity. We tried to confirm this hypothesis by treating cells in the presence of ATP with a variety of phosphatase inhibitors. At concentrations reported to inhibit phosphatase activity in other systems, PP₁, Zn²⁺, 2,3-bisphosphoglycerate and phosphoserine did not by themselves stimulate O₂ consumption, and in fact inhibited the fMLP- or phorbol-ester-stimulated burst, suggesting non-specific effects. In contrast, micromolar concentrations of vanadate produced a marked, sustained, respiratory burst. Permeabilized cells treated with 10 μM-sodium orthovanadate attained a maximal O₂ consumption rate of 4.87 ± 0.38 nmol/min per 10⁶ cells. Similar or even higher concentrations of vanadate had no significant effect when added to intact cells, indicating an intracellular site of action. Though stimulation of a kinase by vanadate cannot be ruled out, these findings are most readily explained by accumulation of phosphoproteins owing to inhibition of phosphatase activity.

In independent experiments, we have observed stimulation of O₂ consumption in electroporated cells treated with GTP[S] [14]. We therefore considered it possible that the stimulation observed in Fig. 3 was the result of contamination of ATP[S] with GTP[S]. However, several lines of evidence argue against this possibility. First, h.p.l.c. analysis of one of the batches of ATP[S] used to produce the 'spontaneous' activation of the oxidase failed to show detectable amounts of GTP[S]. Second, the response was obtained at comparable concentrations of ATP[S] with several batches from two suppliers (Boehringer Mannheim and Fluka). Third, the stimulation of O₂ consumption induced by ATP[S] was markedly inhibited by either ATP or p[NH]ppA (Fig. 3c). In contrast, the effect of GTP[S] is not blocked, and in fact strictly requires the presence of ATP [14]. Conversely, addition of 1 mM-GTP depressed the effect of GTP[S] by over 70 %, whereas the stimulation produced by ATP[S] was essentially unaffected (94.3 % of control). These findings suggest that the activation by ATP[S] results from interaction with an adenine-nucleotide- and not a guanine-nucleotide-binding site, and that contaminating GTP[S] cannot account for the observed stimulation. Stimulation is also unlikely to result from transfer of the thiophosphate group from ATP[S] to GDP by a nucleoside-diphosphate kinase, producing GTP[S] [15]. This conclusion was reached because: (a) addition of GDP does not promote the effect of ATP[S];

(b) GTP is not inhibitory to the response; (c) unlike the response to GTP[S], the effect of ATP[S] does not require ATP; and (d) p[NH]ppA, which is not a substrate of the diphosphokinase, was inhibitory (see above).

In conclusion, the present experiments indicate that ATP is strictly required for the induction of the respiratory burst by chemotactic peptides. The response is specific for adenine nucleotides and requires hydrolysis of the γ -phosphate, probably for the formation of an active phosphoprotein. In addition, the data suggest that kinases capable of inducing a respiratory burst are active even in the absence of exogenous stimuli, but that accumulation of the essential phosphoprotein(s) is prevented by the vigorous ongoing phosphatase activity. It is difficult to rule out that stimulation of kinase activity occurred as a result of the electroporabilization procedure itself. However, even in this event, the endogenous phosphatases were sufficient to preclude spontaneous stimulation when ATP was the energy source. Moreover, the electroporated cells remained susceptible to stimulation by physiological ligands (see above) and by exogenous phorbol esters or diacylglycerol [8], resembling intact cells. These observations, together with the well-established turnover of phosphoproteins in unstimulated cells (e.g. [16,17]), are consistent with ongoing kinase activity in the resting (unstimulated) state. The possible existence of basal kinase activity, together with the presence of robust phosphatases, suggest that control of the respiratory burst may be exerted by inhibition of phosphatase activity, in addition to or instead of the more commonly postulated activation of kinases. Indeed, a slight but significant inhibition of phosphotyrosine phosphatase activity by fMLP or phorbol ester was recently reported in neutrophils [18]. Our findings using vanadate, a phosphotyrosine phosphatase inhibitor, support the possibility that inhibition of the enzyme(s) may suffice to activate the NADPH oxidase.

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REFERENCES

1. Babior, B. M. (1984) *J. Clin. Invest.* **73**, 599–601
2. Curnutte, J. T. & Babior, B. M. (1975) *Blood* **45**, 851–861
3. Cohen, H. J. & Chovaniec, M. E. (1978) *J. Clin. Invest.* **61**, 1088–1096
4. Clark, R. A., Leidal, D. G., Pearson, D. W. & Nauseef, W. M. (1987) *J. Biol. Chem.* **262**, 4065–4074
5. Curnutte, J. T., Kuver, R. & Scott, P. J. (1987) *J. Biol. Chem.* **262**, 5563–5569
6. Ligeti, E., Doussiere, J. & Vignais, P. V. (1988) *Biochemistry* **27**, 193–200
7. Tauber, A. I. (1987) *Blood* **69**, 711–720
8. Grinstein, S. & Furuya, W. (1988) *J. Biol. Chem.* **263**, 1779–1783
9. Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 464–505
10. Boyum, A. (1968) *J. Clin. Lab. Invest.* **21**, Suppl. **97**, 77–98
11. Cassidy, P., Hoar, P. E. & Kerrick, W. G. L. (1979) *J. Biol. Chem.* **254**, 11148–11153
12. Coyne, M. D., Luszczynska, H. M. & Kunzi, N. (1987) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**, 433–444
13. Sklar, L. A. (1986) *Adv. Immunol.* **39**, 95–143
14. Nasmith, P. E., Mills, G. B. & Grinstein, S. (1989) *Biochem. J.* **257**, 893–897
15. Seifert, R., Rosenthal, W., Schultz, G., Wieland, T., Gierschick, P. & Jakobs, K. H. (1988) *Eur. J. Biochem.* **175**, 51–55
16. White, J. R., Huang, C. K., Hill, J. M., Naccache, P. H., Becker, E. L. & Sha'afi, R. I. (1984) *J. Biol. Chem.* **259**, 8605–8611
17. Okamura, N., Curnutte, J. T., Roberts, R. L. & Babior, B. M. (1988) *J. Biol. Chem.* **263**, 6777–6782
18. Kraft, A. S. & Berkow, R. L. (1987) *Blood* **70**, 356–362

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