Phosphorylation of Cytosolic Proteins by Resting and Activated Human Neutrophils

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A study was conducted on the phosphorylation of proteins in the neutrophil cytosol in response to phorbol myristate acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (fMLP). Autoradiography of gel electrophorograms prepared from neutrophils incubated with 32P, in the presence and absence of the activators showed nine proteins whose state of phosphorylation was affected by neutrophil activation. 32P was gained by eight of these proteins and was gone within 0.2 minutes after activation. The other change in the extent of labeling appeared to reach completion by one to two minutes. It was possible to quantitate the changes in 32P content of three of the nine proteins. One of these was the 20-kD protein that lost label when the neutrophils were activated. Quantitation showed that over half the 32P present in this protein in the resting state was gone within 0.2 minutes after activation. The other two were proteins weighing 11 and 69 kD. The phosphorylation characteristics of these two proteins differed, depending on whether activation had been carried out with PMA or fMLP. These differences in protein phosphorylation support other evidence suggesting that PMA and fMLP do not activate neutrophils by identical biochemical pathways. Differences in phosphorylation between resting and activated cells were not affected by dibutyryl cyclic guanosine monophosphate (cGMP), dibutyryl cyclic adenosine monophosphate (cAMP), theophylline, aspirin, hydrocortisone, or colchicine. The differences were abolished, however, by 30 μmol/L trifluoperazine. This finding is consistent with the hypothesis that the calcium/calcmodulin system plays a biochemical role in the activation of neutrophils.

Materials and Methods

Superoxide dismutase (type I, from bovine blood), cytochrome c (type VI, from horse heart), N-formyl-methionyl-leucyl-phenylalanine, 4β-phorbol-12-myristate-13-acetate, acetylsalicylic acid, N\textsuperscript{6},O\textsuperscript{2}-dibutyryl-adenosine 3',5'-cyclic monophosphate, N\textsuperscript{7},O\textsuperscript{2}-dibutyrylguanosine 3',5'-cyclic monophosphate, trifluoperazine, colchicine, theophylline, and hydrocortisone were purchased from Sigma Chemical Company, St Louis. Falcon plastic tissue culture flasks (25-cm² growing surface) were purchased from Scientific Products, McGaw Park, Ill. Diisopropyl fluorophosphate was purchased from Aldrich, Milwaukee. Cyano[G\textsuperscript{3}H]cobalamin, and 32P, (carrier-free) from the Blood Research Laboratory and the Department of Medicine, Tufts-New England Medical Center, Boston. Supported in part by USPHS grant AI-11827 and by grant 1349 from the Council for Tobacco Research, USA, Inc. P.C.A. is the recipient of National Research Service Award AI-06457. Submitted Aug 22, 1983; accepted May 7, 1984. Address reprint requests to Dr Bernard M. Babior, Division of Hematology/Oncology, New England Medical Center, 171 Harrison Ave, Boston, MA 02111.

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were purchased from Amersham, Arlington Heights, Ill. Sodium fluoride and disodium ethylenediamine tetraacetate were obtained from Fisher, Medford, Mass. Macrodex and Ficoll-Paque were purchased from Pharmacia, Piscataway, NJ. Sodium dodecyl sulfate, acrylamide, and N,N'-methylenebisacrylamide (all of electrophoresis purity), molecular weight standards, and Coomassie brilliant blue R-250 were purchased from Bio-Rad, Richmond, Calif. Activated charcoal (powder) was purchased from Mallinckrodt, Paris, Ky.

Preparation and Labeling of Neutrophil Monolayers

Human neutrophils were purified from whole blood by dextran sedimentation followed by Ficoll-Paque density gradient centrifugation, as described elsewhere. The cells were suspended (concentration 2.5 x 10^6 cells/mL) in phosphate-buffered saline containing 0.45 mmol/L Ca^{++} and 0.25 mmol/L Mg^{++}. Neutrophil monolayers were prepared by placing 2-mL portions of the suspension in tissue culture flasks and allowing the cells to attach for 30 minutes at 37 °C. The method for removing nonadherent cells, the composition of the reaction buffer, and the procedure for loading the cells with 32P was as previously described.3

Specific Activity of [γ-32P]ATP

The specific activity of the [γ-32P]adenosine triphosphate (ATP) in the neutrophil monolayers was measured as described by Hamster and Niehaus, except that before incubation with galactose and galactose kinase, the labeled nucleotide was concentrated by treatment with charcoal.14

Activation and Fractionation of Neutrophils

32P-loaded neutrophils were activated at 37 °C with prewarmed reaction buffer (1.5 mL per flask) containing either 100 ng/mL PMA or 0.6 μmol/L fMLP. After the appropriate period of activation, the cells were rinsed in ice-cold reaction buffer that had been supplemented with 0.1 mol/L NaF and 10 mmol/L sodium ethylenediamine tetraacetate (EDTA; pH 7.4) to inhibit further kinase and phosphatase activity. The cells were then incubated for ten minutes at 4 °C in NaF/EDTA reaction buffer containing 0.5 mmol/L diisopropyl fluorophosphate (DFP) in order to inactivate neutrophil proteases. Unreacted DFP was then removed by washing the DFP-free NaF/EDTA reaction buffer. Finally, 1.0 mL of 0.25 mol/L sucrose containing 0.1 mol/L NaF and 10 mmol/L sodium EDTA (pH 7.4) was added to each flask, and the cells in the monolayer were simultaneously detached and disrupted by sonication for 20 seconds at full power, using a Heat Systems W220D sonicator fitted with a cup horn through which ice water was circulated. This procedure released 96.3% ± 0.6% of the myelo-peroxidase and 89.0% ± 2.7% of the alkaline phosphatase from the surface of the dish. Nuclei and whole cells were removed by centrifugation at 300 g for ten minutes. The cytosol and plasma membrane/granule precipitate was recovered by centrifugation at 100,000 g for one hour.

Trifluoperazine Treatment

32P-loaded neutrophils were preincubated for ten minutes at 37 °C with 30 μmol/L TFP. Cells were then either incubated with 100 ng/mL PMA or 30 μmol/L TFP for five minutes (37 °C) or allowed to recover from TFP treatment by incubation for 20 minutes (37 °C) in the absence of TFP and then incubated with 100 ng/mL PMA for five minutes (37 °C). Resting cells were treated with TFP as described, except that the incubation with PMA was omitted. The reaction was terminated and cells fractionated as described above.

For measuring the effect of TFP on O_{2} production and specific granule release, unlabeled cells were treated with TFP as described above, except that incubations with PMA were carried out for ten minutes. O_{2} production and the release of cobalamin-binding protein during exposure to PMA were determined as described below.

Gel Electrophoresis and Autoradiography

The cytosolic proteins were precipitated by adding an equal volume of acetone and incubating for 30 minutes at 4 °C. The precipitate was recovered by centrifugation at 12,000 g for ten minutes in an Eppendorf microtube, dissolved in 75 μL of sample buffer [1% sodium dodecyl sulfate (SDS), 60 mmol/L Tris (pH 6.8), 20% (vol/vol) glycerol], and heated in boiling water for five minutes. The membrane/granule pellet was dissolved directly in 100 μL of sample buffer and boiled for five minutes. The samples were then assayed for protein according to the method of Schaffner and Weismann, and portions containing 25 μg of protein were prepared for electrophoresis by adding dithionitrotetrazolium to 40 mmol/L and boiling for an additional three minutes. The samples were electrophoresed on an 8% to 30% SDS gradient-pore gel with dimensions of 0.75 x 15 x 200 mm. Electrophoresis and autoradiography were carried out as previously described. Quantitation of the autoradiograms was accomplished by the integration of scans, which were obtained using a Quick Scan R & D densitometer. Integration was performed by cutting out and weighing the regions of interest from Xerox copies of the scans. Peak areas were expressed as percent of the area of the total scan.

Determination of O_{2} Production and Cobalamin-Binding Protein Release

For assays of NADPH oxidase activity and specific granule release, neutrophil monolayers were prepared as described above and incubated according to the conditions used for loading with 32P, except that the radioactive label was omitted from the buffer. The cells were then incubated at 37 °C in 1.5-mL portions of reaction buffer, with or without activator (see above). The reaction buffer was replaced at intervals for a total of ten minutes, as indicated in Results. To determine O_{2} production, the portions of reaction buffer contained 1 mg/mL cytochrome c, and O_{2} was measured as previously described. For this assay, 300 μL of the reaction buffer sample was placed in a 1.5-mL Eppendorf tube and mixed with 335 μL of 0.155 mol/L NaCl and 13 μL of 1% (vol/vol) Triton X-100 in water. To this mixture was added 100 μL 0.155 mol/L saline, containing 5 ng CN[^{3}H]Cbl. After vigorous agitation, the mixture was allowed to stand for five minutes at 4 °C. One-half milliliter of a 0.5% (wt/vol) suspension of activated charcoal in 0.155 mol/L NaCl/1% (wt/vol) bovine serum albumin was then added. The final mixture was spun for ten minutes in an Eppendorf microtube, and the quantity of[^{3}H} in 625 μL of supernatant was determined by liquid scintillation counting. To determine the total cellular cobalamin-binding protein, the cells were removed from the plates at ten minutes by sonication into NaF- and EDTA-free 0.25 mol/L sucrose, as described above, and 30-μL portions of the disrupted cells were brought to 300 μL with 0.155 mol/L NaCl. Cobalamin-binding protein was then determined as described above. The total cellular cobalamin-binding protein was taken as the sum of the total amount released from the cell plus the total amount found in the cell sonicate.

RESULTS

Time Course of O_{2} Production and Specific Granule Release by Adherent Cells

The purpose of this study was to investigate protein phosphorylation as it relates to neutrophil activation.
Our approach was to compare changes in protein phosphorylation with the alterations in neutrophil activity induced by PMA and fMLP, two agents whose effects on neutrophils differ sharply with respect to time. As indicators of neutrophil activation, we employed O₂ production and specific granule release. Figure 1 shows the time courses of these two events in neutrophils stimulated with PMA and fMLP. The figure indicates that (a) both PMA and fMLP induced degranulation and O₂ production; (b) when initiated by fMLP, both of these activities slowed sharply four to five minutes after they began, while the PMA-initiated activities proceeded unabated for at least the duration of the incubation; and (c) both degranulation and O₂ production were well under way within two minutes after stimulation.

**Phosphorylation of Cytosolic Proteins**

We previously showed that when ³²P₁-loaded neutrophils were exposed to activating agents, distinct changes occurred in the extent of labeling of a number of proteins.⁴ One possible explanation for these results is that the specific activity of the [γ-³²P]ATP pool in the ³²P₁-loaded neutrophils was altered by stimulation of the cells. To investigate this possibility, we compared the specific activity of the ATP in ³²P₁-loaded resting neutrophils with that in ³²P₁-loaded cells that had been exposed for ten minutes to PMA (100 ng/mL). The ratio of stimulated to resting specific activities was only 1.25 ± 0.14 SE (N = 3), a value too low to account for the observed changes in the labeling of neutrophil proteins.

We have begun to examine the subcellular distribution of the proteins that gain or lose ³²P when neutrophils are stimulated. Our results indicate that some of the changes in phosphorylation affect cytosolic proteins, whereas others affect proteins associated with the particulate fraction of the cells (plasma membranes and azurophil and specific granules). Figure 2 shows the changes that occurred in the phosphorylation patterns of cytosolic and particulate proteins when neutrophils were activated for five minutes with PMA. Of the proteins previously shown to gain or lose labeled phosphate in experiments with whole cells, one (pp 20) was found solely in the cytosol, one (pp 22) appeared only in the particulate fraction, and six (pp 11, 13, 48, 53 through 55, 69, and 80) were divided between the two fractions (as determined by molecular weight comparisons). In an experiment in which protein phosphorylation in response to PMA was followed with time, none of the phosphoproteins was found to move

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*The phosphoprotein pp 11 was not specifically discussed in our previous report on protein phosphorylation in neutrophils.⁵ A review of the figures in that paper, however, showed that pp 11 could be identified on the autoradiograms as a protein that acquired label upon stimulation.
from one fraction to the other during the course of incubation.

In Fig 3 are presented autoradiograms showing the time courses for the phosphorylation of cytosolic proteins when neutrophils were activated with PMA or fMLP. The autoradiograms show nine proteins whose phosphorylation appeared to be altered by activation. The affected phosphoproteins are listed in the figure. In eight of these proteins, the extent of labeling increased during activation; in the ninth, the extent decreased.

These changes in protein phosphorylation were further characterized by a quantitative analysis of incorporated label in neutrophils exposed to the two activators. The analysis was performed on scans of autoradiograms from eight time course experiments: four done with PMA and four with fMLP. Six of the eight autoradiograms were from paired experiments in which portions of a single preparation of neutrophils was used to determine the time course of phosphorylation with each of the two activating agents.

For the five phosphoproteins between pp 40 and pp 55 (Fig 3) and for pp 85, only limited quantitative information could be obtained regarding changes in protein phosphorylation. Both PMA and fMLP appeared to induce two- to threefold increases in the extent of labeling of these six proteins within the first minute or two after the addition of activator. Phosphorylation appeared to reach steady state by this time. However, we were unable to ascertain the detailed configurations of the changes in phosphorylation over time, nor could we establish whether there were any differences in the phosphorylation responses to the two activating agents.

For the other three phosphoproteins, the changes in their phosphorylation during the course of the incubation could be quantified with reasonable accuracy. These changes in phosphorylation are presented in Fig 4. Figure 4 (left graph) shows the results obtained with pp 11. This protein was found to respond only to PMA. When neutrophils were activated with PMA, the protein began to incorporate label after a lag of at least 12 seconds. Labeling peaked at two minutes, reaching about 3/2 times the resting value, then began to decline, until by ten minutes, it had fallen back nearly to resting levels. In contrast to the results with PMA, no change was seen in the phosphorylation of this protein with fMLP.
protein when the neutrophils were activated with fMLP.

Phosphoprotein 69 also responded differently to PMA than to fMLP, but in this case, the difference was in the timing of an increase in labeling that was induced by either stimulus (Fig 4, center). With fMLP as the stimulus, the increase in labeling started between one and two minutes, whereas it was delayed until the five-minute point with PMA. It is of interest that this protein did not begin to accumulate label until after the onset of both degranulation and the respiratory burst (Fig 1).

Phosphoprotein 20 was unique because it was the only protein detected that lost label when the cells were exposed to an activating agent. Treatment of neutrophils with either PMA or fMLP caused this protein to lose approximately 70% of its 32P within the first 12 seconds (Fig 4, right graph).

**Effect of Inhibitors and Metabolic Regulators on Protein Phosphorylation**

To obtain further information about possible connections between neutrophil function and the phosphorylation of cytosolic proteins, we have examined the effects of certain inhibitors and metabolic regulators on the expression of the phosphorylation response. A relationship between cyclic nucleotide metabolism and protein phosphorylation was investigated through studies with theophylline (1 mmol/L) and the dibutyryl analogs of cAMP and cGMP (2 mmol/L). None of these three agents induced detectable changes in the phosphorylation of cytoplasmic proteins when incubated for five minutes with resting neutrophils, nor did they alter the phosphorylation pattern of cells incubated for five minutes with PMA or for two minutes with fMLP. Thus, as far as could be determined from these experiments, the phosphorylation of the major cytosolic phosphoproteins appears to be independent of cyclic nucleotides. A similar lack of effect was seen with 1 mmol/L hydrocortisone, 5 μmol/L colchicine, and 50 μmol/L acetylsalicylic acid. Neutrophils treated with these agents for ten minutes and then incubated for an additional five minutes, either with no addition or after the addition of PMA, showed phosphorylation patterns identical with those of untreated resting or PMA-stimulated controls. These results suggest that the observed phosphorylation events were independent of microtubule assembly (colchicine) and prostaglandin synthesis (acetylsalicylic acid).

**Effects of Trifluoperazine on Neutrophil Function and Protein Phosphorylation**

In agreement with previous work, we found that trifluoperazine was a potent inhibitor of neutrophil function. Cells preincubated for ten minutes with 30 μmol/L trifluoperazine were unable to either manufacture O_2_ or discharge their specific granules in response to PMA (Table 1). This failure to respond to PMA was not due to a toxic effect of the agent on the neutrophils, because cell survival, as measured by trypan blue exclusion, was no different in TFP-treated cells than in normal control cells (94.6% exclusion and 93.5% exclusion, respectively). In addition, we confirmed the reversibility of the TFP effect on O_2_ production reported by Smith et al. TFP-treated cells that had been washed free of TFP and then incubated for an additional 20 minutes in TFP-free buffer were found to have completely recovered their ability to generate O_2_ in response to PMA. Degranulation was only partially restored under these conditions (Table 1).

We then examined the effect of TFP on protein phosphorylation. This examination showed that the phosphorylation patterns of TFP-treated neutrophils that had been exposed to PMA were virtually identical to patterns from control and TFP-treated resting cells (Fig 5). (In the figure shown, the pp 20 band from the TFP-treated cells was not as prominent as in the resting control. In two other experiments, however, the pp 20 band was equally prominent in the control and TFP-treated resting cells. In none of the three experiments was label lost from this protein when the TFP-treated cells were exposed to PMA.) Thus, the inhibitor appeared to prevent all the changes in phosphorylation that were induced in uninhibited cells by PMA. Similar results were obtained using fMLP as the activating agent (data not shown).

Because the effect of TFP on O_2_ production was found to be reversible, although its effect on degranulation was not, we carried out studies to determine the extent to which the effect of TFP on protein phosphorylation could be reversed. The results (Fig 5) showed that the blockade of PMA-induced changes in protein phosphorylation was almost completely relieved when TFP-treated neutrophils were incubated for 20 minutes in TFP-free buffer. Only pp 69 failed to take up any 32P in response to PMA after the removal of

<table>
<thead>
<tr>
<th>Conditions</th>
<th>O_2_ Production (nmol/10 min)</th>
<th>Cobalamin-Binding Protein Released (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>Exp 1 3.8</td>
<td>Exp 1 2.0</td>
</tr>
<tr>
<td>PMA</td>
<td>29.4 25.3</td>
<td>41.6 34.3</td>
</tr>
<tr>
<td>TFP + PMA</td>
<td>3.0 1.1</td>
<td>2.2 4.4</td>
</tr>
<tr>
<td>TFP, wash, PMA</td>
<td>31.9 25.8</td>
<td>20.4 8.2</td>
</tr>
</tbody>
</table>

The experiments were conducted as described in the text.
Fig. 5. Effect of 30 μmol/L trifluoperazine on the phosphorylation of cytosolic proteins in response to PMA. Pair 1 was not exposed to TFP. Pair 2 was exposed to TFP during the final incubation period. Pair 3 was preincubated with TFP, then washed free of the inhibitor before the final incubation period (for details, see text).

of the inhibitor. Removal of TFP also restored fMLP-induced protein phosphorylation, although some residual impairment in the labeling of pp 69 was seen with this activator as well.

DISCUSSION

An early response of the neutrophil to activating agents is a change in the phosphorylation of some of its phosphoproteins.\textsuperscript{3-5} We have now studied the phosphorylation response of the proteins of the neutrophil cytosol and have found this response to be at least partially agent specific. In particular, differences between fMLP-induced and PMA-induced protein phosphorylation have been observed with proteins of subunit molecular weights of 11,000 (pp 11) and 69,000 (pp 69). Phosphorylation of pp 11 is stimulated dramatically by PMA, but is totally unaffected by fMLP (Fig 4). With pp 69, activator specificity is expressed in the duration of the lag before phosphorylation begins; with fMLP, phosphorylation has begun by one minute after the addition of the activator to the cells, whereas PMA-induced phosphorylation does not start until three to five minutes after the activator is added. The other changes in phosphorylation observed in these experiments were seen with both fMLP and PMA and appeared to be complete (or nearly so) within one to two minutes after the addition of activator.

The phosphorylation response was not affected by any of a number of regulators or inhibitors that are known to act on various metabolic and structural systems in the neutrophil. Agents without an apparent effect on the labeling of the major cytosolic phosphoproteins in human neutrophils included dibutyryl cAMP, dibutylryl cGMP, and theophylline (all acting on cyclic nucleotide-dependent systems), aspirin (which inactivates cyclooxygenase), hydrocortisone, and colchicine (which depolymerizes microtubules). One agent, however, did affect the phosphorylation response. This agent, trifluoperazine, completely inhibited all the changes in protein phosphorylation that normally occurred in response to either PMA or fMLP. It is of interest, in connection with the postulated relationship between protein phosphorylation and neutrophil activation, that of all the agents tested, trifluoperazine is the only one that is able to abolish both degranulation and the activation of the respiratory burst in the neutrophil.\textsuperscript{17,18}

In considering these results, it should be borne in mind that we are using the term "cytosol" in an operational sense to refer to the 100,000 g supernatant. We believe that this fraction consists largely of cytosolic proteins, but we cannot exclude the possibility that it contains membrane proteins, such as the PMA receptor\textsuperscript{21} whose attachment to the plasma membrane is salt sensitive or dependent on divalent cations. Conversely, although the 100,000 g pellet consists mainly of plasma membrane, specific granules, and azurophil granules, it would also contain any cytoplasmic proteins susceptible to precipitation by the NaF or EDTA present in the sonication buffer.

To interpret the changes in phosphorylation, we compared their characteristics with those of the degranulation and O\textsubscript{2}-generating responses of neutrophil monolayers. This comparison indicates that neither the phosphorylation of pp 11 nor the phosphorylation of pp 69 are likely to play a role in the initiation of degranulation or of the respiratory burst; pp 11 only responded to one stimulus (PMA), whereas pp 69, though it responded to both stimuli, did not begin to take up \textsuperscript{32}P until after degranulation and O\textsubscript{2} production had begun. Other proteins changed their extents of labeling rapidly, a finding consistent with the possibility that some of them may participate as components of the poorly understood biochemical apparatus responsible for the activation of the neutrophil; to conclusively identify those that perform this function, however, will require additional investigation.
Phosphoprotein 69 is unique in that it acquires most of its label after O₂ production and degranulation have begun. This finding suggests that the phosphorylation of pp 69 is related to the regulation of events that occur during the postactivation period. Many such events have been demonstrated: the depolymerization of microtubules, oxidation of glutathione, and the conversion of NAD to NADP are some examples. It is not yet possible to determine which postactivation events are affected by the phosphorylation of this protein.

Phenothiazines such as trifluoperazine have at least two biochemical effects: they block the activation of calmodulin-dependent protein kinases by binding to calmodulin, thereby preventing the formation of the active kinase-Ca²⁺-calmodulin complex and they inhibit the activity of the calmodulin-independent protein kinase C by binding to the phorbol diester site of that kinase. Our results are therefore consistent with at least two possible mechanisms for activating the neutrophil. According to one mechanism, the Ca²⁺-calmodulin complex is responsible for neutrophil activation, acting at a biochemical level to regulate protein phosphorylation, degranulation, and respiratory burst expression. According to the other mechanism, neutrophil activation results from the phosphorylation of key regulatory proteins by the lipid (or phorbol ester) activatable enzyme, protein kinase C. Calcium would be likely to participate in this mechanism, too, as it can activate protein kinase C through several mechanisms, both direct and indirect. Its participation in kinase C-mediated activation, however, would probably be independent of calmodulin.

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Phosphorylation of cytosolic proteins by resting and activated human neutrophils

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