Activation of protein kinase C in neutrophil cytoplasts

Localization of protein substrates and possible relationship with stimulus–response coupling

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Treatment of enucleated, granule-free neutrophil cytoplasts with the protein kinase C activator phorbol 12-*O*-myristate-13-acetate (PMA) causes an increased ^{32}P-incorporation into a variety of polypeptides. Permeabilization of PMA-stimulated, ^{32}P-labeled cytoplasts by 0.01% digitonin fully releases the majority of these phosphorylated proteins. A statistically significant correlation is found between the extent of PMA-induced activation of generation of superoxide anion (O_2^-) and the phosphorylation of a cytosolic polypeptide with an apparent M_r of 46000, whose ^{32}P-labeling is also enhanced by the treatment of cytoplasts with 1-oleyl-2-acetylglycerol, the Ca^{2+} ionophore ionomycin or latex beads. Furthermore, treatment of cytoplasts with the protein kinase C inhibitor trifluoperazine markedly inhibits the ^{32}P-labeling of proteins in the 40000 M_r range, including the 46 kDa polypeptide, and almost totally abolishes the activation of O_2^- production by PMA.

Neutrophil cytoplast *Protein phosphorylation* *Protein kinase C* . *Superoxide anion* *Phorbol myristate acetate*

1. INTRODUCTION

A major research interest on host-defense is the elucidation of the molecular mechanisms by which chemotaxins, lymphokines and other extracellular signals modulate the functions of macrophages and granulocytes. Elevation of cytosolic Ca^{2+} and cAMP concentration, increased turnover of some lipid classes, and phosphorylation of specific proteins have all been considered as possible intracellular mediators of extracellular signals borne by either soluble or endocytosable particulate agents [1–13]. In particular, we [7] and others [11] have shown that cell activators such as *N*-formyl-Met-Leu-Phe, PMA and opsonized zymosan increase the level of ^{32}P incorporation into a similar set of proteins, with dose–response and kinetic effects comparable to those observed during activation of some neutrophil functions (chemotaxis, secretion, generation of oxygen radicals) by these agents. This has been taken as evidence that phosphorylation of certain proteins may play a role in some aspects of neutrophil activation.

Identification of the subcellular localization of these proteins as well as of the class of kinases (Ca^{2+}-, cAMP-, or cGMP-, or lipid-dependent), catalyzing their phosphorylation, would be crucial to the understanding of where and how diverse activation routes interact to eventually elicit a cell response. We have approached this problem by investigating protein phosphorylation in enucleated, granule-free neutrophil cytoplasts [12,14], thereby restricting the search for protein substrates of

Abbreviations: PMA, phorbol 12-*O*-myristate-13-acetate; TFP, trifluoperazine; OAG, 1-oleyl-2-acetylglycerol; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; $B_{12}BP$, vitamin B_{12} binding protein

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kinase(s) to the plasma membrane, cytosol and cytoskeleton. Furthermore, we have exposed the cytoplasts to known activators of protein kinase C [15,16] in the attempt to identify its substrates and to establish a possible correlation between their phosphorylation and the activation of neutrophil functions.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Carrier-free [^{32}P]orthophosphoric acid in dilute HCl (pH 2–3) and cyano-[^{57}Co]cobalamin were obtained from Amersham (Amersham, UK). PMA, concanavalin A and ferricytochrome *c* (type VI) were products of Sigma (St. Louis, MO, USA). Trifluoperazine dihydrochloride and the ionophore A23187 were purchased from Smith Kline and French Labs. (Herts, UK) and Calbiochem-Behring Corp. (Lucerne, Switzerland), respectively. Ionomycin and OAG were gifts from C.M. Lin of Hoffman-La Roche and R.Y. Tsien of the University of California (Berkeley), respectively. Stock solutions of PMA, A23187, ionomycin and digitonin, were prepared in Me_2SO, whereas TFP was dissolved in methanol.

2.2. *Preparation of neutrophils and neutrophil cytoplasts*

Neutrophils were isolated from bovine blood as in [8,17]. The preparation of cytoplasts [12] was carried out essentially as reported by Roos et al. [14]. Densities of cell and cytoplast suspensions were assessed by hemocytometer counting.

2.3. *Labeling of cytoplasts*

Cytoplasts (3×10^8/ml) in Hepes medium (30 mM Hepes–NaOH, 120 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4) with 5 mM glucose and 0.1% BSA were incubated at 37°C with $^{32}PO_4$ (0.5–1 mCi/ml) until a steady-state incorporation of ^{32}P into the internal phosphate pool was attained (90–120 min). The suspension was then diluted 10-fold with Hepes medium, and the labeled cytoplasts were sedimented for 10 min at $800 \times g$, rinsed once and suspended in Hepes medium (6×10^7/ml).

The ^{32}P-labeled cytoplasts were exposed to the stimulants for 120 s at 37°C, and then rapidly sedimented at $10000 \times g$ in a Janetzki centrifuge model TH 12. The pellet was directly solubilized in the final sample buffer used for the SDS–PAGE [7].

In some experiments, the sedimented cytoplasts were resuspended in Hepes medium, in which 100 mM NaF had replaced an equivalent amount of NaCl, and treated with 0.01% digitonin for 10 min at 0°C. Alternatively, the stimulated cytoplasts were exposed for 10 min at 37°C to Hepes medium containing 1% Triton X-100 and, in addition to 100 mM NaF, 10 mM EGTA and 5 mM Na-pyrophosphate. The detergent-insoluble material was separated from the extracted cytoplast components by centrifugation at $10000 \times g$ for 10 min, and then immediately solubilized in the sample buffer of SDS–PAGE [7].

2.4. *Electrophoresis and autoradiography*

SDS–PAGE of reduced samples was conducted on linear gradient slab gels [7]. After electrophoresis, the gels were fixed and stained in 25% methanol–10% acetic acid with 0.15% Coomassie brilliant blue, destained in 25% methanol–10% acetic acid, and dried under vacuum. Dry gels were autoradiographed using Cronex 7 X-ray films and Lightning plus intensifying screens (Du Pont de Nemours, Wilmington, DE). Occasionally, the relative intensity of bands on the autoradiographs were quantitated by densitometric tracing using a Bio-Rad scanning densitometer, mod.1650 (Bio-Rad Labs., Richmond, CA).

2.5. *Other procedures*

Secretion of vitamin B_{12}-binding protein, reduction of ferricytochrome *c* by O_2^-, the activities of alkaline phosphatase, myeloperoxidase and lactate dehydrogenase, as well as DNA and protein were determined as in [12,17].

3. RESULTS

3.1. *Properties of neutrophil cytoplasts*

Cytoplasts, pinched off from cytochalasin B-treated neutrophils as large cytoplasmic vesicles [14], retain about one-half of the cytosol and 40% of the plasma membrane, as indicated by the recovered activity of lactate dehydrogenase and of the plasmalemmal marker alkaline phosphatase [17], respectively (table 1). The extremely low content in DNA and granule markers (B_{12}BP and

Table 1

Summary of biochemical analysis of enucleated, granule-free neutrophil cytoplasts

	Activity or amount per	
	10^7 cytoplasts	10^7 neutrophils
Alkaline phosphatase (units)	46.4 ± 5.3	126.6 ± 10.4
Lactate dehydrogenase (units)	219.0 ± 20	392.0 ± 32
DNA (μg)	1.5 ± 0.1	103.0 ± 5
Vitamin B_{12}-binding protein (units)	0.35 ± 0.07	21.9 ± 1.5
Peroxidase (units)	18.6 ± 2.9	722 ± 152
Protein (mg)	0.27 ± 0.03	0.87 ± 0.07

Cytoplasts were prepared essentially as described by Roos et al. [14]. Data are means of 14 experiments ± SE. Units of enzyme or binding protein activities: alkaline phosphatase, μmol *p*-nitrophenol/min; lactate dehydrogenase, μmol NAD^+/min; vitamin B_{12} binding protein, ng vitamin bound; peroxidase, μmol *o*-dianisidine oxidized/min

peroxidase) (table 1) indicates that not only nuclei but also granules are essentially absent in the cytoplast population. This is confirmed by SDS–PAGE analysis. In fact, polypeptides with an apparent M_r of 14000–18000, which are the main constituents of isolated granules (not shown), are almost completely removed from cytoplasts (fig.1).

When treated with 0.01% digitonin, which permeabilizes cholesterol-rich membranes like the plasma membrane [18], cytoplasts release 84% of protein and 97% of lactate dehydrogenase, respectively, and generate ghosts which retain 97% of the plasmalemmal alkaline phosphatase. Further, extraction of cytoplasts with the non-ionic detergent Triton X-100, which removes a large fraction of the plasma membrane and all the soluble cytoplasm [19], leaves an alkaline phosphatase-free residue mainly composed of the cytoskeletal proteins actin and myosin (fig.1).

3.2. *Increased phosphorylation of proteins in activated cytoplasts*

Phosphorylation of specific protein substrates in intact neutrophils has been shown to rapidly occur concomitantly with activation of cell functions

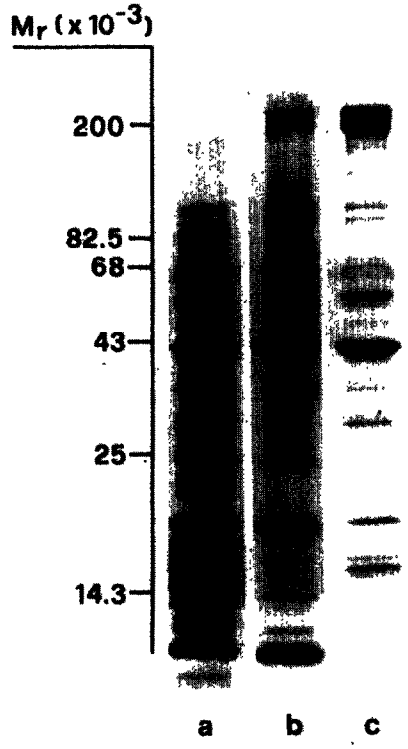

Fig.1. Electrophoretic profiles of the polypeptides of intact neutrophils (a) and of neutrophil cytoplasts before (b) and after (c) extraction with 1% Triton X-100 (10 min, 37°C). The SDS-gel (8–20% acrylamide) was stained with Coomassie brilliant blue.

[7,11,20,21]. An increased level of phosphorylation of polypeptides with apparent M_r from 18000 to about 100000 is also observed when neutrophil cytoplasts are activated by various agents such as PMA, latex beads and ionomycin (figs 2b,3b,4b). In particular, PMA induces a heavy phosphorylation of polypeptides with an apparent M_r of 62000, 46000, 43000 and 18500 (ranges: M_r 61000–64000, 45200–47000, 43000–43500 and 18200–18800, in 4 different experiments).

To study the intracellular localization of these polypeptides, ^{32}P-labeled cytoplasts were first activated with PMA and then permeabilized with digitonin or extracted with Triton X-100. The majority of phosphorylated proteins, including those with apparent M_r 46000, 43000 and 18500 are recovered in the fraction solubilized by either digitonin or Triton X-100 (fig.2f,l), and thus ap-

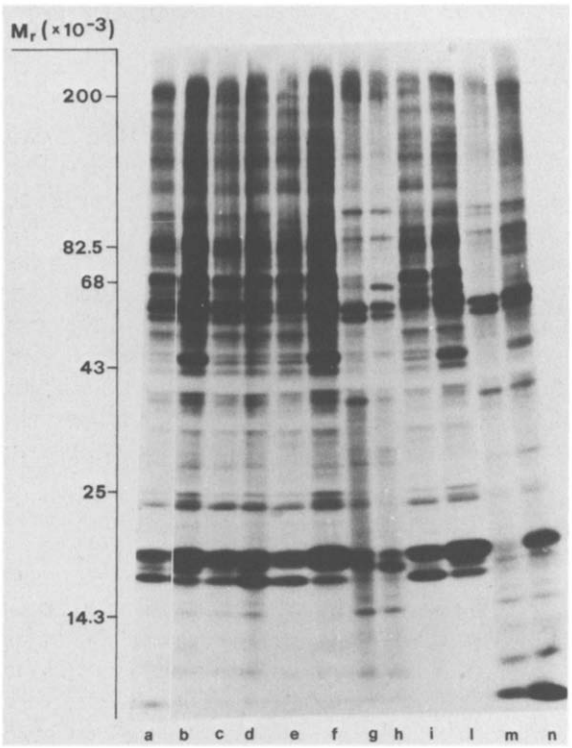

Fig.2. Autoradiograph showing phosphorylation and cellular localization of proteins of cytoplasts treated with PMA. ^{32}P-labeled cytoplasts (1.5×10^7) in 0.25 ml Hepes medium with 5 mM glucose were treated with 90 nM PMA (in the absence or presence of 50 μM TFP) or 100 μg/ml concanavalin A for 2 min at 37°C. After sedimentation, cytoplasts were either processed immediately or extracted with 1% TritonX-100 (10 min, 37°C) or 0.01% digitonin (10 min, 0°C). Boiled samples were subjected to SDS–polyacrylamide (8–16%) gel electrophoresis, stained, dried and autoradiographed. Lane a, control cytoplasts; lane b,c, cytoplasts treated with PMA without (b) or with (c) TFP; lane d, cytoplasts treated with concanavalin A; lanes e–h, Triton X-100 extracts (e,f) or insoluble residues (g,h) from control (e,g) and PMA-treated (f,h) cytoplasts; lanes i–n, cytosols (i,l) or ghosts (m,n) derived from control (i,m) or PMA-treated (l,n) cytoplasts, permeabilized with digitonin.

pear to be cytosolic. One exception is the protein with an apparent M_r of 62000, which is recovered in the Triton X-100-insoluble material when cytoplasts are extracted under conditions preserving the integrity of cytoskeleton. One possibility is that this protein is vimentin, which has been shown to undergo increased ^{32}P-labeling in rabbit neutrophils reacting with chemotactic factors [20].

3.3. *Correlation between protein phosphorylation and activation of neutrophils*

To investigate whether the increased phosphorylation of cytosolic proteins may be linked to the activation of neutrophils, we followed two approaches. First, we attempted to correlate the effect of various stimulants on the generation of O_2^- and on the extent of ^{32}P-labeling of the 46-kDa polypeptide. This protein was selected among the complex set of peptides which undergo an increased phosphorylation in activated cytoplasts, because its labeling by ^{32}P can be easily quantified by densitometric analysis of the autoradiographs. As shown in fig.3, a gradual increase of the concentration of PMA, to which the cytoplasts are exposed, also causes a gradual increase in the extent of ^{32}P-incorporation into the 46-kDa polypeptide. In two experiments, in which the activation of O_2^- generation and ^{32}P-incorporation into the 46-kDa protein by increasing concentrations of PMA were followed in parallel, the two events showed a strict correlation

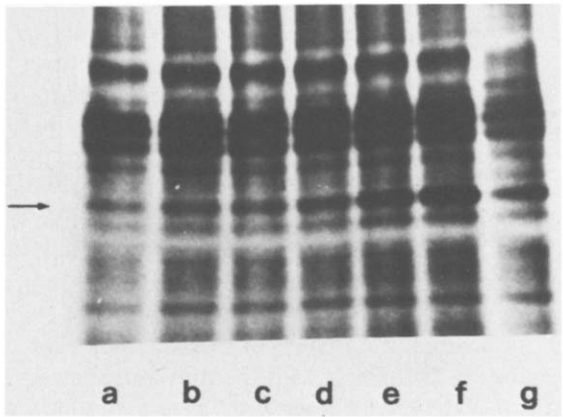

Fig.3. Increased phosphorylation of the 46-kDa protein in cytoplasts treated with increasing doses of PMA or with latex beads. Cytoplasts were incubated with PMA or latex beads (diameter 1.1 μm) and processed as indicated in fig.2. Lane a, control cytoplasts; lanes b–f, 1×10^{-8}, 3×10^{-8}, 1×10^{-7}, 3×10^{-7}, 1×10^{-6} M PMA; lane g, 3 mg/ml latex beads. Only the relevant portion of the autoradiograph is shown with an arrow pointing to the gel position of the protein with an apparent M_r of 46000.

with regression coefficients (r^2) of 0.87 and 0.92.

Another interesting result is that the combination of ionomycin (1 μM) and OAG (30 μg/ml) appears to provide a parallel enhancement in phosphorylation of the 46-kDa polypeptides (fig.4) and O_2^- generation (1.6 ± 1.6, 6.0 ± 2.1, 9.1 ± 4.6 nmol/min per 5 × 10^6 cytoplasts, with ionomycin, or OAG, or ionomycin + OAG, respectively; means of 3 experiments ± SE). Finally, concanavalin A, which causes aggregation of cytoplasts and secretion of B_{12}BP from intact neutrophils (not shown), causes no activation of O_2^- generation by cytoplasts or intact cells (the absence of response of the O_2 reduction system to the activating effect of concanavalin A is a peculiarity of bovine neutrophils, not found with neutrophils of other animal species [25]) (not shown), and produces only a minimal enhancement in the phosphorylation of the 46-kDa protein (fig.2d), as compared with PMA.

The second approach followed for studying the possible correlation between protein phosphorylation and activation of neutrophils was that of employing the protein kinase inhibitor, TFP [21,22] which fully inhibits the phosphorylation of the 46- and 43-kDa proteins in PMA-stimulated cytoplasts (fig.2c).

Unlabeled neutrophils or cytoplasts were incubated at 37°C for 5 min in the absence or presence of 50 μM TFP at inhibitor/protein ratio comparable to those of fig.2c. Reduction of oxygen to O_2^- by intact cells or cytoplasts was then activated by addition of 20–100 nM PMA, whereas stimulation of secretion of vitamin B_{12}-binding protein from intact neutrophils was induced by either 20–100 nM PMA or 10 μM A23187 or 100 μg/ml concanavalin A. These experiments showed that TFP, which is an efficient inhibitor of endogenous protein phosphorylation, also markedly (86–100%) inhibits O_2^- generation and B_{12}BP secretion.

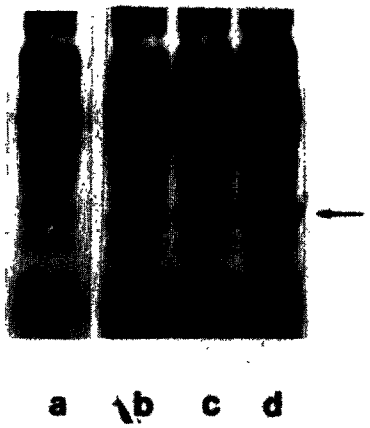

Fig.4. Combined effect of ionomycin and OAG on the extent of phosphorylation of the 46-kDa protein. Cytoplasts were incubated with ionomycin and/or OAG and processed as indicated in fig.2. Lane a, control cytoplasts; lane b, 1 μM ionomycin; lane c, 30 μg/ml OAG; lane d, 1 μM ionomycin + 30 μg/ml OAG. Only the relevant portion of the autoradiograph is shown with an arrow pointing to the protein with an apparent M_r of 46000.

4. DISCUSSION

A brief exposure of ^{32}P-labeled neutrophil cytoplasts to PMA produces increased ^{32}P-labeling of a number of polypeptides. Since the phorbol ester does not increase the cytosolic level of Ca^{2+} [12] or cAMP [23], but is a potent activator of protein kinase C [16], it is very likely that these polypeptides are substrates of this enzyme. Among the ^{32}P-labeled proteins there is one with an apparent M_r of 46000, which becomes heavily phosphorylated also after exposure of cytoplasts to OAG or ionomycin, which both cause activation of protein kinase C in platelets [16,24], as well as to latex beads.

An interesting characteristic of cytoplasts is that they essentially contain only 3 compartments: the plasmalemma, the cytosol and the cytoskeleton, which can be easily separated by use of appropriate detergents. By exploiting this possibility, we have shown that practically all the polypeptides, whose labeling by $^{32}PO_4$ increase in PMA-treated cytoplasts, are released concomitantly with lactate dehydrogenase upon cytoplast exposure to the plasma membrane-permeabilizing agent, digitonin. This strongly indicates that they are cytosolic components.

The complex pattern of ^{32}P-containing bands in autoradiographs of stimulated cytoplasts does not allow an unambiguous and general correlation between activation of protein kinase C and of functions, characteristic for a cell such as the neutrophil. However, a statistically significant cor-

relation is found between the extent of ^{32}P-labeling of one of the protein kinase C substrates, the 46-kDa protein, and the activation of O_2^- generation. Furthermore, TFP inhibits both the phosphorylation of this protein (as well as of other polypeptides in the 40000 range), and the O_2^- production by cytoplasts (or neutrophils) and the secretion of granule content from intact neutrophils.

White et al. [21] have also recently found that TFP inhibits granule enzyme release from rabbit neutrophils as well as the phosphorylation of a cytosolic protein, which on the basis of two-dimensional PAGE has an apparent M_r of 50000.

We have previously seen [17] that in PMA-treated human neutrophils ^{32}P-labeling of a 47-kDa polypeptide follows a kinetic similar to that of activation of neutrophil metabolism and secretion. Furthermore, increased phosphorylation of a cytosolic 47-kDa protein by protein kinase C is characteristic of the process of platelet activation [16,22]. We are thus tempted to speculate that in neutrophils, as in platelets, increased phosphorylation of a cytosolic 46–47 kDa polypeptide might be a necessary event in the process of stimulus–response coupling.

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