SURFACE-REACTIVE STIMULI SELECTIVELY INCREASE PROTEIN PHOSPHORYLATION IN HUMAN NEUTROPHILS

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1. Introduction

Phosphorylation of proteins is an important regulatory mechanism in metabolic pathways [1]. A sudden change in the turnover of some membrane phosphoproteins can be the critical event in the coupling of stimulation by extracellular ligands with appropriate cell response [2]. A coordinated and transient rise in the activity of protein kinase(s) and phosphatase(s) may thus represent one of the key mechanisms of regulation of the functions of cells responsive to external stimuli.

One such cell is the blood neutrophil. In fact, exposure of neutrophils to a variety of particulate as well as surface-reactive soluble stimuli (formyl-peptides, phorbol esters) elicits a complex set of metabolic and functional changes. These include activation of O_2 reduction to O_2^- [3–5], a transient rise in cyclic AMP concentration [6–8] and in protein methylation [9], translocation of calcium pools [10], Mottola and D. R., in preparation), enhanced phospholipid turnover [11,12], and activation of random and oriented locomotion [7–10,13–16] and of secretion [17–19]. Thus, neutrophils present an interesting system with which to investigate protein phosphorylation as an intracellular regulatory mechanism.

We present here evidence that interaction of ^{32}P-labeled human neutrophils with known activators of their functions, such as *N*-formyl-methionyl–leucyl–phenylalanine (FMLP) and phorbol 12-myristate-13-acetate (PMA) [4,5,8,10,13,15,16,19], results in an immediately increased incorporation of ^{32}P into 4 polypeptides with app. M_r 59 000, 47 000, 45 000 and 27 000.

Abbreviations: FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate-13-acetate; PM, phorbol 12-myristate; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

2. Materials and methods

2.1. *Preparation of neutrophil suspensions and ^{32}P labeling*

Neutrophil suspensions containing >98% neutrophils were prepared from citrate-anticoagulated venous blood obtained from healthy adult donors. Standard techniques (dextran sedimentation and Hypaque/Ficoll gradients) were used [20,21], followed by hypotonic lysis of contaminating erythrocytes. The cells were suspended in Krebs–Ringer–Hepes buffer, containing 30 mM Hepes (brought to pH 7.4 with NaOH), 110 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$. One ml neutrophil suspension, containing ~1×10^8 cells, was warmed at 37°C in the presence of 10 mM glucose and 2 mg BSA. Carrier-free $H_3{}^{32}PO_4$ (HCl-free water solution, New England Nuclear MD) (0.5–1 mCi) were then added and the incubation continued for 150 min. Neutrophils were sedimented for 10 min at $100 \times g$, washed once and suspended in warm (37°C) $H_3{}^{32}PO_4$-free fresh medium at 1×10^7 cells/ml.

2.2. *Cell stimulation*

Neutrophils ($3\text{-}5 \times 10^6$) in 0.5 ml Krebs–Ringer–Hepes medium with 10 mM glucose and 2 mg BSA/ml, were exposed to 1×10^{-8}–1×10^{-7} M FMLP or 0.1 μg/ml PMA or 0.1 μg/ml phorbol 12-myristate (PM) for 10–90 s. These reagents (purchased from Sigma MD) were delivered in a few μl of a stock solution in dimethyl sulphoxide, and an identical volume of the solvent was added to the control samples. The reaction was stopped by precipitating the cells with 0.1 ml 36% (w/v) trichloroacetic acid. After standing

in ice for 1 h, the acid-treated neutrophils were collected by centrifugation in an Eppendorf centrifuge model 3200, and solubilized in the 'final sample buffer' [22] (if necessary, 0.1 NaOH was added to restore the pH of the buffer). Proteins of the samples were dissociated by boiling for 1–2 min.

2.3. *SDS–polyacrylamide gel electrophoresis*

Linear gradient slab gels (8–20%) 25 cm long and 1 mm thick, were used [22]. Proteins of known M_r (monomeric M_r in parentheses) were run as standards. These were: myosin heavy chain (220 000); sarcoplasmic reticulum Ca^{2+}-ATPase (100 000); BSA (68 000); aldolase (40 000); and lysozyme (14 700). Solubilized sample protein (~200 μg) was electrophoresed.

After electrophoresis at constant current (25–30 mA), the gels were fixed and stained in 30% (w/v) trichloroacetic acid with 0.15% Coomassie blue, destained in 7.5% acetic acid/5% methanol, and dried under vacuum. Dry slab gels were subjected to autoradiography.

2.4. *Autoradiography*

Screen-type X-ray films (Kodak X-OMAT R) were preflashed as in [23] and enclosed between the dry gel slab and an intensifying screen (Trinax 2, 3M or Quanta III, Dupont De Nemours, USA) in a radiographic cassette. After exposure at −70°C for 12–36 h, the film was developed according to the manufacturer's instructions. As shown [23], the intensity levels of the images obtained on pre-exposed films using a screen at −70°C is proportional to the amount of radioactivity causing the image on the film.

Quantitation of the band intensity on the autoradiographic film was made by using a computerized image-processing system [24]. This system, currently in use at the Institute of Electrical Technology and Electronics of the University of Trieste [25], utilizes a minicomputer (HP 2100 A) with 32 000 memory words. Its input and output peripherals are a TV camera and a cathode ray tube display, respectively. In this system the image intensity is evaluated within 0–63 units, with the upper limit corresponding to the highest level of darkness of the image. Comparison between image intensities of different lanes of the autoradiographic film was made only when a reference peptide band, usually the more radioactive one, showed identical darkness values. This permits one to exclude that a change in band intensity is due to a difference in the amount of electrophoresed material.

3. Results

By incubating the cells at 37°C with $^{32}P_i$ for 2–3 h the phosphate pool of neutrophils is extensively labeled without loss of viability, as indicated by the exclusion of the dye trypan blue. After removal of extracellular $^{32}P_i$ by cell centrifugation, neutrophils were exposed to stimulants to study their effect on cell protein phosphorilation. This study was done by separating the polypeptides by high-resolution SDS slab gel electrophoresis.

Fig.1(a–d) shows the electrophoretic distribution

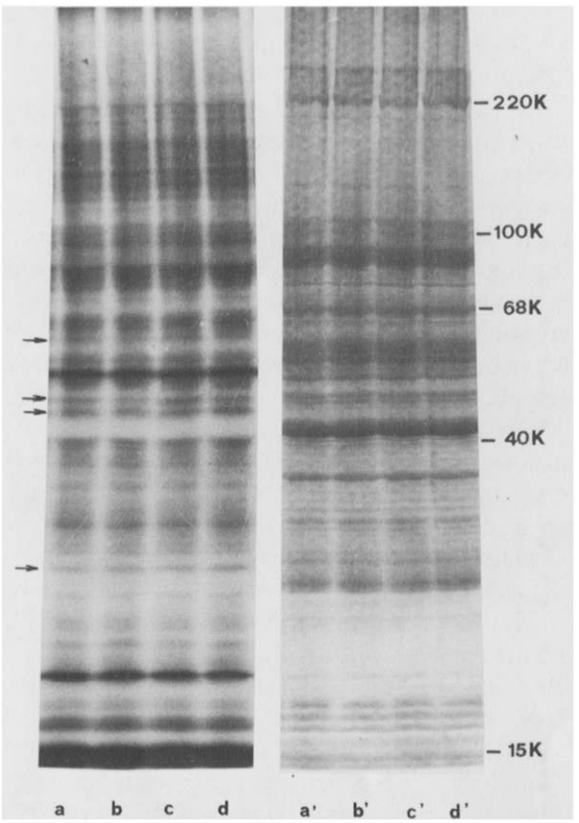

Fig.1. SDS–polyacrylamide gel electrophoresis of ^{32}P-labeled FMLP-treated human neutrophils. Whole ^{32}P-labeled neutrophils were exposed to FMLP (a,a', 1×10^{-8} M FMLP, 20 s; c,c' and d,d', 1×10^{-7} M FMLP, 20 and 60 s, respectively). Untreated cells (b,b') served as control. Cell proteins were acid-precipitated, then solubilized in electrophoresis sample buffer and analyzed in 8–20% gradient acrylamide slab gels, as in section 2. (a–d) Autoradiograph; (a'–d') Coomassie blue stain. Numbers at right indicate M_r of standards listed in section 2. Arrows at left indicate polypeptide bands of stimulated cells which by autoradiography give a more intense image on the film.

of the ^{32}P-labeled proteins of control as well as of FMLP-treated neutrophils, as detected by autoradiography, and fig.1(a′–d′) shows the distribution of total proteins detected by Coomassie blue staining. As expected, the Coomassie blue patterns of resting and stimulated neutrophils are identical. In particular, they show a heavily-stained band of 43 000 app. M_r, which is very likely to be actin, and is not labeled by $^{32}P_i$. However, several other polypeptides take up the label. Of these, 4 can be seen to become increasingly labeled after neutrophil exposure to FMLP for 20–60 s. These peptides have app. M_r 59 000, 47 000, 45 000 and 27 000.

Also when neutrophils are exposed to PMA for very short times, there is no change in the distribution of total proteins (not shown), whereas more label is found associated with the 4 polypeptides described above (fig.2(a–c)). PM, the other phorbol ester used in these studies, is almost inactive in promoting an increased ^{32}P-labeling (fig.2(e,f)).

A quantitative evaluation of the enhanced ^{32}P-labeling by a computerized system of image processing is reported in table 1. Cell stimulation by PMA results in the most marked enhancement in ^{32}P association to the 4 peptides, particularly to that with 47 000 app. M_r. Further, FMLP is more active at 1×10^{-7} M than of 1×10^{-8} M.

It is evident from fig.1,2 that the increased ^{32}P-labeling of the 4 neutrophil proteins is already detectable after a few seconds from the addition of the stimulants to the cells. Also in this case, a more precise quantitative evaluation of the kinetics of ^{32}P-labeling of the peptides can be obtained by the computerized method of image processing. Fig.3 shows the gradual increase of ^{32}P-binding to the 4 peptides, with a peak reached at ~60 s, followed by a decrease of labeling.

Table 1
Increased ^{32}P-labeling of 4 protein subunits of stimulated human neutrophils

	Autoradiographic image intensities of peptide bands ($\times 10^{-3}$)			
	59	47	45	27
PMA (0.1 μg/ml, 60 s)	42	52	43	17
PM (0.1 μg/ml, 60 s)	27	31	33	11
Control cells	28	25	25	11
FMLP (1×10^{-8} M, 20 s)	31	24	26	a
FMLP (1×10^{-7} M, 20 s)	33	30	28	a
Control cells	22	19	19	a

[a] Images too faint to be quantitatively evaluated by the image processing system

After a short exposure of ^{32}P-labeled human neutrophils to surface-reactive agents, cell proteins were acid-precipitated and separated by SDS–PAGE. Autoradiography and evaluation of the image intensities on the films were done as in section 2

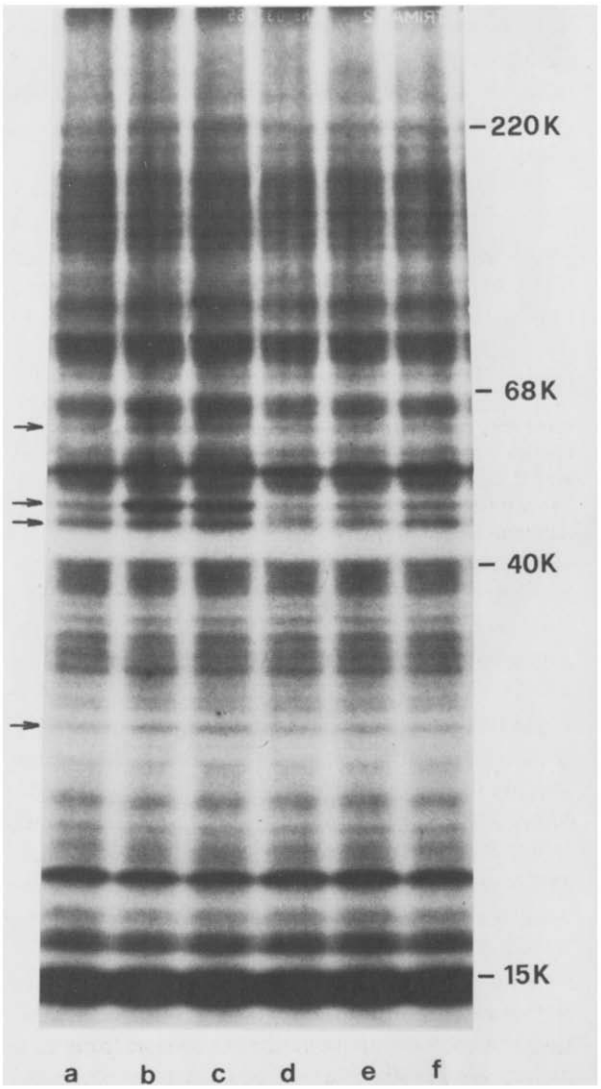

Fig.2. Autoradiography of an SDS–polyacrylamide gel electropherogram of ^{32}P-labeled human neutrophils, treated with either PMA or PM. Whole ^{32}P-labeled neutrophils were exposed to PMA (a,b,c, 30, 60 and 90 s, respectively) or PM (e,f, 30 and 60 s). Untreated cells (d) served as controls. For other details see fig.1.

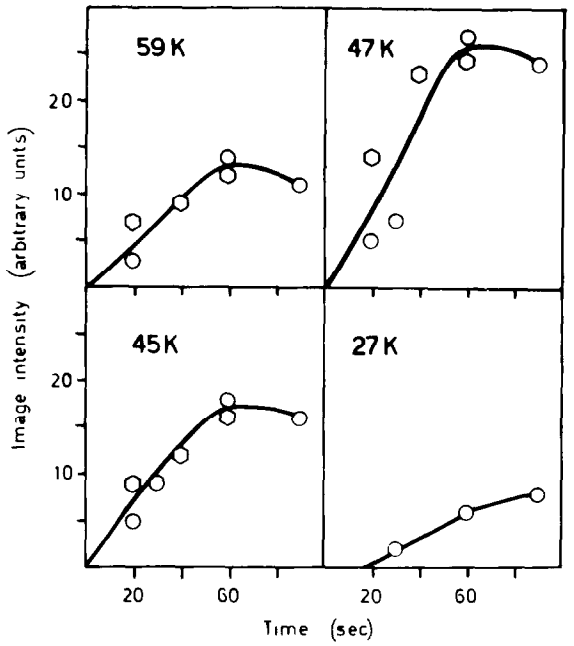

Fig.3. Time course of ^{32}P-labeling of 4 polypeptides of human neutrophils exposed to PMA for different times. The 4 panels show results of 2 expt, in which the image intensities of the peptides with app. M_r 59 000, 47 000, 45 000 and 27 000 were analyzed. Ordinates are increments in autoradiographic image intensities (see section 2) of the peptide bands on the film with respect to the intensities of the same peptides in untreated cells (reference intensities: 59 000, 24–28 units; 47 000, 25–29 units; 45 000, 17–25 units; 27 000, 11 units).

4. Discussion

PMA and FMLP are 2 stimulants of neutrophil functions, such as locomotion, secretion and generation of cytotoxic O_2 derivatives [4,5,8,13,15,16,19], which greatly differ for their chemical structure and possibly also for mode of binding to the cell surface. FMLP is a peptide which binds to specific surface receptors of the human neutrophil with an equilibrium $K_d = 1.2–1.4 \times 10^{-8}$ M [26]. PMA is a lipophilic compound, which might also specifically combine to surface receptors [27,28]. Although these receptors have not yet been characterized in the neutrophil, their interaction with the phorbol esters is likely to occur by very specific recognition mechanisms. In fact, absence of the acetate group in position 13 of PMA (as in PM) results in loss of stimulatory activity of the ligand, as detected by failure of activating O_2 reduction and translocation of Ca^{2+} pools in neutrophils (Mottola and D. R., in preparation).

In spite of the difference in their chemical and probably binding properties, both FMLP and PMA can activate an increased transfer of ^{32}P from neutrophil phosphate pool to 4 proteins with app. M_r 59 000, 47 000, 45 000 and 27 000. Substantial evidence exists that the increased peptide labeling is related to the stimulation of the neutrophil functions:

(i) FMLP is more effective in the stimulation of ^{32}P binding to the 4 proteins at a concentration (1×10^{-7} M) similar to that known to maximally activate the oxidative metabolism of human neutrophils [4] and to induce the highest transient elevation in intracellular levels of cyclic AMP [8];

(ii) PM, an inactive analogue of PMA, does not enhance the peptide phosphorylation;

(iii) The PMA-induced increase in ^{32}P-labeling of the 4 proteins peaks at ~60 s, just about the time when increased production of O_2^- by neutrophils is detected [29].

Increased phosphorylation of proteins requires the activation of a kinase. Neutrophils have been shown to have both cyclic AMP-dependent and cyclic AMP-independent kinases [6,30]. Furthermore, a few seconds after the exposure to stimulants the concentration of cyclic AMP in the neutrophil cytosol increases [6–8] and Ca^{2+} are mobilized from intracellular stores (Mottola and D. R., in preparation). Thus, either a cyclic AMP-dependent or a Ca^{2+}-dependent protein kinase could be responsible for the increased phosphorylation of proteins in stimulated neutrophils.

We are attempting to clarify this point. Furthermore, experiments are in progress concerning the intracellular localization of the protein substrates of the kinase(s).

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