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CO-ACTIVATION OF PROTEIN KINASE C AND NADPH OXIDASE IN THE PLASMA MEMBRANE OF NEUTROPHIL CYTOPLASTS

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Enucleated, granule-free neutrophil cytoplasts, which in hypotonic media fully release cytosolic components and generate ghosts, have been used to study the cell localization of protein kinase C (PK-C). Treatment of cytoplasts with phorbol myristate acetate, a potent activator of neutrophil functions, triggers translocation of PK-C from the cytosol to the plasma membrane, with an activity recovery of 83 ± 16%. In the ghost fraction, PK-C catalyzes the phosphorylation of polypeptides with an apparent mol. wt. of 115K, 89K, 79K, 62K, 47K and 19K. From the plasma membrane PK-C can be extracted in an active form by Triton X-100 but not by EGTA. Translocation of PK-C is already evident at 5 sec and plateaus at about 50 sec. Activation of plasmalemmal, O_2^- generating NADPH oxidase by the phorbol ester is delayed by about 20 sec with respect to the activation of PK-C. Dose/response experiments show that the pattern of activation of O_2^- generation by cytoplasts strictly superimposes with the pattern of PK-C translocation.

The involvement of protein kinase C (PK-C) in neutrophil activation is strongly suggested by the stimulatory activity (1-3) of 1-oleyl-2-acetyl-glycerol and of PMA, which are known to substitute for unsaturated diacylglycerol, the PK-C physiological activator (4-6), as well as by the strict correlation between increased phosphorylation of some PK-C protein substrates and stimulation of secretion of granule content (7) and of generation of O_2^- (3,8). However, the mechanism of intracellular activation of the PS-requiring PK-C, and its topological relation with the plasmalemmal NADPH oxidase (9,10), which upon stimulation generates O_2^-, has not been yet clearly established. Here we show that

Abbreviations: PMA, phorbol 12-myristate, 13-acetate; PS, phosphatidyl serine; LDH, lactate dehydrogenase; PBS, phosphate buffered saline.

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PMA triggers activation of cytosolic PK-C by inducing its translocation to the plasma membrane, with a $t_{1/2}$ of a few sec and dose/response effects identical to those required for the activation of the NADPH oxidase. Identification of the plasma membrane as the recipient membrane was made possible by the use of enucleated, granule free neutrophil cytoplasts (3,11,12), virtually devoid of internal membranes.

MATERIALS AND METHODS

Neutrophil cytoplasts. Cytoplasts were prepared from neutrophils of bovine blood by treatment of intact cells with cytochalasin B, followed by centrifugation through a Ficoll gradient (11,12). To determine the distribution of enzymes between the cytosol and the plasma membrane, control and PMA-treated cytoplasts were lysed by incubation at 0°C for 20 min in 5 mM Na-phosphate, pH 8, with 50 μg/ml leupeptin and 0.5 mM phenyl methyl sulfonyl fluoride, under mild stirring. A ghost fraction was then separated from the released cytosolic protein by centrifugation at 4°C and 100,000g for 20 min. When required the pellet was solubilized in lysis buffer with 0.25% Triton X-100, followed by a brief sonication.

Bioassays and other analytical techniques. To study the phosphorylation of ghost proteins catalyzed by membrane-associated kinase(s), cytoplast ghosts (7 μg protein) were incubated at 30°C for 5 min in a reaction medium (67 μl) containing 2 μmol Tris/HCl at pH 7.5, 0.5 μmol of Mg acetate, 5 μmol of EGTA and 1.2 nmol of [γ-^{32}P]ATP (8.5 x 10^6 cpm) (Amersham, U.K.). The reaction was stopped with 33 μl of sample buffer of SDS-PAGE (3), which was then conducted on a 8-18% linear gradient slab. After fixation, staining with Coomassie blue, destaining and drying (3), the dry gels were autoradiographed using Cronex 7 X-ray film and Lightning plus intensifying screens (Du Pont de Nemours, Wilmington, DE).

Incorporation of ^{32}P into H1 histone from [γ-^{32}P]ATP, catalyzed by PK-C, was assayed at 30°C in a reaction mixture (0.25 ml) containing 5 μmol Tris/HCl at pH 7.5, 50 μg of H1 histone, 1.25 μmol of Mg acetate 2.5 μg of leupeptin, 3.3 nmol of [γ-^{32}P]ATP (3-6x10^5 cpm) and either 5 mM EGTA (ghost fraction, 5-20 μg of protein, final concentration of Triton X-100 lower than 0.04%) or 5 mM EGTA, 40 μg/ml of sonicated PS and 20 nM PMA (cytosol, 5-20 μg of protein). The kinase reaction was stopped after 5 min by the addition of 25% trichloroacetic acid; acid precipitable materials were collected on Millipore membrane filters (pore size, 0.45 μm) and the radioactivity of ^{32}P was measured by liquid scintillation.

Activities of Ca^{2+}/calmodulin-and of cAMP-dependent protein kinases were measured as above, with 0.5 mM Ca^{2+} (no EGTA) or 10 μM cAMP replacing PS + PMA. Reduction of ferricytochrome c by O_2^-, produced by 0.6-1x10^7 PMA-treated cytoplasts, the activities of LDH, alkaline phosphatase and myeloperoxidase, vitamin B_{12}-binding capacity, DNA and protein were determined as previously reported (3,12).

RESULTS

Cytoplasts of bovine neutrophils contain about one-half of the cytosol and about 40% of the plasma membrane, according to recovery of LDH and of the plasmalemmal marker alkaline phosphatase, and are contamined by about 1% of nuclei (DNA) and 1.5-2.5% of granules (vitamin B_{12} binding protein and myeloperoxidase)(3).When incubated at 0°C under hypotonic conditions, they exhibit a lower osmotic resistance than intact neutrophils (11), and release > 97% of cytosolic LDH, thereby generating ghosts which fully retain the plasmalemmal marker alkaline phosphatase (data not shown).

Release of LDH in the hypotonic medium is accompanied by release of PK-C activity, measured by the incorporation of ^{32}P into HI histone from [$\gamma^{32}P$]ATP in the presence of PS and PMA (or Ca^{2+} and diolein), thereby indicating that in resting neutrophil cytoplasts PK-C is a cytosolic enzyme. Other protein kinases, such as those activated by Ca^{2+}/calmodulin or by cAMP, are also fully released by hypotonic lysis of cytoplasts.

Treatment of cytoplasts with PMA induces an activation of PK-C by promoting its rapid association to the plasma membrane, as monitored by an increased phosphorylation of ghost proteins in the presence of EGTA. In fact, in ghosts derived from PMA-treated cytoplasts and incubated with [γ-^{32}P]ATP there is a significant enhancement of phosphorylation of polypeptides with an apparent mol. wt. of 115K, 89K, 79K, 62K, 47K and 19K (Fig.1).

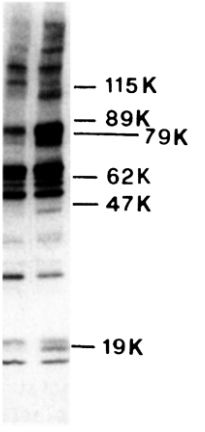

Fig. 1: Increased phosphorylation of endogenous proteins by PK-C in ghosts derived from PMA-treated cytoplasts. Ghosts were prepared by hypotonic lysis of control cytoplasts (a) or cytoplasts treated at 37°C for 5 min with 100 nM PMA (b).

The membrane-associated PK-C can be solubilized in an active form by 0.25% Triton X-100 (but not by Ca^{2+}-chelating agents such as EGTA). The availability of a soluble form of active PK-C, catalyzing the phosphorylation of H1 histone, permits a precise determination of the kinetics and a quantitation of the translocation process. To follow the time course of the PMA-induced translocation of PK-C two methods were applied. In the first one, samples of cytoplasts exposed to PMA for 5-300 sec were rapidly diluted into ice-cold PBS (12) and pelleted by centrifugation. After dispersion of the pellets in lysis medium with 0.25% Triton X-100 and 0.5 mM EGTA, the solubilized PK-C was assayed without addition of PS (Fig. 2a). Under these conditions, the measured PK-C activity virtually corresponds to that of the activated, membrane-associated PK-C. Alternatively, after treatment with PMA for 5-300 sec, cytoplasts were diluted in ice-cold PBS, sedimented and lysed in hypotonic medium. The generated ghosts were dispersed in lysis medium with 0.25% Triton X-100, and then assayed for PK-C activity in the presence of 0.5 mM EGTA (Fig. 2b). In both cases translocation of PK-C is already evident at 5-10 sec from addition of PMA to the cytoplasts, and reaches a maximum at about 50 sec. The actual rate of enzyme translocation is likely to be faster than shown in Fig. 2, since one has also to consider the time required for PMA interaction with the plasma membrane.

Activation of PK-C by association to the plasma membrane clearly precedes the activation of plasmalemmal NADPH oxidase. In fact, in pa-

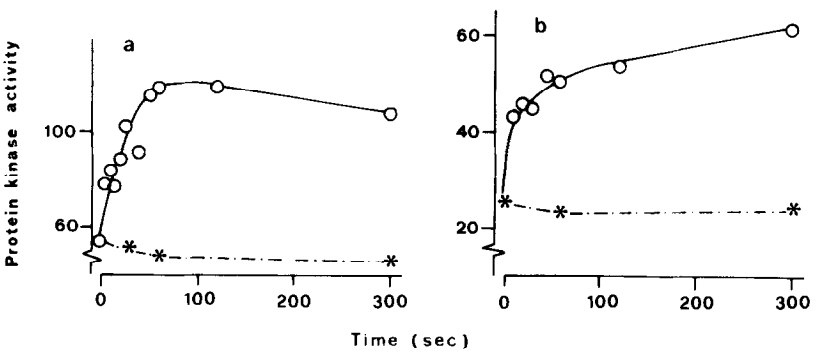

Fig. 2 Time course of increased association of PK-C to the plasma membrane of neutrophil cytoplasts. Cytoplasts ($6x10^7$/ml) were incubated at 37°C in the absence (*) or in the presence (o) of 100 nM PMA. PK-C activities were measured in whole cytoplasts (a) or in cytoplast ghosts (b). The kinase activity is expressed as pmol Pi/min/$1x10^7$ cytoplasts (a) or pmoles Pi/min/ghosts derived from $1x10^7$ cytoplasts (b). Activities at time = 0 are basal kinase activities.

rallel experiments, PMA causes a detectable activation of the O_2^--generating system of cytoplasts at 27 ± 5 sec, with maximal rate of O_2^- production being attained at 61 ± 5 sec (means of 4 experiments ± S.E.M.).

The correlation between activation of cytosolic PK-C by binding to the plasma membrane and activation of the plasmalemmal NADPH oxidase was also studied by comparing dose/response curves of the process of PK-C translocation with the stimulation of O_2^- production by cytoplasts. When cytoplasts are treated with increasing concentrations of PMA and then subjected to hypotonic lysis,the amount of PK-C released gradually decreases (Fig. 3). Concomitantly PK-C becomes increasingly associated with the ghost fraction. Quantitation of PK-C translocation at various PMA concentrations indicates that 83 ± 16% (mean of eleven values ± S.E.M.) of the PK-C activity lost from the cytosol can be accounted for by its association with the plasma membrane.

Concurrently with the increased association of PK-C to the plasma membrane, there is a gradually increasing activation of NADPH oxidase. In fact, in four different experiments (Fig. 3), the pattern of PMA-induced stimulation of O_2^- generation strictly superimposes with the pattern of PK-C translocation.

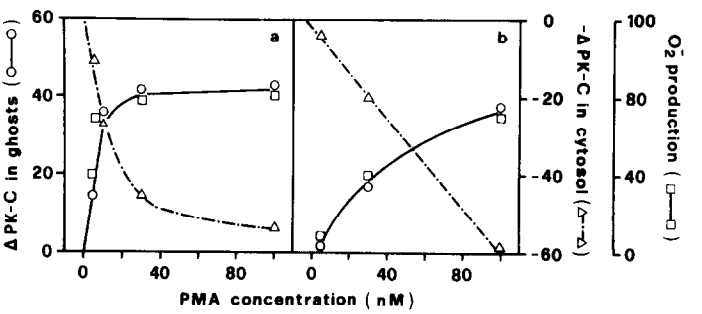

Fig. 3 Dose/response curves of PMA effects on association of PK-C to and production of O_2^- by the plasma membrane of neutrophil cytoplasts. Four different batches of cytoplasts were incubated at 37°C for 5 min in the absence or the presence of PMA, collected by centrifugation (10,000g, 30 sec) and lysed hypotonically. The ghost fraction was separated from the released cytosolic protein by centrifugation, and solubilized in lysis medium with 0.25% Triton X-100. PK-C activities in cytosol were calculated by deduction of basal kinase activities measured in the presence of only EGTA. Each graph (a and b) represents average results of experiments carried out with two cytoplast preparations, showing a similar pattern of response to PMA. PK-C activities are decrements or increments with respect to activities measured in untreated samples (cytosol: 57.6 and 103.6; ghosts: 3.0 and 10.2, in a and b, respectively) and are expressed as pmoles Pi/min/cytosol or ghosts derived from $1x10^7$ cytoplasts. The O_2^- production is expressed as nmol O_2^-/min/$1x10^7$ cytoplasts.

DISCUSSION

Activation of neutrophil O_2^- production, as well as of secretion of granule content, has long been considered to be associated and possibly preceded by an increase in cytosolic Ca^{2+} concentration (1,12). However, with some agents, such as PMA, activation of neutrophil functions occurs without a Ca^{2+} rise (12) and can even be induced at 10^{-8}M cytosolic Ca^{2+} (13), thereby suggesting that other mechanisms may trigger the response of neutrophils to surface stimulation. The data here reported indicate that a rapid translocation (or tighter association) of cytosolic PK-C to the plasma membrane, followed by its activation, may provide one of such mechanisms. This conclusion is consistent with the observation that activation of O_2^- generation and of secretion by neutrophils is causally related to the phosphorylation of specific cytosolic proteins by PK-C (3,7,8).

Triggering by PMA of a marked decrease in PK-C activity in the cytosol has been shown in various cell types to be accompanied by a partial recovery of the enzyme in a "particulate" fraction, sedimented at 100,000g (14-16). Association of PK-C to this fraction, which collects all the cell organelles and cell membrane-derived vesicles, does not provide a precise indication on the tropism of PK-C translocation. In fact, redistribution of PMA during cell homogenization might cause unspecific binding of PK-C to various cell organelles. To overcome this difficulty, we have used enucleated, granule-free cytoplasts of neutrophils (3,11,12). Cytoplasts respond to a PMA treatment with a rapid activation of the plasmalemmal, O_2^--generating NADPH oxidase, thereby permitting the precise determination of the correlation between translocation/activation of PK-C and activation of the NADPH oxidase.

The mechanism by which the PK-C translocation and activation occurs is still obscure. Phorbol esters are thought to exert their effects while retained at the cell surface (17). In particular, the phorbol moiety of PMA and the upper part of myristate, close to the ester bond, appear to reside in the hydrophilic surface region of the plasma membrane, while only the tail of the acyl chain dips into the lipid bilayer (18). Insertion of an increasing number of PMA molecules into the plasma

membrane might thus cause a gradually increasing perturbation of its structure, as revealed by displacement of Ca^{2+} from membrane binding sites (1), thereby leading to exposure of phospholipids and "recruitment" of PK-C from the cytosol by PS (19). On the other hand, PMA is able to replace in vitro the PK-C requirement for unsaturated diacylglycerol (5), which is known to dramatically increase the enzyme affinity for PS (4). It would thus be possible that in resting cytoplasts PK-C is loosely bound to the plasma membrane and becomes "anchored" to it after PMA insertion into the membrane.

An alternative mechanism would be that in PMA-treated cytoplasts PK-C is activated in an irreversible manner by limited proteolysis catalyzed by a Ca^{2+}-dependent protease, similar to that exsisting in brain (6,20). This enzyme, which preferentially reacts with membrane-associated protein kinase, has an apparent Ka value for Ca^{2+} of about 20 μM and is only slightly active at 1 μM Ca^{2+} (20). However, irreversible activation of PK-C by this mechanism seems to be unlikely, because a) in neutrophil cytoplasts PMA does not increase the cytosolic Ca^{2+} beyond its basal value of about 70 nM (12) and b) the catalytically active PK-C fragment, derived from proteolysis, is unable to bind either phospholipid or membrane (20) and should thus be found in the extruded cytosol and not in the ghost fraction.

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