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Immunocytochemical localization of protein kinase C in resting and activated human neutrophils

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An immunocytochemical method was used to determine possible changes in the subcellular distribution of protein kinase C (PKC) in human neutrophils in response to opsonized latex beads and zymosan. While in resting cells most of the PKC immunoreactivity was localized in the cytoplasm, a redistribution of PKC to the plasma and phagosomal membranes was observed in cells treated with latex beads or zymosan for 5-20 min, suggesting a participation of PKC in endocytosis.

Protein kinase C; Neutrophil; (Human)

1. INTRODUCTION

The chemotactic peptide FMLP and opsonized latex beads are representative stimuli of the receptor-mediated and phagocytic routes of neutrophil activation. Both FMLP and opsonized particles provoke a variety of responses, including the activation of the plasma membrane-bound NADPH oxidase (superoxide generation) and release of enzymes from cytoplasmic granules [1–7]; both processes participate in the bactericidal function of neutrophils.

Another potent activator of some of these functions is the tumor promoter TPA, which effectively substitutes for endogenous diacylglycerol to activate PKC [8–11]. As assayed by subcellular fractionation techniques, it has been shown that TPA induces a stable interaction of PKC with neutrophil membranes [12–15]; the activated enzyme appears to mediate the TPA actions [14–16]. On the other hand, FMLP promotes the interaction of only a tiny portion of total PKC activity with the membrane fraction [17] and with opsonized latex beads we were also unable to show significant membrane association of the enzyme (unpublished). Unfortunately, unless there is a strong binding of PKC to membranes, such as promoted by TPA, all techniques which require cell disruption and lengthy fractionation procedures may lead to artefactual redistribution of PKC among the isolated subcellular fractions. To circumvent this problem, in the present study we used a previously well-characterized [18–20] immunocytochemical method to determine changes in the subcellular distribution of PKC in response to opsonized latex beads and zymosan. We found that phagocytic stimuli caused translocations of PKC to the plasma and phagosomal membrane regions.

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Abbreviations: FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C

2. MATERIALS AND METHODS

2.1. *Materials*

Rhodamine-conjugated latex beads (2.7 μm average diameter) were purchased from Polyscience; cytochalasin B, zymosan, FMLP, and

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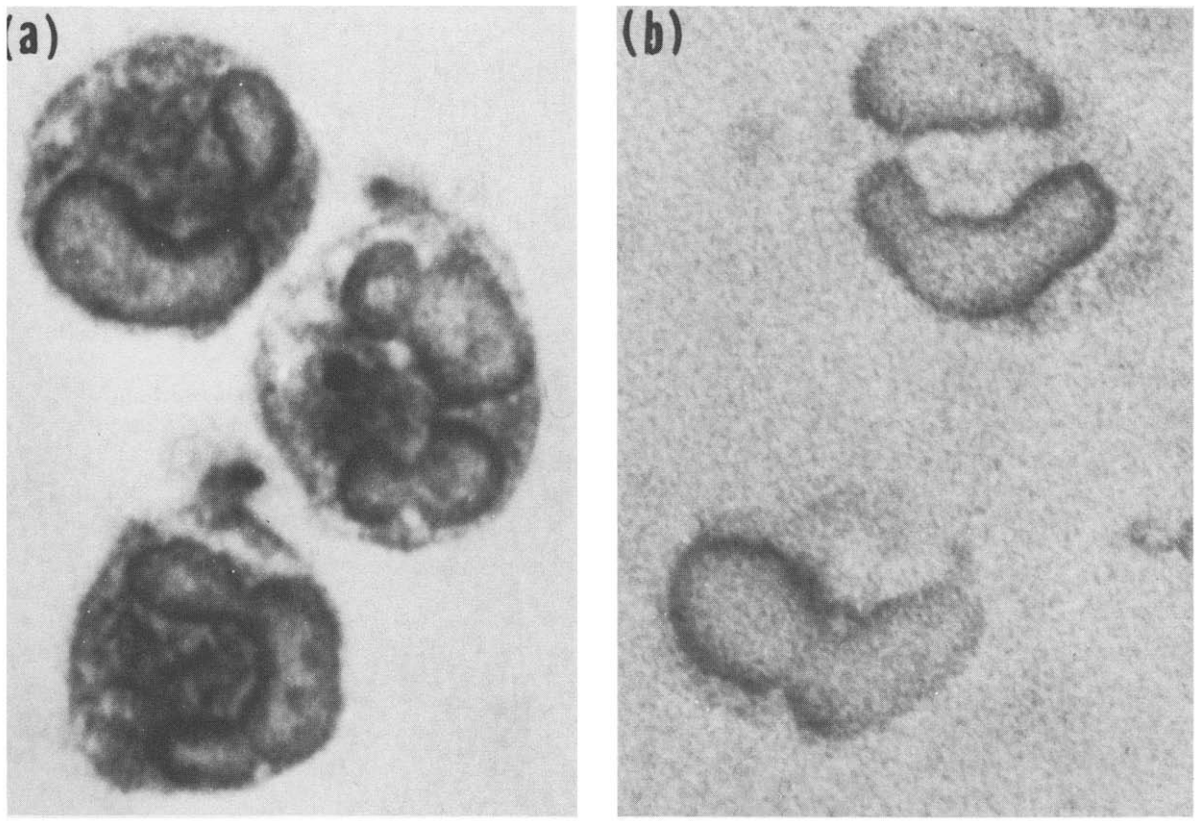

Fig.1. Immunocytochemical localization of PKC in human neutrophils. Polyclonal antisera raised against PKC in rabbit (a) or preimmune serum (b) were used to localize PKC in human neutrophils as described in section 2.

leupeptin from Sigma; and hespan (6% hetastarch in 0.9% NaCl) from American Critical Care Division of American Hospital Supply Corp. Zymosan and latex beads were opsonized as described [21].

2.2. *Methods*

2.2.1. Isolation of human neutrophils

Human neutrophils were obtained by continuous flow leukapheresis from normal adults. Residual erythrocytes were removed by hypotonic lysis with a resulting purity of >95% neutrophils [22]. Alternatively, peripheral blood was obtained by phlebotomy and neutrophils were isolated by sedimentation of erythrocytes through hespan followed by centrifugation through lymphocyte separation medium (9.4% sodium diatrizoate, 6.2% Ficoll), and finally by hypotonic lysis of residual erythrocytes [23]. Isolated cells were resuspended in phosphate-buffered saline-glucose solution, consisting of 0.6 mM $CaCl_2$, 2.6 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 136 mM NaCl, 8 mM Na_2HPO_4 and 5.5 mM glucose.

2.2.2. Immunocytochemical localization of protein kinase C

Polyclonal antisera against protein kinase C were raised in rabbits [18]. Neutrophils were kept in petri dishes (9 cm diameter) and diluted to 2×10^5/ml with 50 mM phosphate buffer, pH 7.5; cover glasses were placed into the dishes and neutrophils were allowed to spread on glasses for 30 min in an incubator. Cover glasses were then transferred into another dish containing 50 mM phosphate buffer, pH 7.5, and latex beads (5×10^6/ml). After incubation for various lengths of time (up to 20 min), cells were fixed (30 min) with 4% (w/v) paraformaldehyde and 0.1%

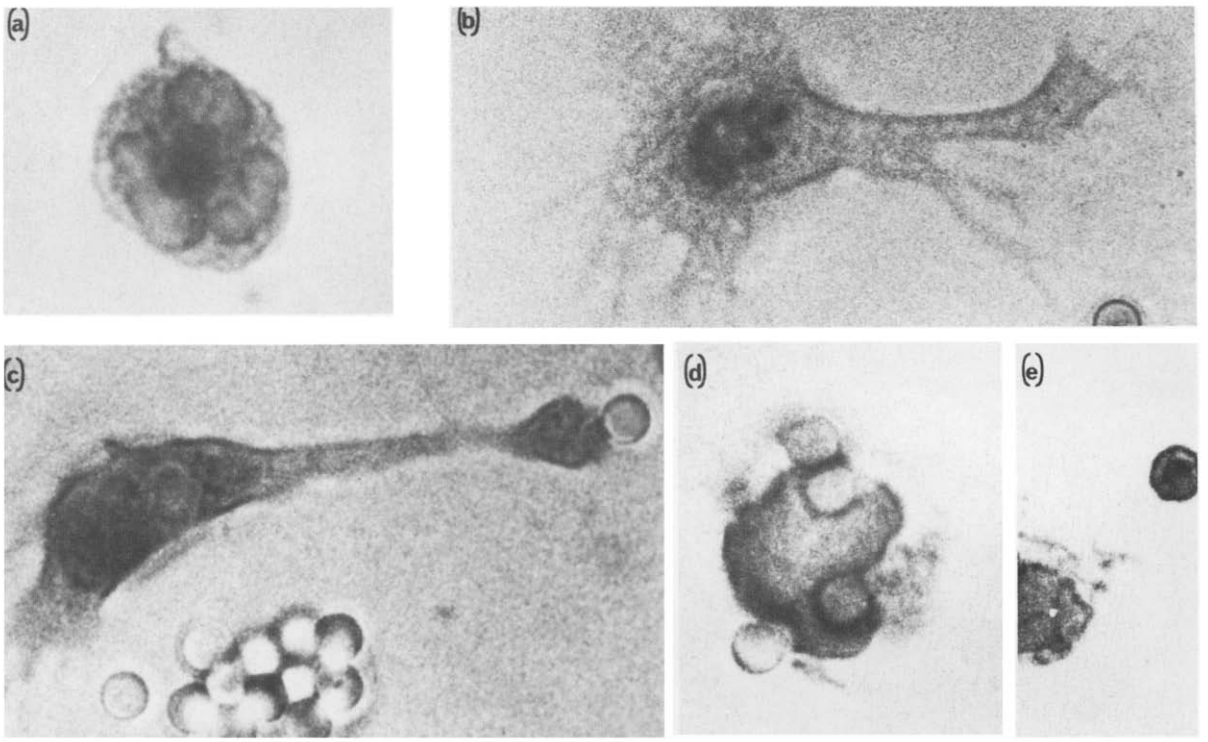

Fig.2. Latex bead-induced change in the subcellular localization of PKC. The immunostaining procedure was performed on untreated human neutrophils (a) or on cells incubated with latex beads (5×10^6/ml) for 5 min (b) or 20 min (c,d) as described in section 2. A single phagocytic vacuole situated in the close vicinity of a broken cell is also shown (e); this preparation was fixed after incubation of cells with latex beads for 20 min.

glutaraldehyde in 50 mM Tris-HCl, pH 7.5, and washed (7.5 min) with 0.2% (w/v) Triton X-100. In other experiments cells (2×10^5 ml) were incubated with zymosan (0.2 mg/ml) in an incubator for 5 or 20 min, followed by pelleting on a slide using a cytospin (3 min) and fixation as before. The immunostaining procedures were essentially the same as described by Shoji et al. [19] for the promyelocytic HL60 cells. Briefly, cells were successively incubated with 1% horse serum (in 50 mM Tris-HCl, pH 7.5) and 1% goat serum in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, and 0.9% NaCl) for 20 min each. Incubations with 1:500 dilutions of primary antisera were performed for 2 h at room temperature; this step was followed by washing with Tris-buffered saline 3 times and incubation with 1% horse serum for 10 min. Further steps were carried out according to instructions in the Vectastain biotin-avidin-peroxidase kit (Vector Laboratories, Burlingame, CA). The last wash step was performed at 4°C and the peroxidase was developed for 7–10 min with diaminobenzidine (25 mg) and 8% hydrogen peroxide (10 μl) in 50 ml ice-cold Tris-buffered saline. The specificity of antisera was previously verified using monospecific antibodies and various immunocytochemical controls [18].

Photographs were taken with Kodak Tri-X pan films processed to ASA 400, using Leitz Vario Orthomat photomicroscope with (fig.2) or without phase contrast (figs 1,3).

3. RESULTS AND DISCUSSION

3.1. *Immunostaining in control cells*

Antisera raised against pig brain PKC in rabbit and used in previous studies [18–20] were employed for immunocytochemical localization of

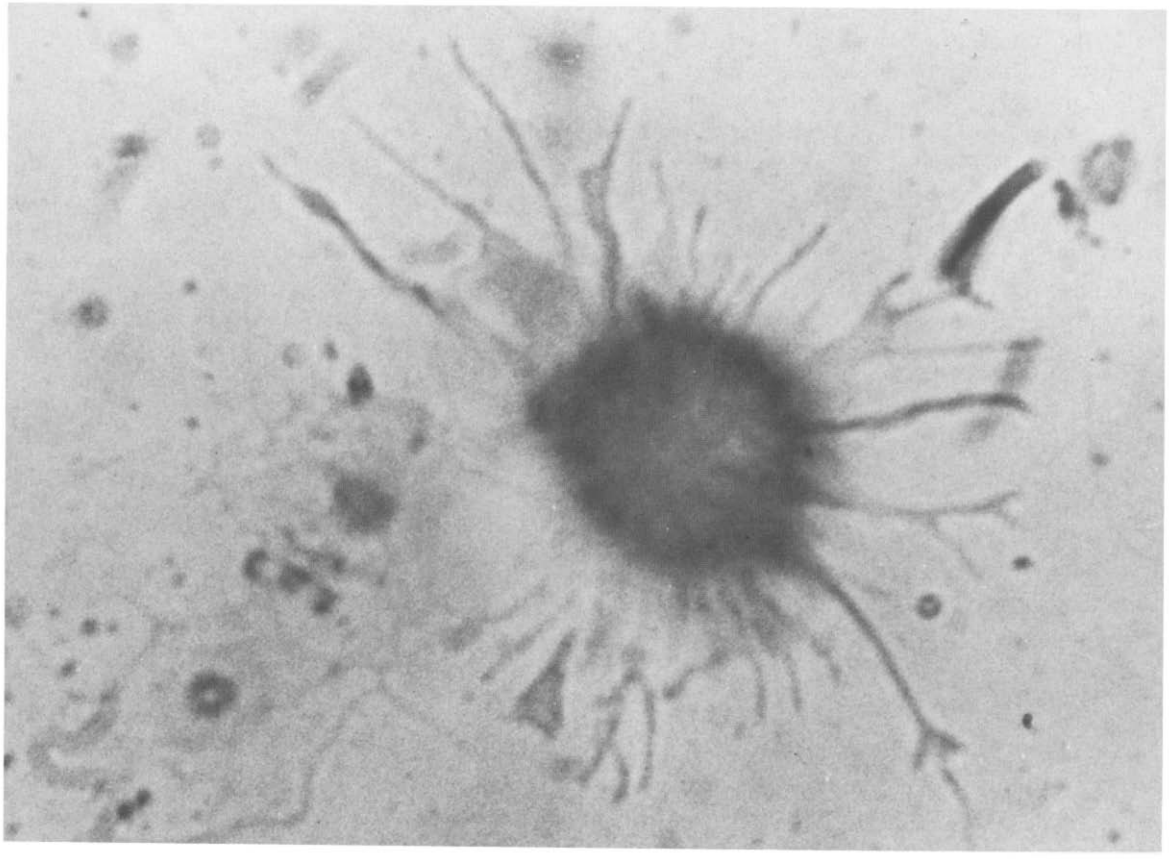

Fig.3. Zymosan-induced change in the subcellular localization of PKC. Human neutrophils were stained after incubation with zymosan (0.2 mg/ml) for 5 min. See section 2 for details.

the enzyme in human neutrophils (fig.1a). Compared to the staining pattern obtained with preimmune serum (fig.1b), it is clear that PKC was mainly localized in the cytoplasm. With preimmune serum we observed some staining of the nuclear membrane, but not the cytoplasmic compartment or plasma membrane region. As can be seen from the subsequent experiments, phagocytosis was associated with PKC translocation from the cytoplasmic compartment to plasma and phagosomal membranes and staining of nuclear membrane remained unaltered. Therefore, we did not investigate further the nature of the staining observed with preimmune serum. The plasma membrane region did not appear to contain appreciable PKC staining in the resting neutrophil. These data are consistent with cell fractionation studies [12–15] showing that PKC is primarily in the soluble fraction in unstimulated cells.

Phagocytosis of opsonized particles resembles the physiologic function of bacterial ingestion by neutrophils. Addition of opsonized latex beads to resting (fig.2a) neutrophils resulted in rapid formation of pseudopods. Fig.2b shows a cell developing numerous pseudopods after a 5 min treatment. These structures did not appear to contain significant immunostains at this stage; instead, PKC remained concentrated in the cytosol. After a 20 min incubation, cells either began to establish contacts with the particles (fig.2c) or were in an advanced stage of ingesting them (fig.2d). In the former case (fig.2c), immunoreactive material was present in both the cell membrane and cytosol, as well as in the terminal portion of the pseudopod membrane. In the latter case (fig.2d), immunoreactivity accumulated in the plasma membrane, with the

heaviest staining around the phagocytic vesicles. Some of the cells did not remain intact during the staining procedures; accordingly we often observed single phagocytic vacuoles, always in close proximity to broken cells coated with heavy immunostain (fig.2e). When cells were stimulated with zymosan particles for 5 min (fig.3), most immunostain was localized near the plasma membrane with little staining in the pseudopodia. After 20 min (not shown), we could not detect zymosan-containing cells, possibly due to the extreme fragility of such cells: due to the giant size of these particles.

In summary, using immunocytochemical techniques on intact neutrophils stimulated by opsonized particles, we were able to demonstrate distinct, time-dependent translocation of cytoplasmic PKC to the plasma membrane, the terminal portion of pseudopod and the surface of ingested particles during phagocytosis. It is not clear at present what triggers the observed redistribution of PKC. In neutrophils, both diacylglycerol and arachidonic acid stimulate translocation of PKC to plasma membrane [24]. It is likely, therefore, that opsonized particles exerted their effects by activating either phospholipase C or phospholipase A_2. We are presently investigating these possibilities as well as the activation state of PKC during phagocytosis.

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