Influence of Calcium on the Subcellular Distribution of Protein Kinase C in Human Neutrophils

EXTRACTION CONDITIONS DETERMINE PARTITIONING OF HISTONE-PHOSPHORYLATING ACTIVITY AND IMMUNOREACTIVITY BETWEEN CYTOSOL AND PARTICULATE FRACTIONS


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Activation of the neutrophil respiratory burst is thought to involve a translocation and activation of protein kinase C. We report that the presence of Ca\(^{2+}\) during the disruption of unstimulated human neutrophils and cytoplasts resulted in an increase in protein kinase C activity (histone phosphorylation) and immunoreactive protein kinase C species in the particulate (membrane) fraction and a reduction in such activities in the cytosol. This Ca\(^{2+}\)-induced translocation of activity was concentration-dependent and occurred at physiologically relevant concentrations of Ca\(^{2+}\) (30–500 nM). The Ca\(^{2+}\)-induced membrane association of protein kinase C could be reversed by removal of Ca\(^{2+}\). These findings indicate that the Ca\(^{2+}\) concentration of the extraction buffer can determine the subcellular distribution of protein kinase C in disrupted cells and suggest that the observed location of this enzyme activity in cell fractions may not necessarily reflect the localization in intact cells. These results also raise the possibility that the distribution of protein kinase C between cytosol and membrane is a dynamic equilibrium controlled by levels of free Ca\(^{2+}\). Thus, Ca\(^{2+}\) might regulate distribution as well as activation of protein kinase C.

Protein kinase C, a ubiquitous enzyme requiring phosphatidylserine (PS), Ca\(^{2+}\), and diacylglycerol (DG) for maximal activity, appears to play an integral role in signal transduction (1, 2). A function for protein kinase C has been proposed in the activation and/or regulation of the neutrophil respiratory burst (3–6). The mechanism of involvement of protein kinase C in neutrophil activation is not fully understood but is thought to involve the redistribution of protein kinase C from a cytosolic location in resting cells to a membrane-associated site during stimulation (3, 7). Stimulation of the neutrophil is also associated with a rise in intracellular Ca\(^{2+}\) (8, 9). Ca\(^{2+}\) and protein kinase C are believed to act synergistically in activating the neutrophil respiratory burst, but the mechanism underlying this interaction has not been defined.

We report here studies of the role of Ca\(^{2+}\) in the translocation of protein kinase C in the human neutrophil. Physiologic levels of calcium were found to induce a reversible translocation of protein kinase C activity from the cytosol to the particulate fraction. This redistribution could be demonstrated both as translocation of PS/DG/Ca\(^{2+}\)-dependent histone-phosphorylating activity as well as translocation of a polyepitope of 80 kDa that was immunoreactive to an antibody raised to a consensus sequence of protein kinase C (10).

MATERIALS AND METHODS

Reagents—All chemicals and supplies were purchased and prepared as indicated or as described previously (9). Extraction buffer A (Ca\(^{2+}\)-depleted) consisted of a 50 mM Tris-HCl buffer (pH 7.5, 25 °C) containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 50 µg/ml leupeptin (Sigma), 2 mM EGTA (Sigma), and 50 mM 2-mercaptoethanol (Bio-Rad) and was prepared fresh each day. Extraction buffer B (Ca\(^{2+}\) present) consisted of a 50 mM Tris-HCl buffer (pH 7.5, 25 °C) containing 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml leupeptin, and 50 mM 2-mercaptoethanol and contained approximately 500 nM Ca\(^{2+}\) (measured with fura-2 free acid (Molecular Probes, Inc., Junction City, OR) (11)). For experiments in which precise levels of Ca\(^{2+}\) were required, extraction buffer C was prepared using Ca\(^{2+}\) buffers to replace the EGTA in buffer A. The Ca\(^{2+}\) buffers were prepared using stock solutions of 50 mM EGTA (pH 7.5, 25 °C) and of 50 mM EGTA with 50 mM CaCl\(_2\) (pH 7.5, 25 °C) diluted in extraction buffer to a final EGTA concentration of 10 mM (11). Final Ca\(^{2+}\) concentrations were calculated by iterative calculation of the equilibrium for H\(^{+}\), Mg\(^{2+}\), and Ca\(^{2+}\) using the dissociation constants given in Martell and Smith (12). Bovine serum albumin and 3,3’-diaminobenzidine were purchased from Sigma. Horseradish peroxidase conjugate and goat anti-rabbit IgG were purchased from Cappel.

Cell Preparation—Human neutrophils were prepared using a discontinuous plasma-Percoll gradient (13). Lipopolysaccharide-free (<0.1 ng/ml by amoebocyte lysate assay (Sigma)) (13) reagents and plasticware and sterile technique were used throughout. Cells prepared using this method were >95% neutrophils with 1–2% erythrocytes, 0–5% eosinophils, and <0.5% mononuclear cells (13).

Cytoplast Preparation—Cytoplasts were prepared from human neutrophils using discontinuous Ficoll gradients as described by Roos et al. (14).

Preparation of Subcellular Fractions—Cells were resuspended to a concentration of 5 × 10\(^{6}\)/ml in the appropriate ice-cold extraction buffer and immediately disrupted (>99% disruption) by sonication for 5–10 s with an Ultrasonic sonicator using a stepped microtip (whole sonicate fraction). The sonicate was centrifuged at 10,000 × g (60 min, 4 °C). The supernate (cytosolic fraction) was collected, and the pellet was resuspended by sonication (10 s) to the original volume in extraction buffer (particulate fraction). Fractions were stored at 4 °C and assayed within 48 h.

Protein Kinase C Assay—Protein kinase C was assayed in the presence of Ca\(^{2+}\), PS, and DG by measuring the incorporation of \(^{32}\)P
Ca$^{2+}$ and Protein Kinase C Distribution

into histone type III-S as described previously (3, 9). Aliquot equivalent to fractions from 1.26 x 10$^5$ cells were incubated in a 250-μl reaction mixture consisting of 55 mM Tris-Cl, pH 7.5, 0.01% Triton X-100, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.4 mM EGTA, 10 mM MgCl$_2$, 0.6 mM CaCl$_2$, 20 μg/ml phosphatidylerine (Avanti Polar Lipids), 2 μg/ml of the specific antisem (YGC, 1.2-dioleoylglycerol (Sigma), 160 μg/ml histone type III-S (Sigma), and 50 μl/l μl Clx) [γ-32P]ATP (ICN Radiochemicals). Incubation was at 30 °C for either 30 min (cytosol fraction) or 60 min (particulate fraction). The reaction was stopped by addition of 1 ml of ice-cold 25% trichloroacetic acid, followed by 25 μl of bovine serum albumin (20 mg/ml) (Sigma, fraction V) as a carrier. Precipitates were collected on 0.45-μm type HA Millipore filters by vacuum filtration. Filters were then counted for 32P using liquid scintillation spectroscopy. Activity was measured as picomoles of 32P incorporated per min/10$^5$ cells. The non-Ca$^{2+}$- and phospholipid-dependent phosphorylations were subtracted from the total amount of 32P incorporated in order to determine the total specific protein kinase C activity.

Electrophoresis and Immunoblotting—Cytosol and particulate fractions from neutrophil cytoplasts were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide reduced gel with a 12% stacking gel (15) and transferred to nitrocellulose by the method of Towbin et al. (16). The nitrocellulose was incubated overnight at 4 °C in 3% (w/v) bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl) to block nonspecific binding sites, followed by a 1-h incubation with a polyclonal antibody to protein kinase C. Anti-peptide antiserum specific for a conserved region of protein kinase C was the kind gift of Dr. Ora Rosen, Memorial Sloan-Kettering Cancer Center, New York. The antigenic peptide (residues 381-394) of the deduced amino acid sequence lies C-terminal to the ATP binding site (10). The nitrocellulose blot was incubated with horseradish peroxidase conjugate/goat anti-rabbit IgG (diluted 1/200) for 30 min and then washed extensively with Tris-buffered saline. Immunoreactive bands were visualized with 3,3'-diaminobenzidine as a substrate for horseradish peroxidase.

Data Analysis—Experiments were carried out in duplicate or triplicate. Results are expressed as the mean ± S.E., and the statistical significance was analyzed using Student’s t test.

RESULTS

When unstimulated neutrophils prepared in lipopolysaccharide-free conditions were disrupted in Ca$^{2+}$-depleted buffer, the protein kinase C activity was found to be predominantly (>95%) in the cytosolic (soluble) fraction. However, disruption in the presence of Ca$^{2+}$ resulted in a 3-fold increase in activity in the particulate fraction, associated with a 70% reduction in cytosolic activity (Fig. 1). These results raised the possibility that Ca$^{2+}$ could influence the subcellular distribution of protein kinase C in the neutrophil.

The reversibility of the Ca$^{2+}$-induced association of protein kinase C with the particulate fraction was investigated. The particulate fraction prepared in the presence of Ca$^{2+}$ was resuspended in either a Ca$^{2+}$-containing buffer (buffer B, 500 nM Ca$^{2+}$) or a Ca$^{2+}$-depleted (2 mM EGTA) extraction buffer (buffer A) and reincubated to determine released activity. After resuspension in the presence of 500 nM Ca$^{2+}$, 7 ± 3% of the original histone-phosphorylating activity (62 ± 11 pmol of 32P/min/10$^5$ cell equivalents) was released into the supernatant in contrast, after resuspension in a Ca$^{2+}$-depleted medium (2 mM EGTA), 42 ± 3% of the previously particulate-associated associated activity was released into the soluble fraction (n = 3). This EGTA-induced release of substantial kinase activity from the particulate fraction mirrors the Ca$^{2+}$-induced loss of cytosolic kinase activity demonstrated previously (Fig. 1). These results suggest that the Ca$^{2+}$-induced association of protein kinase C with the particulate fraction is a reversible interaction in which the distribution is regulated by the concentration of free Ca$^{2+}$.

To determine if physiological levels of free Ca$^{2+}$ could influence protein kinase C distribution, we used a Ca$^{2+}$-buffering system to accurately control the level of free Ca$^{2+}$ in the extraction buffer. A loss of activity from the cytosol and an increase in activity in the particulate fraction became apparent between 30-300 nM (Fig. 2), which approximates the physiological range (8, 9). At 27 nM Ca$^{2+}$, the cytosolic protein kinase C activity, expressed as a percentage of the levels observed in the presence of 10 mM EGTA without added calcium, was 98 ± 3% (n = 6), and at 54 nM Ca$^{2+}$, a level that is close to resting cytosolic Ca$^{2+}$, the cytosolic protein kinase C activity was 93 ± 6% (n = 3). In the presence of 270 nM Ca$^{2+}$, the level of cytosolic protein kinase C had declined to 52 ± 4% (n = 6), a level that was significantly different (p < 0.0002) from that observed in the presence of excess EGTA. This loss in cytosolic protein kinase C activity in the presence of 270 nM Ca$^{2+}$ was accompanied by an increase in pellet-associated activity to 220 ± 56% (n = 6, p < 0.01). These findings suggest that changes in intracellular levels of free Ca$^{2+}$ during stimulation of the neutrophil could be sufficient to regulate association of protein kinase C with the membranes of the intact cell.

Exposure of neutrophils to phorbol myristate acetate, 1 μg/ml, resulted in a rapid reduction in protein kinase C activity in the cytosol and an increase in such activity in the particulate fraction as reported previously (3, 9). Manipulation of...
the Ca\(^{2+}\) content of the extraction buffers as described here did not influence protein kinase C activity in particulate fractions from these phorbol ester-stimulated cells (data not shown).

It has been suggested that association of protein kinase C with the cell membrane may be accompanied by a proteolytic processing of the enzyme to a form that functions independently of Ca\(^{2+}\) and PS (17). Therefore, the particulate fractions were isolated in the presence of Ca\(^{2+}\) and anti-proteinases (phenylmethylsulfonyl fluoride and leupeptin) and solubilized by brief sonication in extraction buffer containing 0.1% Triton X-100. Analysis of the kinase activity of these preparations revealed a dependence upon the presence of PS, DG, and Ca\(^{2+}\) for maximal activity. An activity of 18.2 pmol of \(^{32}\)P/min/10\(^7\) cell equivalents obtained in the presence of PS, DG, and Ca\(^{2+}\) was reduced to 6.7 pmol of \(^{32}\)P/min/10\(^7\) cell equivalents by the removal of Ca\(^{2+}\); when all three activators were removed, the histone-phosphorylating activity was further reduced to 2.9 pmol of \(^{32}\)P/min/10\(^7\) cell equivalents. Thus, no evidence for the formation of a Ca\(^{2+}\)- and PS-independent form was observed (n = 3).

A similar analysis of the kinase activity remaining in the cytosolic fraction after the Ca\(^{2+}\)-induced translocation of protein kinase C to the particulate fraction demonstrated that this enzyme was dependent upon PS and DG for maximal activity (Fig. 3); however, the cytosolic kinase activity remaining after Ca\(^{2+}\)-induced translocation did not require Ca\(^{2+}\)

because removal of Ca\(^{2+}\) by chelation with EGTA did not inhibit kinase activity (Fig. 3). The PS/DG-dependent, Ca\(^{2+}\)-independent activity in the cytosol of cells extracted in the absence of Ca\(^{2+}\) was 130 pmol of \(^{32}\)P/min/10\(^7\) cell equivalents, as compared to 125 pmol of \(^{32}\)P/min/10\(^7\) cell equivalents in cytosol prepared in the presence of Ca\(^{2+}\).

In addition to inducing association of protein kinase C with
Table 1

Effect of Ca\(^{2+}\) during extraction on localization of protein kinase C in cytoplasts

<table>
<thead>
<tr>
<th>Extraction buffer</th>
<th>Protein kinase C activity*</th>
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<tr>
<td></td>
<td>Whole sonicate</td>
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<tr>
<td>Ca(^{2+})-depleted</td>
<td>159 ± 23</td>
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<tr>
<td>Ca(^{2+})-present</td>
<td>123 ± 53</td>
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*Activity expressed as picomoles of \(^{32}\)P incorporated per min/3 × 10\(^7\) cytoplasts, mean ± S.E. (two experiments in triplicate).

Fig. 4. Calcium-dependent translocation of immunoreactive protein kinase C in cytoplasts demonstrated by immunoblot. Cytoplasts were disrupted by sonication in extraction buffer depleted of Ca\(^{2+}\) (buffer A) or in the presence of 500 nM Ca\(^{2+}\) (buffer B) run on 7.5% reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see "Materials and Methods"). Each lane contains 30 μl of sample equivalent to 9 × 10\(^7\) cytoplasts. Whole sonicates (lanes 1 and 2), cytosol (lanes 3 and 4), and particulate fraction (lanes 5 and 6) were prepared in the presence and in the absence of calcium as indicated. Molecular mass standards are shown on the right margin, and protein kinase C (PKC) is indicated by an arrow at the left.

The particulate fraction, the presence of Ca\(^{2+}\) during extraction also resulted in a partial loss of total (whole sonicate) protein kinase C activity in our assay (see Figs. 1 and 2). Because of the possibility that Ca\(^{2+}\)-dependent granular proteases or a reported protein kinase C inhibitor found in the specific granules (18) could be contributing to this loss, we used organelle-depleted cytoplasts to investigate the effect of Ca\(^{2+}\) during the extraction process.

Disruption of the cytoplasts in the presence of 500 nM Ca\(^{2+}\) resulted in an association of protein kinase C activity with the particulate (membrane) fraction that was equivalent to that shown for intact neutrophils (Table I). The decreases in cytosol and whole sonicate protein kinase C activities noted in conjunction with the Ca\(^{2+}\)-induced association of protein kinase C with the particulate fraction (Table I) were also similar to that observed in intact cells.

Thus, translocation of protein kinase C activity, measured as histone phosphorylation, was observed to be a Ca\(^{2+}\)-sensitive event. To demonstrate physical translocation of protein kinase C in response to changes in Ca\(^{2+}\) concentration, we used an anti-peptide antibody specific for a highly conserved amino acid sequence of the α, β, and γ isoforms of protein kinase C (10, 19). Cytoplasts, and cytosol and particulate fractions from cytoplasts, were prepared in the presence (buffer B) and in the absence (buffer A) of Ca\(^{2+}\) and separated by electrophoresis. Immunoblots of these gels demonstrated the presence of an immunoreactive species of approximately 80 kDa, putative protein kinase C (Fig. 4). In the absence of Ca\(^{2+}\), this band was predominantly in the cytosol; in the presence of 500 nM Ca\(^{2+}\), there was a loss of this immunoreactive band from the cytosol and an increase in protein kinase C immunoreactivity in the particulate fraction (Fig. 4). Thus, protein kinase C, measured as immunoreactivity and as histone-phosphorylating activity, was translocated from cytosol to particulate fraction in the presence of Ca\(^{2+}\).

Discussion

Using a cell-free model system, it has been reported that Ca\(^{2+}\) in the sub-micromolar range can induce the association of purified rat brain protein kinase C with inside-out erythrocyte membrane vesicles (20). This association could be reversed by reducing the concentration of Ca\(^{2+}\), suggesting that the distribution of protein kinase C in the intact cell might be regulated by the intracellular concentration of free Ca\(^{2+}\) (21). Ca\(^{2+}\) has been reported to induce association of protein kinase C with neutrophil membranes but at concentrations significantly above the physiological range (17).

We have shown that, at concentrations of Ca\(^{2+}\) equivalent to the intracellular Ca\(^{2+}\) levels in resting neutrophils (50-60 nM) (8), protein kinase C is found predominantly in the cytosolic fraction. However, a small rise in Ca\(^{2+}\) to concentrations easily attained intracellularly during stimulation (250-500 nM) (8, 9) resulted in a translocation of protein kinase C to the membrane fraction. This was measured as translocation of PS/DG/Ca\(^{2+}\)-dependent histone-phosphorylating activity as well as the physical translocation of an immunoreactive protein kinase C species demonstrated by Western blot technique (10). It is, therefore, possible that changes in intracellular Ca\(^{2+}\) during neutrophil stimulation could induce a redistribution of protein kinase C from the cytosol to membranes.

Our results indicate that the Ca\(^{2+}\)-induced association of protein kinase C with cellular membranes is a reversible interaction. Irrespective of the location of protein kinase C in the intact cell, as soon as the cells are lysed, the observed localization will be determined by the Ca\(^{2+}\) concentration in the buffer. This is in agreement with other workers who demonstrated that protein kinase C translocation in response to fMet-Leu-Phe could only be detected by application of a freeze clamp technique and if the concentration of Ca\(^{2+}\) chelators in the lysing buffer was low (22). However, this Ca\(^{2+}\)-sensitivity does not hold true for the translocation induced by phorbol esters, which was not readily reversed in the presence of EGTA, perhaps because of the ability of phorbol esters to stabilize the membrane-bound protein kinase C-PS-Ca\(^{2+}\) complex (20, 23).

The kinase activity remaining in the cytosol after Ca\(^{2+}\)-induced translocation of protein kinase C to the membrane was found to be independent of Ca\(^{2+}\), although it retained its phospholipid dependence (Fig. 3). Phospholipid-dependent, Ca\(^{2+}\)-independent protein kinases have been described in rabbit reticulocytes (24) and rat brain tissue (25), and a Ca\(^{2+}\)-inhibited, PS-dependent kinase activity has been described in guinea pig peritoneal macrophages (26). However, the relationship between this Ca\(^{2+}\)-independent activity and protein kinase C is not clear. The Ca\(^{2+}\)-independent activity may be a separate enzyme distinct from protein kinase C or a molecular variant of protein kinase C (27). Alternatively, protein kinase C may be metabolically processed to a Ca\(^{2+}\)-independent form. We could find no evidence for the proteolytic formation of the Ca\(^{2+}\)- and PS-independent kinase reported by others (17) following the Ca\(^{2+}\)-induced translocation of protein kinase C. This is not surprising because our extraction buffer contained leupeptin which has been shown to inhibit Ca\(^{2+}\)- and PS-independent kinase formation (17); however, it is possible that the Ca\(^{2+}\)-independent kinase that we detected could be derived from protein kinase C by a limited proteolytic reaction or by autophosphorylation (28).

The buffer conditions that permitted Ca\(^{2+}\)-induced associ-
Stimulation of the neutrophil results in increased concentrations of cytosolic Ca\(^{2+}\) (8, 9) to levels similar to those required to induce membrane association of protein kinase C. We predict that stimulation of the neutrophil results in a translocation of protein kinase C to the cell membrane. Furthermore, we have recently demonstrated that priming of the neutrophil by lipopolysaccharide, ionomycin, or platelet-activating factor for an enhanced respiratory burst is associated with an increase in intracellular free Ca\(^{2+}\) (9, 31, 32). The reduced lag time might arise because the elevated cytosolic Ca\(^{2+}\) induces association of the protein kinase C with the membrane of primed cells, reducing the time required for assembly of the active protein kinase C complex. Thus, a redistribution of protein kinase C, induced by raised levels of intracellular Ca\(^{2+}\), may be involved in the priming of the neutrophil for an enhanced respiratory burst.

REFERENCES