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Biochemistry

Identification of the proteolytically activated form of protein kinase C in stimulated human neutrophils

(cell activation/protein kinase C down-regulation/protein kinase M)

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ABSTRACT **The proteolytically activated form of protein kinase C has been identified in human neutrophils by using a monoclonal antibody that recognizes both the native kinase and the catalytically active proteolytic fragment (protein kinase M). Stimulation with fMet-Leu-Phe results in the conversion of ≈30% of native protein kinase C to protein kinase M, with little evidence of further degradation. Stimulation with phorbol 12-myristate 13-acetate, on the other hand, causes only a transient formation of protein kinase M, with complete loss of total kinase activity. These differences are related to the differences in biochemical responses, reported earlier, in neutrophils exposed to these two activators.**

Protein kinase C (PKC) is present in a variety of cells and tissues and is known to play an important role in the complex mechanisms of signal transduction (1–5). Early studies established that the native kinase contains regulatory and catalytic domains and requires Ca^{2+} and phospholipids (PL) as well as diacylglycerol for full activity (4, 5). Digestion with the Ca^{2+}-requiring proteinase calpain releases the catalytic domain, which is fully active in the absence of Ca^{2+} and lipid cofactors (4, 6, 7).

In previous reports (8–12), we have provided evidence for the formation of the proteolytically modified form (PKM; protein kinase M) during activation of human neutrophils as an essential step in the response characterized by exocytosis of granule contents. This conclusion was based on the following observations: (*i*) upon stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA) or with the chemotactic factor fMet-Leu-Phe, the disappearance of native PKC was accompanied by the appearance of a Ca^{2+}/PL-independent kinase (8, 10, 11); (*ii*) the rate and extent of formation of this Ca^{2+}/PL-independent kinase were found to be significantly reduced when the activity of neutrophil calpain was inhibited by leupeptin or by introduction of a monoclonal anti-calpain antibody (8, 11, 12) into the cells; and (*iii*) inhibition of endogenous calpain resulted in comparable decreases in the rate and extent of disappearance of native PKC and appearance of the Ca^{2+}/PL-independent kinase as well as of granule exocytosis (8). However, the evidence that the Ca^{2+}/PL-independent kinase was PKM, derived from proteolysis of native PKC, remained circumstantial.

In the present study, we have employed a monoclonal antibody that recognizes both PKC and the released catalytic domain, PKM, to demonstrate the formation of PKM in human neutrophils stimulated by fMet-Leu-Phe or PMA. We provide direct proof that the Ca^{2+}/PL-independent kinase formed in stimulated cells is indeed PKM.

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MATERIALS AND METHODS

Neutrophils were prepared from freshly drawn blood as described (9). PMA, phenylmethylsulfonyl fluoride, leupeptin, and fMet-Leu-Phe were from Sigma. Calpain was purified from human neutrophils as reported (13). Anti-PKC monoclonal antibodies were prepared by intraperitoneal injection of purified native neutrophil PKC following procedures identical to those used in the preparation of anti-calpain monoclonal antibodies (12). In brief, the monoclonal antibodies were obtained by fusing spleen cells from immunized mice with myeloma cells (14). The hybrids were tested for antibody production with a solid-phase binding assay (15), and the positive hybrids were cloned as described (16). Clones 8.17, 27.2, and 27.9 were expanded as described (12).

Neutrophil stimulation was carried out by incubating the cells for 10 min at 37°C at a density of 5×10^6 cells per ml in 10 mM Hepes (pH 7.4) containing 0.15 M NaCl, 5 mM KCl, and 5 mM glucose as well as PMA (10 ng/ml) or 0.1 μM fMet-Leu-Phe. As indicated below, aliquots (0.5 ml) of the cell suspension were removed and the cells were collected by centrifugation at 1000 × *g* for 2 min and resuspended in 10 mM Hepes (pH 7.4) containing 0.25 M sucrose, 5 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM leupeptin, and 2 mM phenylmethylsulfonyl fluoride. The cells were then lysed by sonication (6 bursts, 10 sec each), and the particulate fraction was removed by centrifugation at 200,000 × *g* for 10 min. The clear supernatant was treated with 5% (vol/vol) trichloroacetic acid (final concentration). The precipitated proteins were collected by centrifugation and dissolved in 0.2 ml of 0.06 M Tris·HCl (pH 6.8) containing 1% SDS, 10% (wt/vol) glycerol, and 2% 2-mercaptoethanol and heated for 2 min at 100°C. Aliquots (0.05 ml) were analyzed by PAGE. The proteins were transblotted from the gels onto nitrocellulose sheets, and PKC was identified by using monoclonal antibody 8.17 and an ^{125}I-labeled anti-mouse IgG antibody.

Native PKC and the Ca^{2+}/PL-independent protein kinase form were purified as described (8). Purified native PKC was digested with calpain as reported (8).

RESULTS

Characterization of the Monoclonal Antibody. Of a large number of monoclonal antibodies raised against native PKC purified from human neutrophils, only three (designated as 8.17, 27.2, and 27.9) were found to recognize both PKC and PKM (Fig. 1, lanes 1 and 2). Immunoautoradiographic analysis carried out during digestion of purified PKC with a purified preparation of neutrophil calpain revealed the pro-

Abbreviations: PKC, protein kinase C (Ca^{2+}/phospholipid/diacylglycerol-dependent protein kinase); PKM, protein kinase M (proteolytically formed catalytic fragment of PKC); PL, phospholipid; PMA, phorbol 12-myristate 13-acetate.

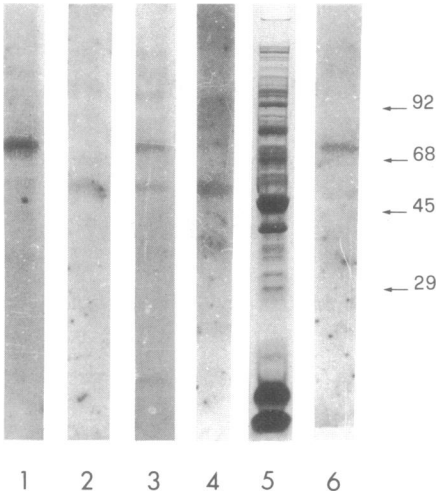

FIG. 1. Identification of native PKC and PKM with monoclonal antibody 8.17. Samples of purified native PKC (lane 1), purified PKM (lane 2), and purified PKC digested with calpain for 2 (lane 3) or 10 (lane 4) min and also aliquots (0.5 mg) of the neutrophil cytosolic fraction (lanes 5 and 6) were subjected to SDS/PAGE and transblotted on a nitrocellulose sheet. The cytosolic fraction was also stained with Coomassie blue (lane 5). Arrows indicate the positions of marker proteins (in kDa).

gressive conversion of native PKC to a smaller species corresponding to PKM (Fig. 1, lanes 3 and 4). With the conditions employed, complete conversion was observed after 10 min (Fig. 1, lane 4). Of the multiplicity of protein bands present in the unstimulated neutrophil lysate (Fig. 1, lane 5), only a single band corresponding to PKC was recognized by the monoclonal antibody (Fig. 1, lane 6). By comparison with the electrophoretic mobilities of standard proteins, the molecular masses of PKC and PKM were estimated as ≈75 kDa and 50 kDa, respectively, which is in agreement with previous reports (5, 10).

Formation of PKM in Human Neutrophils Stimulated with fMet-Leu-Phe. With a concentration of fMet-Leu-Phe that promotes a maximal rate of granule exocytosis (Fig. 2*A*), we observed a decrease in the quantity of native PKC and a concomitant appearance of PKM, which appeared to persist (Fig. 2 *B* and *C*), even after 10 min, when the degranulation process is known to be complete (10–12). Even at this time, however, the immunoautoradiographic analysis shows that native PKC, although reduced in amount, was still present. The amount of PKM present under these conditions was ≈30% of the total immunoreactivity. Thus, a characteristic of neutrophil stimulation with fMet-Leu-Phe is the simultaneous presence of both native PKC and the irreversibly activated form (PKM). This appears to be an essential condition for the release of the contents of both primary and secondary granules. The results reported in Fig. 2 document the formation of PKM and its accumulation following activation of neutrophils with fMet-Leu-Phe, which confirms earlier indications based on the changes in level of PKC and the appearance of the Ca^{2+}/PL-independent kinase activity.

Formation and Disappearance of PKM in Human Neutrophils with PMA. PMA is a well-known neutrophil activator that increases O_2^- production at low concentrations and induces granule exocytosis involving only secondary granules at higher concentrations (10–12, 17–20). After exposure of neutrophils to low concentrations of PMA (10 ng/ml), we observed a rapid and complete loss of native PKC, with the concomitant appearance of PKM (Fig. 3 *A–C*). This is in contrast to the effect of fMet-Leu-Phe, where significant quantities of PKC persist. However, if the concentration of PMA was raised to that required to elicit a maximal rate of

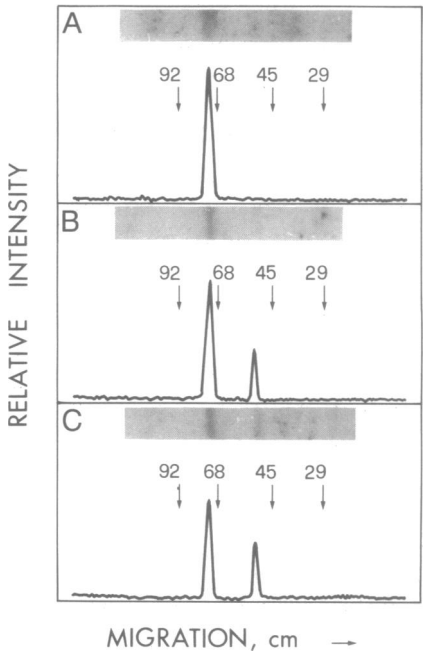

FIG. 2. PKC forms in neutrophils stimulated with fMet-Leu-Phe. Neutrophil stimulation, electrophoresis, and transblotting were performed as described in *Materials and Methods*. Monoclonal antibody 8.17 was used to identify PKC forms. (*A*) An aliquot was taken at zero time (before addition of fMet-Leu-Phe). Samples were taken after exposure to the stimulus for 2 min (*B*) and 10 min (*C*). Densitometer scans, shown below each gel, were carried out with a Shimadzu densitometer model CS-9000. Arrows indicate the positions of marker proteins (in kDa).

granule exocytosis (100 mg/ml), then we observed the almost complete disappearance of both PKC and PKM within the 10-min interval (Fig. 3*D*).

DISCUSSION

The proteolytic activation of PKC, originally described by Nishizuka and coworkers (4, 7), was later discarded as a physiological mechanism after the discovery of the activation of native PKC by Ca^{2+} and PL. PKM was assigned the role of an intermediate in the proteolytic degradation of PKC and related to the down-regulation of the kinase (21–25) that was observed in cells stimulated by PMA (8).

In studies designed to identify the biochemical events involved in the responses of human neutrophils to various external stimuli, we observed a progressive decline in native PKC activity and the concomitant appearance of a Ca^{2+}/PL-independent kinase (10, 11). In cells stimulated with fMet-Leu-Phe, both the disappearance of PKC and the appearance of the Ca^{2+}/PL-independent kinase reached plateau levels, with the accumulation of an apparently stable form of the latter (8). On the other hand, with stimulation of neutrophils by PMA, only a transient accumulation of the independent kinase was observed; with concentrations of PMA usually employed to elicit the maximum response, both PKC and the Ca^{2+}/PL-independent kinase are rapidly degraded (8).

On the basis of the observation that the disappearance of PKC, the formation of the Ca^{2+}/PL-independent kinase, and granule exocytosis are all related to the translocation and activation of endogenous neutrophil calpain (26), we suggested that the formation of PKM was indeed occurring in activated neutrophils. We also propose that events leading to the activation of calpain and the formation of PKM are

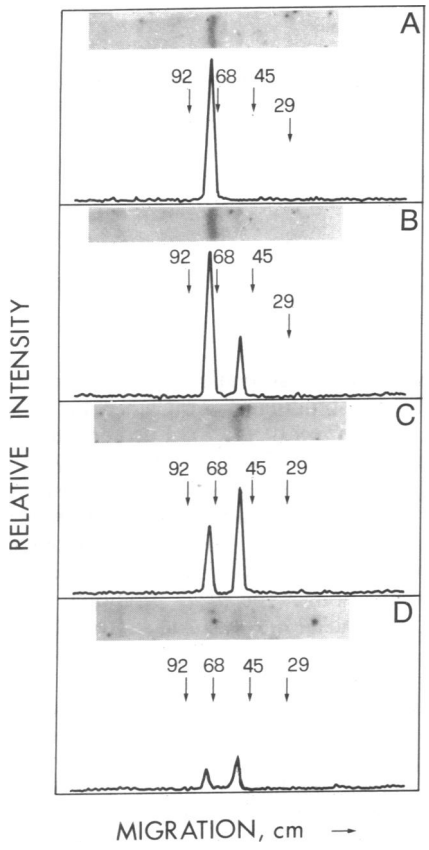

FIG. 3. PKC forms in neutrophils stimulated with PMA. Neutrophil stimulation, electrophoresis, and transblotting were performed as described in *Materials and Methods* and the legend to Fig. 2. (*A*) An aliquot was taken before addition of PMA. Aliquots were taken after exposure to PMA (10 ng/ml) for 2 min (*B*) and 10 min (*C*). (*D*) An aliquot was taken after exposure to PMA (100 ng/ml) for 10 min. Arrows indicate the positions of marker proteins (in kDa).

essential steps in the response characterized by exocytosis of granule contents (8, 10, 11).

The differences in biochemical responses of neutrophils to fMet-Leu-Phe and PMA may be related, at least in part, to the differences in the effects of these activators on PKC. With fMet-Leu-Phe, the characteristic response is exocytosis of both primary and secondary granules (11), in contrast to PMA, which causes exocytosis only of secondary granules (11). This difference may be related to the more rapid formation of PKM and its accumulation in cells stimulated by fMet-Leu-Phe as compared to the complete loss of both activities in cells stimulated by PMA.

In the present work we have identified the Ca^{2+}/PL-independent kinase generated in activated neutrophils as PKM and confirmed the differences in its intracellular accumulation in response to fMet-Leu-Phe and PMA, which may be related to the differences in biochemical responses observed with these reagents.

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