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DIFFERENTIAL MECHANISMS OF TRANSLOCATION OF PROTEIN KINASE C TO PLASMA MEMBRANES IN ACTIVATED HUMAN NEUTROPHILS

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Three classes of activators of human neutrophils that induce the intracellular translocation of protein kinase C from the cytosol to the particulate fraction were compared for their effects on the properties of the particulate (membrane-bound) enzyme. In cells stimulated with 10 ng/ml of phorbol-12-myristate-13-acetate (PMA) the particulate enzyme is almost fully active in the absence of added Ca^{2+} or phospholipids and this activity is not released by the Ca^{2+}-chelator EDTA. In contrast, binding of protein kinase C to the particulate fraction in cells treated with the chemotactic factor f-Met-Leu-Phe (fMLF) or with the ionophore A-23187 plus Ca^{2+} is observed only when the cells are lysed in the presence of 1 mM Ca^{2+}. With these stimuli the particulate enzyme retains a nearly absolute requirement for Ca^{2+} and phospholipids. Thus only the full intercalation of protein kinase C caused by PMA, which is resistant to removal by chelators stabilizes an active form of protein kinase C in the neutrophil membrane. In confirmation of this conclusion, in isolated plasma membranes loaded with partially purified protein kinase C by incubation with 5 μM Ca^{2+} further incubation with PMA, but not with fMLF, caused a significant fraction of the bound PKC to become resistant to removal by chelators, and to be nearly fully active in the absence of added activators.

It is generally accepted that intracellular mobilization of Ca^{2+} promotes association of cytosolic protein kinase C (PKC) with the plasma membrane and that this association with the cell membrane is an obligatory first step in the activation of protein kinase C by natural activators such as diacylglycerol and phospholipids or by tumor promoting phorbol esters (1-3) such as phorbol-12-myristate-13-acetate (PMA). PMA, in constrast to diacylglycerol, is metabolized very slowly and intercalated into the cell membrane where it appears to induce the formation of an active complex containing the kinase, phospholipids, Ca^{2+} and PMA (1, 3-7). This particulate activity is active in the absence of Ca^{2+} and phospholipids, but the requirement for these activators

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reappears when the activity is extracted from the particles with Triton X-100 (8). An additional mechanism for activation of neutrophil PKC involves the cytosolic Ca^{2+}-activated thiol proteinase, calpain, which is also translocated to the plasma membranes in the presence of low concentrations of Ca^{2+} (8). Binding of both PKC and calpain to the neutrophil plasma membrane results in conversion of PKC to the proteolytically modified form that no longer binds to the cell membranes and is fully active in the absence of Ca^{2+} and phospholipids (9-12). Stimulation of neutrophils by fMLF elicits responses that are significantly different form those observed following stimulation with low concentration of PMA, including mobilization of intracellular Ca^{2+} (13), degranulation (14, 15), and respiratory burst (16). We have observed the release of a membrane-bound serine proteinase in neutrophils stimulated by low or by high concentrations of PMA, but not by fMLF (unpublished observations). In these experiments, only the latter conditions caused the exocytosis of granule contents and a net increase in intracellular free Ca^{2+}. These observations are consistent with recent reports of synergistic effects of Ca^{2+} mobilization and protein kinase activation (2, 6, 17, 18).

We now describe some significant differences in the properties of particulated PKC formed in response to PMA, Ca^{2+}, and fMLF. In these experiments, the calpain inhibitor, leupeptin, was added to prevent the proteolysis of PKC, which could have resulted in activation of PKC by proteolysis and loss of its ability to remain bound to the cell membrane (11, 12).

MATERIALS AND METHODS

Human neutrophils and partially purified PKC were prepared as previously described (11). The purified enzyme had a specific activity of 10^5 units/mg, when assayed in the presence of Ca^{2+} and lipids. PMA, f-Met-Leu-Phe, A-23187, cytochalasin B, phosphatidyl serine, dioleoylglycerol, ATP, histone, leupeptin and phenylmethylsulphonyl fluoride were from Sigma Chemical Co., St. Louis, Mo. $|\gamma\text{-}^{32}P|$ATP (3000 Ci/mmol) was from Amersham International, Amersham, England. Percoll was obtained from Pharmacia, Uppsala, Sweden.

PKC activity was assayed as previously described (11). The unit of activity was the amount that catalyzed the incorporation of 1 nmol of ^{32}Pi into histone III-S. Activation of neutrophils was performed as follow: neutrophils ($5x10^6$ cells/ml) were incubated at 0°C in 10 mM HEPES, pH 7.4, containing 0.15 M NaCl and 5 mM glucose (medium A) with 0.1 mM leupeptin. After 10 min either 10 ng/ml of PMA, or 0.1 μM f-Met-Leu-Phe with 5 μM cytochalasin B, or 0.1 μM A-23187 with 5 or 50 μM Ca^{2+}, were added and the incubation continued for 10 min at 37°C. The cells were then collected by centrifugation for 5 min at 400xg, resuspended in the original volume of a 0.25 M sucrose solution containing 10 mM HEPES, pH 7.4, 5 mM EDTA and 10 mM ß-mercaptoethanol (medium B). Preloading

of the cells with leupeptin was performed in order to inhibit the proteolytic conversion of PKC and to stabilize its association to the membrane (12). Other methods were as described (11, 12).

Preparation of the particulate fraction. Neutrophils suspended in medium B at $5x10^6$ cells/ml were lysed by sonication with an MSE model MK2 sonicator, using six 10-second bursts, and the suspension centrifuged at 100,000xg for 30 min. The supernatant was collected and used as the "soluble fraction". The pellet was suspended in medium B and used as the "particulate fraction".

Preparation of plasma membranes. Neutrophils ($25x10^6$ cells/ml) were suspended in 0.25 M sucrose and lysed with 40-60 strokes in a glass-Teflon homogenizer. When approximately 80% of the cells were broken, the homogenate was loaded on to a 55% Percoll solution (8 ml) containing 0.25 M sucrose and 5 mM $MgCl_2$. The gradient was centrifuged at 100,000xg for 15 min and fractions of 1 ml were collected from the top of the gradient. The fractions containing cell particles were diluted 10 times with isotonic sucrose and centrifuged for 45 min at 200,000xg to remove Percoll. The fractions containing the highest 5'-nucleotidase activity (19) were used as "plasma membrane fraction". This fraction was free of granules and mitochondria and only 5% contaminated by endoplasmic reticulum, as revealed by the assay of the specific markers: 5'-nucleotidase (20) for plasma membranes, lysozyme (21, 22) for granules, succinate dehydrogenase (23) for mitochondria and NADH-ferricyanide reductase (24) for endoplasmic reticulum.

RESULTS

Translocation of PKC from the soluble to the particulate fraction. When human neutrophils are lysed and the particulate fraction prepared in the presence of EDTA, PKC activity is recovered almost quantitatively in the cytosolic fraction in a form that is inactive in the absence of Ca^{2+}, phospholipids and diacylglycerol. Treatment with PMA causes a substantial fraction of the total PKC activity (~30%) to become translocated to the particulate fraction, where its activity is almost fully expressed (~70%) in the absence of added effectors (Table I). Very little activity was recovered in particulate fraction after exposure of neutrophils to f-Met-Leu-Phe or to ionophore plus Ca^{2+} (Table I). These results provide direct evidence for the hypothesis that tumor promoters cause a tight intercalation of protein kinase C into the plasma membranes in a manner that exposes the active site to protein substrates (3, 7). With other activators, such as f-Met-Leu-Phe or ionophore plus Ca^{2+}, which have also been reported to cause activation of PKC (2) but promote a different set of biochemical responses (13-16) this EDTA-resistant binding to plasma membranes was not observed.

Effect of Ca^{2+} on the translocation of PKC from the soluble fraction in activated neutrophils. When the particulate fraction was isolated from cells sonicated in the presence of 1 mM Ca^{2+} the bulk of the PKC activity (80-85%) was

TABLE I

Effect of various stimuli on the association of catalytically active PKC with the particulate fraction of human neutrophils

Treatment[a]	Total PKC activity[b] (nmol/min/mg)	PKC activity (nmol/min/mg) in soluble fraction[c]	PKC activity (nmol/min/mg) in particulate fraction[d]	
			+ effectors	- effectors
none	4.42	4.09	0.02	0.004
PMA	4.06	2.62	1.32	0.92
f-Met-Leu-Phe	4.18	4.02	0.03	0.006
A-23187+5 uM Ca^{2+}	4.36	4.15	0.04	0.006
A-23187+50 uM Ca^{2+}	4.20	4.00	0.05	0.007

[a] Human neutrophils ($50x10^6$ cells) were incubated at 37°C with the indicated stimuli as described in Methods. Following incubation the cells were lysed by sonication and the particulate fractions were prepared from cells sonicated in buffer B, containing EDTA (see Methods).

[b] Aliquots (0.02 ml) of the sonicated cell suspensions were collected and used for the assay of PKC activity in the presence of Ca^{2+} and lipids (11).

[c] Aliquots (0.02 ml) of the soluble fraction obtained after centrifugation of the lysed cells were assayed for PKC activity (11). In assays carried out in the presence of 5 mM EDTA, without Ca^{2+}, phospholipids and diacylglycerol (see 11), the activity was only 5% of that observed in the presence of effectors.

[d] Aliquots of the particulate fraction (0.05 ml) were used for the assay of PKC activity as described (11) in the presence of Ca^{2+} and lipids (+ effectors) or of 5 mM EDTA (-effectors.

recovered in the particulate fraction (Table II). Except for the cells stimulated with PMA, this activity remained fully dependent on the presence of Ca^{2+} and lipids. Following stimulation with PMA, more than 30% of the total activity was recovered in the particulate fraction in a Ca^{2+}/phospholipid-indepen-

TABLE II

Effect of Ca^{2+} on the association of catalytically active PKC with the particulate fraction from stimulated neutrophils

Treatment[a]	Total PKC activity[b] (nmol/min/mg)	PKC activity (nmol/min/mg) in soluble fraction[b]	PKC activity (nmol/min/mg) in particulate fraction[b]	
			+ effectors	- effectors
none	4.27	0.53	3.58	0.18
PMA	4.18	0.63	3.40	1.36
f-Met-Leu-Phe	4.08	0.50	3.44	0.28
A-23187+5 μM Ca^{2+}	4.12	0.49	3.26	0.24
A-23187+50 μM Ca^{2+}	4.06	0.52	3.22	0.27

[a] Neutrophils were exposed to the stimuli as in Table I. After 10 min of incubation, the cells were collected, suspended in 0.25 M sucrose containing 10 mM mercaptoethanol, 1 mM leupeptin, 2 mM phenylmethylsulphonyl fluoride and 1 mM Ca^{2+} and lysed by sonication.

[b] The soluble and particulate fraction were prepared (see Methods) and the PKC activity measured in the presence of Ca^{2+}, phospholipids and diacylglycerol (+ effectors) or in the presence of 5 mM EDTA (- effectors).

TABLE III

Effect of activators of human neutrophils on the insertion of catalytically active PKC into isolated neutrophil membranes

Addition	PKC activity (total units) recovered	
	+ effectors	- effectors
none	0	0
PMA	11	9.7
f-Met-Leu-Phe	1	0.16

Partially purified PKC (25 units) and 0.1 mg of the neutrophil plasma membrane fraction, prepared as described in Methods, were incubated at 4°C in 0.5 ml of 50 mM sodium borate, pH 7.5, containing 5 μM Ca^{2+}. After 5 min, the membranes were collected by centrifugation for 10 min at 25,000xg and suspended in 0.5 ml of the same buffer solution. Under these conditions approximately 80% of the total PKC activity was taken up by the membranes (see 11). PMA final concentration 10 ng/ml or fMLF plus cytochalasin B (final concentration 0.1 μM and 5 μM respectively) were then added and the suspensions incubated at 37°C. After 10 min the membranes were collected, washed with 1 ml of 0.1 mM EDTA and the PKC activity present in the membranes evaluated as described footnotes to Table I. Purified PKC express 10% and 2% of its maximum activity when exposed to 10 ng/ml PMA or 0.1 μM fMLF, respectively, in the absence of plasma membranes.

dent form. Thus only PMA, and not f-Met-Leu-Phe or Ca^{2+}, can induce formation of the fully intercalated catalytically active form of PKC.

Binding of partially purified PKC to isolated neutrophil membranes. When isolated plasma membranes from human neutrophils were incubated with purified PKC in the presence of 5 μM Ca^{2+}, nearly 80% of the PKC activity became adsorbed to the membranes (11), but this activity was completely removed by 0.1 mM EDTA (Table III). However, when the membranes containing PKC wer exposed to 10 ng/ml of PMA, more than half of the bound PKC became resistant to release by EDTA in a form that was almost fully active in the absence of Ca^{2+} and lipid effectors. Very little activity became tightly inserted into plasma membranes following exposure to f-Met-Leu-Phe and this activity remained fully dependent on the presence of Ca^{2+} and lipids. Thus, neither Ca^{2+} nor f-Met-Leu-Phe was able to induce the conversion of the membrane-bound PKC to the tightly intercalated form.

DISCUSSION

The role of PKC in signal transduction has been firmly established by the classical studies of Nishizuka and his coworkers (reviewed in 1), who were the first to describe its binding to membranes and its activation by diacylglycerol in the presence of Ca^{2+} and phospholipids (25). Earlier observations of the activation of PKC by a Ca^{2+}-requiring proteinase (10) were considered to

represent a non-physiological mechanism (1, 6). However, we have recently reported that simultaneous binding of PKC and calpain to neutrophil plasma membranes, induced by low concentrations of Ca^{2+}, results in conversion of the native form of PKC to the proteolytically activated form (11) and that this conversion takes place in intact neutrophils exposed to low concentrations of PMA (11). We have recently obtained evidence (manuscript submitted) for two distinct activation mechanisms, both involving PKC in human neutrophils. The first, exemplified by the response to low concentrations of PMA, involves the membrane-bound form of PKC and is characterized by O_2^- production and the release of the membrane-bound proteinase, which together may account for the cytolytic effects of PMA-activated neutrophils (26). On the other hand, the response of human neutrophils to activation by f-Met-Leu-Phe plus cytochalasin, which is characterized by the release of granule enzymes is related to the activation of PKC by its conversion by calpain to the irreversibly activated form that is released into the cytosol. This response is also associated with an increase in the level of intracellular free Ca^{2+}, an increase that is not observed in cells activated with low concentrations of PMA (manuscript submitted). A synergistic effects of Ca^{2+} and phorbol esters described by others (6, 17) may be related to this observed mechanism of PKC activation.

The results reported here provide a biochemical basis for the differences in responses reported for PMA and f-Met-Leu-Phe. In either case the initial event appears to be the translocation of PKC from cytosol to plasma membranes mediated by PMA or the intracellular mobilization of Ca^{2+}, or both. This insertion of PKC into the membrane bilayer may be permanent, as with PMA, or transient, as with Ca^{2+} or f-Met-Leu-Phe. In either cases, association may be followed by proteolytic modification of the membrane bound PKC and its release from the plasma membrane. The large loss on total PKC activity following activation of human neutrophils by PMA observed by Wolfson et al (8) may be attributed to the conversion of PKC to the Ca^{2+}/phospholipid-independent form. We suggest that this proteolytic conversion is slow in removing tightly bound PKC and more rapid in the removal of the transiently bound PKC.

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