

Regulation of arachidonate-mobilizing phospholipase A₂ by phosphorylation via protein kinase C in macrophages

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Stimulation of ³²P-labeled macrophages with phorbol ester caused an increase in phosphorylation of the intracellular, high molecular weight phospholipase A₂. This increase in phosphorylation was accompanied by an increase in enzyme activity, but led to no detectable shift in the concentration dependence for Ca²⁺-induced activation. The phosphorylated phospholipase A₂ could be dephosphorylated by treatment with acid phosphatase, and such treatment also reduced its catalytic activity. Together with previous data, these results indicate that the arachidonate-mobilizing phospholipase A₂ is dually regulated by Ca²⁺ (membrane interaction) and by phosphorylation (catalytic activity).

Arachidonic acid; Phospholipase A₂ (intracellular); Protein phosphorylation; Protein kinase C

1. INTRODUCTION

The generation of prostaglandins, leukotrienes and other eicosanoids is initiated by a phospholipase A-type cleavage of membrane phospholipids, which mobilizes arachidonate, and there is now increasing evidence that an intracellular, high *M*_r phospholipase A₂ (PLA₂-85) [1–8] is responsible for this cleavage. A role for protein kinase C (PKC) in the activation of this process has been documented in several cell types, including macrophages [9]. While activation of PKC is sufficient for mobilization of arachidonic acid in macrophages, it is insufficient in some cell types, such as platelets [10] and renal mesangial cells [11], where it potentiates the response to Ca²⁺-ionophore.

We previously demonstrated a stable increase in the activity of arachidonate-mobilizing phospholipase A₂ after stimulation of mouse peritoneal macrophages with 4β-phorbol 12-myristate 13-acetate (PMA) [2]. A similar stable increase in phospholipase activity has been observed in bone-marrow-derived macrophages in response to PMA [12] and in renal mesangial cells in response to PMA, vasopressin and epidermal growth factor [13,14]. The present study addresses the question whether activation of PLA₂-85 in response to PMA reflects a regulatory phosphorylation of the enzyme and the possible relation between this activation and that

exerted by submicromolar concentrations of Ca²⁺ [2,4,7,8,15].

2. EXPERIMENTAL

Mouse peritoneal macrophages were isolated as previously described [9], and cultured (approx. 24 × 10⁶ cells) in 80 cm² tissue culture flasks (Nunc, Nunc). On the second day of culture, cells were labeled for 1 h with 2.2 mCi carrier-free ³²PO₄ (Amersham International, UK). Stimulation of the cells with 150 nM PMA was made during the final 10 min of the labeling period. Then cells were washed three times with Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline and scraped off the flasks in 1.3 ml of buffer A (80 mM KCl, 1 mM EDTA, 2.5 mM dithioerythritol, 10 mM NaF, 100 μM ammonium vanadate and 10 mM HEPES, pH 7.4). Cells were homogenized and centrifuged at 700 × *g* for 5 min and the supernatant was further centrifuged at 10⁴ × *g* for 1 h to obtain a cytosol fraction. After addition of glycerol to 10% (by vol.) the fraction was loaded onto a column of Sephadex G-200 superfine (1 × 48 cm) equilibrated in buffer A containing 10% glycerol (by vol.). Fractions were assayed for PLA₂-85 activity using 50 pmol (54 mCi/mmol) of 1-stearoyl-2-[¹⁴C]arachidonoyl-phosphatidylcholine (Amersham International, UK), as previously described [2]. Fractions containing PLA₂-85 were combined, adjusted to 0.5 M NaCl and loaded onto a phenyl-Superose HR 5/5 column (FPLC system) equilibrated in buffer B (5 mM KCl, 1 mM EDTA, 10% glycerol (by vol.) and 10 mM HEPES, pH 7.4), containing 0.6 M NaCl. After washing with 7 ml of equilibration buffer the column was eluted with a 2 ml gradient from 0.6–0 M NaCl in buffer B followed by 10 ml of buffer B. Fractions of 1.5 ml were collected in tubes containing 100 μg bovine serum albumin (to diminish losses of enzyme by adsorption) and assayed for enzyme activity. Samples for electrophoresis were precipitated by the addition of 6 vols. of ice-cold acetone/13 M NH₄OH (30:1.7, by vol.) as described [16]. Precipitated samples were then dissolved in electrophoresis buffer, boiled for 5 min and subjected to SDS-PAGE (6.5% acrylamide) according to Laemmli [17]. After staining, the gels were dried and autoradiographed.

3. RESULTS AND DISCUSSION

To address the question whether the increase in activ-

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Abbreviations: PLA₂-85, 85 kDa phospholipase A₂; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate.

ity of PLA₂-85 in macrophages responding to PMA [2] is associated with a change in the phosphorylation of the enzyme, we stimulated ³²P-labeled macrophages with PMA and examined both the degree of phosphorylation of PLA₂-85 and the change in activity of the enzyme. The cytosol fraction from macrophages was fractionated by gel chromatography followed by phenyl-Superose chromatography and revealed a 1.7 ± 0.1 -fold (mean \pm SD, $n = 9$) increase in enzyme activity from PMA-stimulated as compared to control cells. The same increase was seen also in unfractionated cytosol and after gel chromatography only. This confirms and extends the previous finding of a 1.6-fold increase in the activity of PLA₂-85 in macrophages exposed to PMA [2]. Gel electrophoresis of the phenyl-Superose fractions followed by autoradiography, demonstrated that PLA₂-85, migrating as a 100 kDa protein on SDS/PAGE [5,7,8], was detected as a phosphoprotein band in both control and PMA-stimulated cells (Fig. 1). The labeling of this phosphoprotein was clearly increased in PMA-stimulated as compared to control cells. A similar, although somewhat more variable, increase in phosphorylation of the 100 kDa band and in enzyme activity (1.5 ± 0.3 -fold; mean \pm SD, $n = 16$) was seen in macrophages stimulated with zymosan particles. We feel confident that the 100 kDa band represents PLA₂-85, since the enzyme activity coeluted with the 100 kDa phosphoprotein from phenyl-Superose and

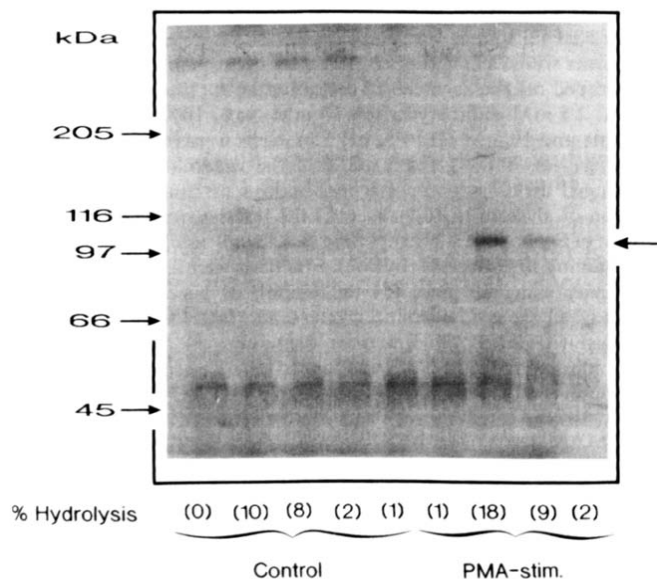


Fig. 1. Phosphorylation of PLA₂-85 in macrophages in response to PMA. PLA₂-85 from control and PMA-stimulated ³²P-labeled macrophages was prepared as described in Experimental and 25 μ l of phenyl-Superose fractions were assayed for enzyme activity using 80 pmol of 1-stearoyl-2-[¹⁴C]arachidonoyl-phosphatidylcholine as substrate. One-third of the selected fractions were prepared for electrophoresis and subjected to SDS/PAGE. The gel was dried and autoradiographed. The enzyme activity in each fraction, expressed as percent hydrolysis, is indicated under each lane. (Left side) Migration of standard proteins. (Right side) Migration of PLA₂-85.

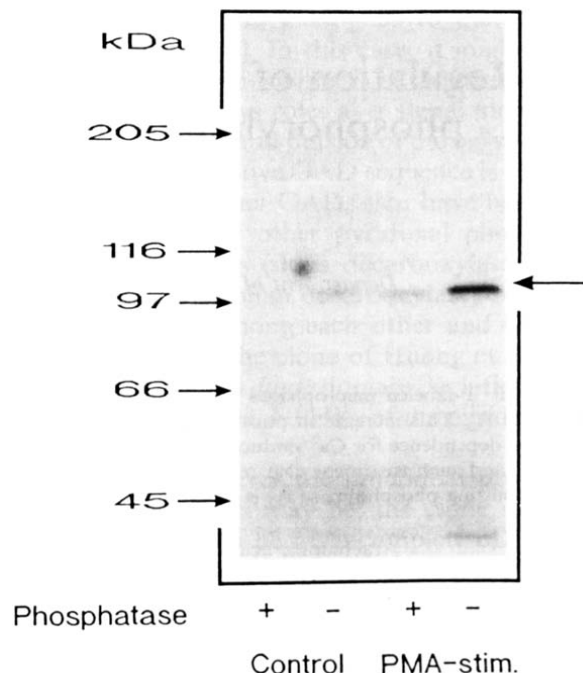


Fig. 2. Dephosphorylation of PLA₂-85 from PMA-stimulated cells. PLA₂-85 from control and PMA-stimulated ³²P-labeled macrophages was prepared as described (see Experimental) and equal aliquots of phenyl-Superose fractions were then incubated in 80 mM KCl, 10 mM MgCl₂, 1 mM EDTA and 10 mM HEPES, pH 6.8, with or without acid phosphatase (6 U/ml; Type VI, Sigma) at 37°C for 40 min in a final volume of 800 μ l. Reactions were terminated with 6 vols. of acetone/13 M NH₄OH and the samples were processed for SDS/PAGE followed by autoradiography.

since purified PLA₂-85 from the macrophage cell line J774 [8] comigrated exactly with the 100 kDa phosphoprotein upon electrophoresis.

In initial experiments, the activity of PLA₂-85 from both control and PMA-stimulated cells was found to be unaffected by treatment with an alkaline phosphatase (not shown). However, treatment with acid phosphatase resulted in extensive dephosphorylation of PLA₂-85, in particular that from PMA-stimulated cells (Fig. 2). The enzyme activity of PLA₂-85 from PMA-stimulated cells was found to be reduced by 30–40% after preincubation with acid phosphatase (Fig. 3). The remaining activity was similar to that of enzyme from control cells, the activity of which was only slightly reduced by phosphatase treatment.

Thus, activation of PKC in macrophages by PMA leads to an increase in phosphorylation of PLA₂-85 that is accompanied by an increase in enzyme activity. However, as previously demonstrated [8], direct (in vitro) phosphorylation of PLA₂-85 by the two predominant isoforms of PKC found in mouse spleen occurred without any detectable change in enzyme activity. It is therefore more likely that a kinase downstream of PKC carries out the regulatory phosphorylation.

The activity of PLA₂-85 is also dramatically affected by Ca²⁺, with half-maximal activation at sub-micromo-

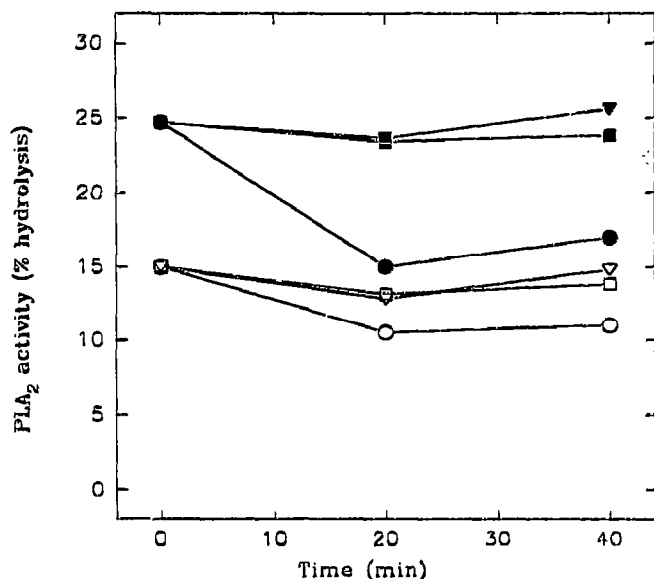


Fig. 3. Effect of dephosphorylation of PLA₂-85 on its enzyme activity. Phenyl-Superose fractions containing PLA₂-85 from control (open symbols) and PMA-stimulated (filled symbols) macrophages were preincubated at 37°C in 80 mM KCl, 10 mM MgCl₂, 1 mM EDTA and 10 mM HEPES, pH 6.8. Preincubation mixtures contained either no addition (○,●), acid phosphatase (5 U/ml) (○●,●●) or acid phosphatase plus 100 mM β-glycerophosphate (□,■). At the indicated time incubations were assayed for phospholipase activity using 40 pmol 1-stearoyl-2-[¹⁴C]arachidonoyl phosphatidylcholine as substrate in the presence of 50 mM β-glycerophosphate. Data represent means of duplicate incubations from a representative experiment.

lar concentration [2,4,7,8,15]. Ca²⁺ binds to the enzyme [18] and is considered to cause activation primarily, or exclusively, through membrane association of the enzyme [6,15,19], since Ca²⁺ is not necessary for the catalytic activity [18]. The phosphorylation-induced activation, although detectable at saturating concentrations of Ca²⁺, could therefore potentially be exerted at either the membrane-binding or the catalytic step. We therefore compared the Ca²⁺-dependent activation of PLA₂-85 from control and PMA-stimulated cells. As shown in Fig. 4, there was no detectable difference in the concentration dependence for Ca²⁺-induced activation; i.e. the phosphorylated enzyme showed the same relative increase in activity irrespective of the concentration of Ca²⁺. This suggests that Ca²⁺ and the regulatory phosphorylation act independent of each other and that phosphorylation affects the catalytic site of the enzyme rather than that involved in Ca²⁺-dependent membrane binding.

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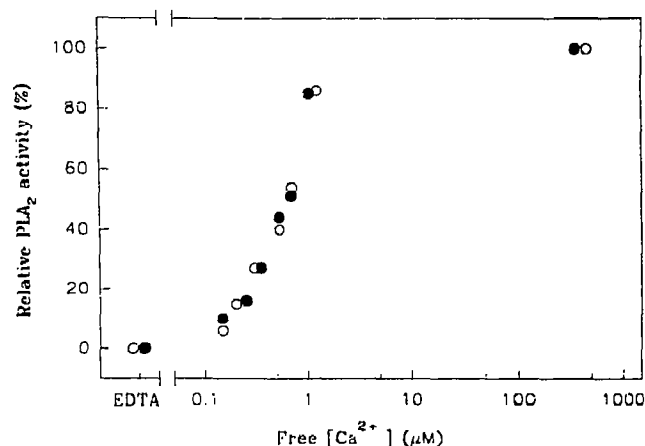


Fig. 4. Ca²⁺-dependence of PLA₂-85 from control and PMA-stimulated macrophages. The activity of PLA₂-85 from control (open symbols) and PMA-stimulated (filled symbols) macrophages was determined as a function of the concentration of free Ca²⁺, using conditions earlier described [2]. The activity is expressed as relative activity (percent of the activity at 400 μM Ca²⁺) to facilitate a comparison. Each point represents the mean of duplicate incubations and is representative of 2–5 separate experiments.

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