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Arachidonic and *cis*-unsaturated fatty acids induce selective platelet substrate phosphorylation through activation of cytosolic protein kinase C

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The ability of arachidonic acid and other fatty acids to induce phosphorylation of endogenous substrates and the role of protein kinase C in mediating these effects were examined. In a cell-free cytosolic system derived from human platelets, arachidonic, oleic, and other *cis*-unsaturated fatty acids induced a dose-dependent phosphorylation of several endogenous substrates. These substrates form a subset of phorbol ester-induced phosphorylations. Multiple lines of evidence suggested the direct involvement of protein kinase C in mediating fatty acid-induced phosphorylations. These observations suggest that arachidonic acid and other unsaturated fatty acids are capable of activating protein kinase C in a physiologic environment resulting in the phosphorylation of multiple endogenous substrates.

Protein kinase C; Arachidonic acid; Phosphorylation; Platelet

1. INTRODUCTION

Protein kinase C has emerged as a key mediator in transmembrane signaling and appears to regulate many cellular functions and responses [1,2]. The finding that protein kinase C is activated by diacylglycerol (DAG) at physiological concentrations of Ca^{2+} [3] led Nishizuka and his co-workers to link the regulation of this enzyme with phosphatidylinositol turnover. In addition, protein kinase C has been identified as the receptor for tumor-promoting phorbol esters [4,5]. This implicated protein kinase C activation in tumor promotion, oncogenesis, and other biological effects of phorbol esters [1].

Protein kinase C is also activated in vitro by arachidonic acid and other *cis*-unsaturated fatty acids [6,7]. The physiologic significance, however, of regulation of protein kinase C by fatty acids has not been determined. This question is particularly important in platelets because arachidonic acid is generated in response to agonist activation of phospholipase A_2 [8]. In human platelets, addition of high concentrations of arachidonic acid led to phosphorylation of 40 kDa and 20 kDa polypeptides. This, however, was accompanied by a two-fold increase in DAG levels and by elevation of intracellular Ca^{2+} [9]. The addition of arachidonic acid to platelet cytosol also resulted in phosphorylation of a 40 kDa protein [9]. These studies do not distinguish between direct effects of arachidonic acid on protein kinase C or indirect effects through the generation of endogenous DAG. We, therefore, asked whether arachidonic acid can induce phosphorylation of endogenous proteins through direct activation of platelet protein kinase C, independent of PI turnover and DAG generation. Demonstrating such an effect is a prerequisite for delineating the physiologic relevance of protein kinase C-activation by arachidonic acid.

Abbreviations: DAG, diacylglycerol; PS, phosphatidylserine; EGTA, ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetra acetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-*N*-2-ethanesulphonic acid; PMSF, phenylmethyl sulfonyl fluoride; PMA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulphate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiprazine; Ab, protein kinase C antibodies; H8, *N*-[2-(methylamino) ethyl]-5-isoquinolinesulfonamide; PKI, protein kinase inhibitor (Type II, bovine heart).

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2. EXPERIMENTAL

2.1. *Materials*

Oleic, acid, linoleic acid, linolenic acid, stearic acid, H7, trypsin, α-chymotrypsin, PGI_2, ATP, calf thymus histone Type III-S, PMSF, PKI and PMA were purchased from Sigma Chemicals. H8 and Pansorbin were from Calbiochem. Arachidonic acid was from Biomol Inc. Leupeptin was from Peptide Institute, Osaka. Triton X-100 was from Research Products International Corp. SDS-PAGE reagents and molecular weight standard markers were from Bio-Rad. [^{32}P]Orthophosphate and [γ^{32}P]ATP were from New England Nuclear. Protein kinase C antibodies were a kind gift from Dr. K.P. Huang, NIH, Bethesda.

2.2. *Preparation of human platelets*

Human platelets were prepared from freshly-drawn blood from healthy volunteers as described by Siess et al. [10]. The platelets were diluted with modified Tyrode's buffer to a final concentration of $2.5\text{-}3.5\times10^8$/ml.

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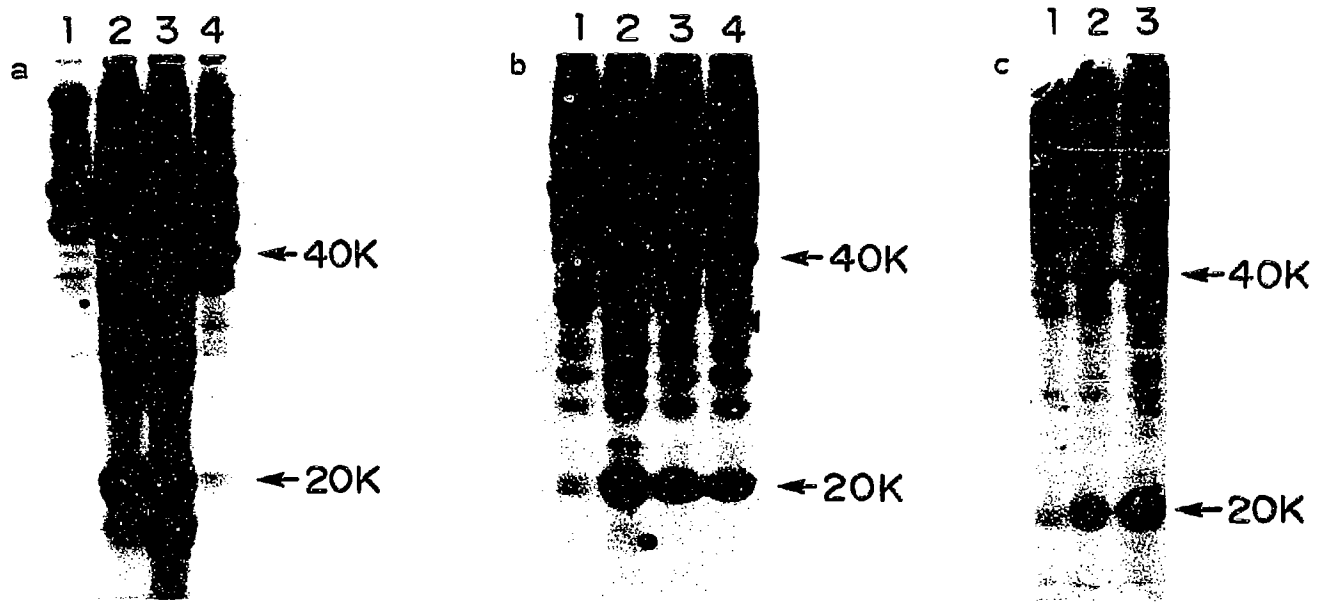

Fig. 1. Phosphorylations in platelet cytosol and in whole platelets. (A) Protein phosphorylation in platelet cytosol. Cytosol from fresh platelets was prepared, and endogenous substrate phosphorylation was carried out as described in section 2. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 0.1 mM Ca^{2+}, 2 μM ATP, 10 mM $MgCl_2$ and 40 μl cytosol in a total volume of 100 μl. (Lane 1) blank (reaction mixture + 10 mM EGTA); (lane 2) 0.1 mM Ca^{2+}; (lane 3) 100 nM PMA + (0.1 mM Ca^{2+} + 10 mol% PS in 3% Triton X-100); (lane 4) 50 μM arachidonic acid (+ 10 mM EGTA). (B) Phosphorylation in whole platelets. Platelets were labeled with [^{32}P]orthophosphate (0.5 mCi/ml) and were treated with PMA or arachidonic acid for indicated time periods. The reaction was stopped by adding an equal volume of 2× sample buffer and subjected to SDS-PAGE as described. (Lane 1) Control platelets; (lane 2) PMA (100 nM, 2 min); (lane 3) arachidonic acid (50 μM, 2 min); (lane 4) arachidonic acid (50 μM, 4 min). (C) γ-Thrombin-induced protein phosphorylation in whole platelets. Platelets were treated with 30 nM γ-thrombin and the reaction was stopped as described for Fig. 1B. (Lane 1) Control platelets; (lane 2) 0.1 min; (lane 3) 0.5 min.

2.3. *Protein phospho ylation in whole platelets*

Phosphorylation studies in intact platelets were carried out as described [11].

2.4. *Protein phosphorylation in platelet cytosol*

Platelets (5.0–7.5 × 10^9) were suspended in 2 ml of homogenizing buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM EGTA, 250 mM sucrose, 1 mM PMSF and 0.02% leupeptin and homogenized by sonication on ice. The homogenate was centrifuged at 100 000 × g at 4°C for 60 min. The resulting supernatant was termed 'platelet cytosol' and was used as the endogenous source of protein kinase C and endogenous substrates. Fatty acid-induced phosphorylation of substrates was carried out in a typical reaction mixture containing 20 mM Tris-HCl pH 7.5, 10 mM EGTA, 2 μM ATP (1.5–2.2 × 10^6 cpm [γ-^{32}P]ATP, spec. act. 75×10^4 cpm/nmol), 10 mM $MgCl_2$, and 40 μl of enzyme/substrate source in a final volume of 100 μl. Incubations with PMA were carried out in the presence of 0.1 mM Ca^{2+} and 3% Triton X-100-mixed micelles containing 10 mol% PS. The reaction was started by the addition of [γ-^{32}P]ATP and incubation was carried out at 30°C for 10 min. Under these conditions, the reaction was linear with time and less than 0.1% of ATP was consumed. Also, no degradation of protein kinase C was detected by Western Blot analysis (data not shown). The reaction was terminated by the addition of 100 μl of 2× sample buffer. Proteins were separated on SDS-PAGE on a 12.5% gel according to the method of Laemmli [12]. The phosphorylated polypeptides were identified by autoradiography, and the appropriate bands were excised and counted in 10 ml of liquid scintillation fluid.

2.5. *Immunoprecipitation of protein kinase C from platelet cytosol by polyclonal antibodies*

Immunoprecipitation of cytosolic protein kinase C was carried out by the procedure of Huang and Huang [13].

2.6. *Peptide mapping of 40 kDa Protein by limited proteolysis*

Peptide mapping of a 40 kDa protein was carried out essentially according to the procedure of Cleveland et al. [14].

2.7. *DAG measurements*

Platelet DAG was measured as described [15].

All results are representative of 3 or more independent experiments.

3. RESULTS AND DISCUSSION

3.1. *Induction of multiple protein phosphorylation in response to arachidonic acid and other fatty acids in platelet cytosol*

To investigate the direct effects of arachidonic acid on platelet substrate phosphorylation and the role of protein kinase C in these phosphorylations, a cell-free system composed of platelet cytosol was utilized. This simplified system offers a closer approximation of physiologic conditions since it contains protein kinase C and physiologic substrates (see below). It also has the advantage of lacking other interfering bio-active lipids.

Under conditions appropriate to assay protein kinase C activity (0.1 mM Ca^{2+} and Triton X-100-mixed micelles containing 10 mol% PS), the addition of PMA resulted in the phosphorylation of several cytosolic proteins that were separated on SDS-PAGE (Fig. 1A, lane 3). Protein phosphorylation in response to PMA was dependent on Ca^{2+} and PS. These proteins were also phosphorylated in intact platelets activated with PMA or

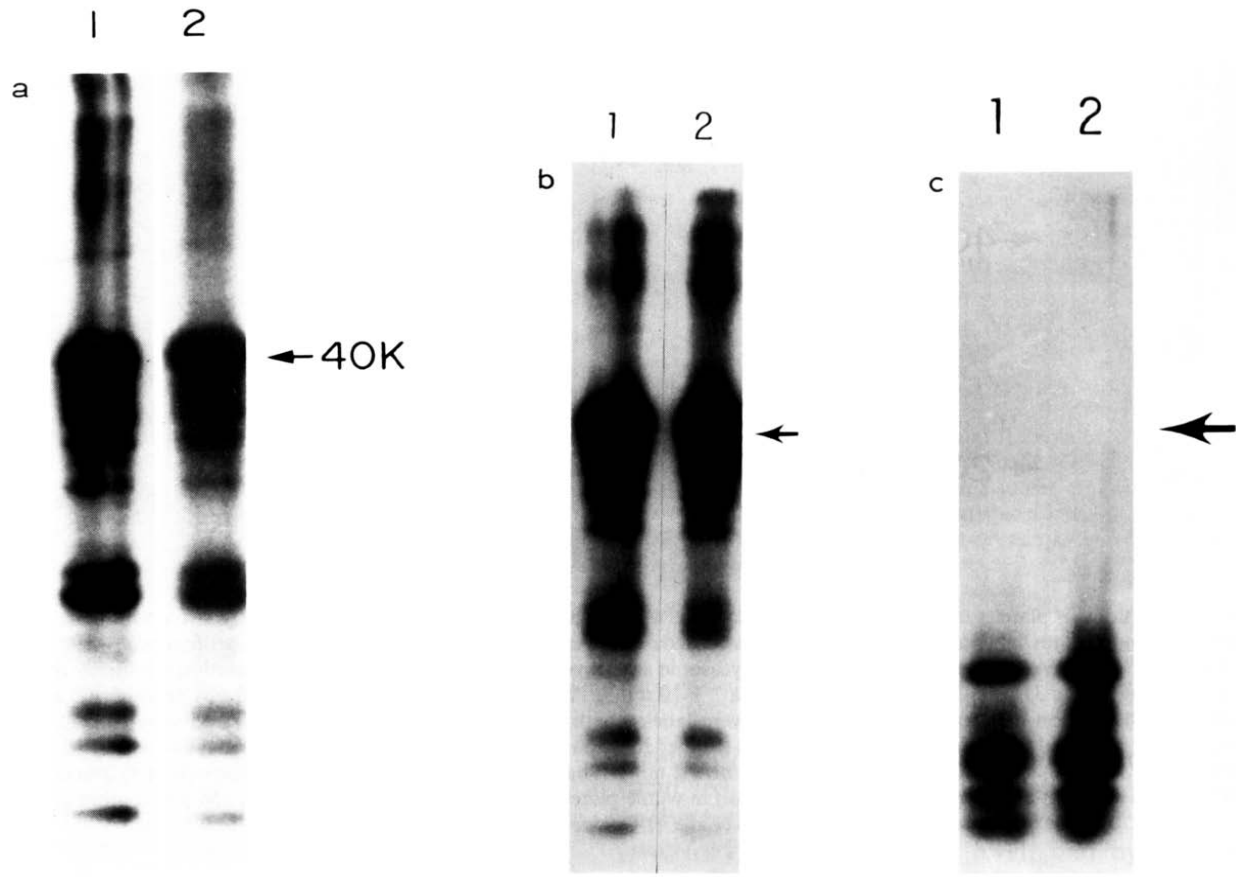

Fig. 2. Partial proteolysis of ^{32}P-labeled 40 kDa substrate by trypsin or α-chymotrypsin. A 40 kDa substrate band phosphorylated in response to PMA (100 nM), arachidonic acid (50 μM) or 50 μM oleic acid in platelet cytosol was excised from the gel and subjected to proteolysis by trypsin or α-chymotrypsin as described in section 2. (A) (Lane 1) PMA; (lane 2) arachidonic acid. (B) (Lane 1) PMA; (lane 2) oleic acid; (C) proteolysis by α-chymotrypsin; (Lane 1) PMA; (lane 2) oleic acid. Visualization of peptide fragments in arachidonic acid-induced 40 kDa substrate was accomplished after 4 days exposure of the film, as compared to 2 days in the case of PMA.

thrombin (Fig. 1B and C) indicating that the in vitro cytosolic system contains all the relevant kinases and substrates.

Since arachidonic acid-induced activation of protein kinase C is independent of phospholipid [7], arachidonic acid was added to platelet cytosol in the absence of phospholipid or mixed micelles. The addition of arachidonic acid (50 μM) to platelet cytosol induced phosphorylation of several proteins (Fig. 1A, lane 4); particularly prominent was the 40 kDa polypeptide known to be an in vitro substrate of protein kinase C [16]. Importantly, these phosphorylations occurred in an environment where no DAG was detected in baseline or arachidonic acid-activated cytosol suggesting a direct involvement of arachidonic acid in activation of protein kinase C. Arachidonic acid-induced phosphorylation was somewhat enhanced by the addition of Ca^{2+}. Calcium, however, was omitted in all further experiments with fatty acids because of its ability to activate calcium calmodulin-dependent kinases.

Proteins phosphorylated in response to arachidonic acid formed a subset of those induced by PMA. Proteins of mol.wt. values of 150, 62, 50, 40 and 20 kDa were phosphorylated by both PMA and arachidonic acid, while proteins of mol.wt. values of 80, 38, 35, 25 and 18 kDa were phosphorylated primarily by PMa only (Fig. 1A). (These protein substrates do not include the phosphorylations mediated by Ca^{2+}.) The extent of the 40 kDa protein phosphorylation in response to arachidonic acid was 40–50% of that effected by PMA.

Arachidonic acid-induced phosphorylation of the 40 kDa protein was found to be dose-dependent with an initial response seen at 1 μM and peaking at 12.5 μM. Other fatty acids, including oleic acid, linoleic acid, linolenic acid, and stearic acid, induced partial phosphorylation of the 40 kDa substrate to 40, 54, 55, and 15% of the phosphorylation seen with PMA.

3.2. *Role of protein kinase C in mediating arachidonic acid-induced phosphorylations*

Because arachidonic acid has multiple targets including calmodulin-dependent kinases [17] and ion channels [18], the role of protein kinase C in mediating the observed effects of arachidonic acid on cytosolic protein phosphorylation was investigated. First, the dose-dependence for induction of phosphorylation of the 40

kDa protein by arachidonic acid in platelet cytosol paralleled the dose-dependence for activation of purified protein kinase C by arachidonic acid in vitro [19]. Second, a kinase-domain inhibitor of protein kinase C, H7 [20] inhibited phosphorylation of the 40 kDa protein induced by PMA as well as that induced by arachidonic acid (Table I). H8, another kinase-domain inhibitor also equally inhibited the PMA or arachidonic acid-induced 40 kDa protein phosphorylation (Table I). PKI (1–50 μg/ml), a specific inhibitor of cAMP-dependent protein kinase, did not inhibit 40 kDa protein phosphorylation induced by either PMA or arachidonic acid. These studies with inhibitors support a role for protein kinase C as a target for arachidonic acid.

To establish the identity of arachidonic acid-induced and PMA-induced phosphorylations, proteolytic fragments of the 40 kDa substrate were studied. The 40 (47) kDa polypeptide is the best studied substrate of protein kinase C in platelets [21–25]. Nishizuka and co-workers showed that the phosphorylated 40 kDa polypeptide in thrombin-activated platelets has a similar tryptic map as purified 40 kDa substrate phosphorylated by protein kinase C in vitro [16]. As shown in Fig. 2, the 40 kDa protein phosphorylated in response to PMA showed identical tryptic fragments as those seen when a 40 kDa protein was phosphorylated by arachidonic acid (Fig. 2A) or oleic acid (Fig. 2B). Proteolysis by α-chymotrypsin showed identical fragments in response to PMA and oleic acid (Fig. 2C). These results demonstrate that PMA, arachidonic acid and oleic acid induce the phosphorylation of the same 40 kDa protein. Moreover, these phosphorylations are likely to be mediated by the same kinase since they show identical phosphorylated proteolytic fragments.

Finally, and to further demonstrate that the arachidonic acid-induced phosphorylations are mediated by protein kinase C, platelet cytosol was depleted of protein kinase C with the use of polyclonal polyspecific

Table I

Comparison of inhibition by H7 or H8 of phosphorylation of a 40 kDa protein in platelet cytosol induced by PMA or arachidonic acid

Inhibitor	Concentration (μM)	Inhibition (%)	
		PMA	Arachidonic acid
None	–	0	0
H7	1	10	5
	10	35	20
	50	65	50
H8	1	27	14
	10	52	73
	50	82	96

Phosphorylation in platelet cytosol was performed as described for Fig. 1A.

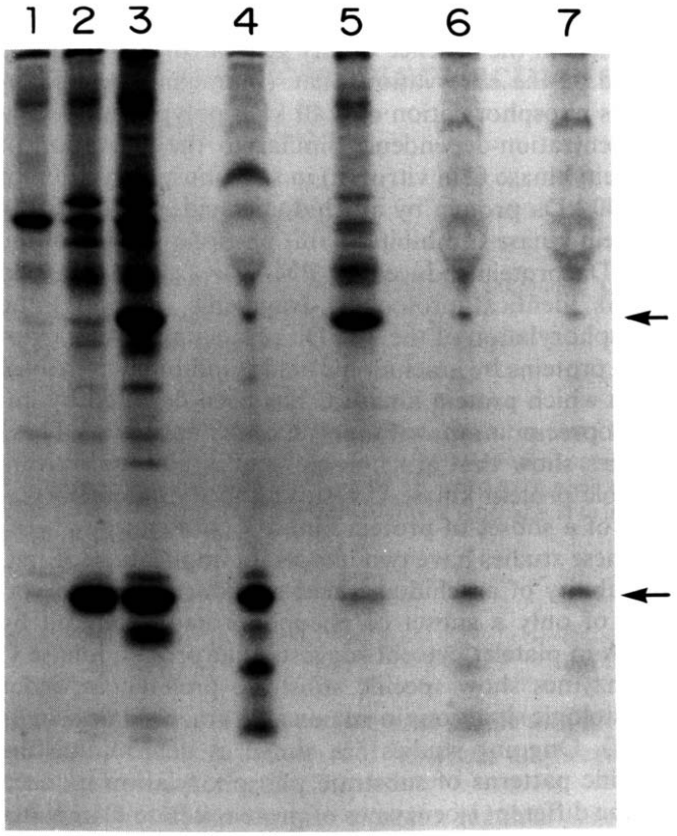

Fig. 3. Antibody inhibition of the 40 kDa substrate phosphorylation induced by PMA or arachidonic acid in platelet cytosol. Platelet cytosol (20 μl) was incubated with 100 μg of antiserum protein on ice for 2 h. After precipitating the antigen–antibody complex by Pansorbin the supernatant was taken out for phosphorylation as described in section 2. (Lane 1) Blank (+ 10 mM EGTA); (lane 2) 0.1 mM Ca^{2+}; (lane 3) 100 nM PMA + Ca^{2+} + PS; (lane 4) 100 μg Ab + PMA + Ca^{2+} + PS; (lane 5) 50 μM arachidonic acid, (lane 6) 100 μg Ab + arachidonic acid; (lane 7) duplicate of lane 6. The arrows on the top and the bottom of the gel indicate the position of the 40 kDa and 20 kDa substrate, respectively.

antibodies. These antibodies inhibited the activity of platelet protein kinase C in vitro assays with near-complete immunoprecipitation of protein kinase C activity using 25–100 μg of antibody as previously shown for rat brain enzyme [13]. When platelet cytosol was preincubated with polyspecific antibody (100 μg) against protein kinase C for 2 h on ice, the phosphorylation of platelet cytosolic proteins in response to PMA was nearly completely inhibited (Fig. 3). Under these conditions, the response to arachidonic acid was also nearly completely inhibited with only 10% phosphorylation of the 40 kDa protein remaining (Fig. 3). Control preimmune serum had no such effect. These results indicate that the phosphorylation of platelet cytosolic proteins in response to arachidonic acid involves the activation of protein kinase C.

In the present study, we evaluated the possible regulation of protein kinase C by arachidonic acid in a physiologic environment with endogenous substrates. We show that arachidonic acid can directly activate

protein kinase C in a cell-free cytosolic preparation of platelets in the absence of any generated DAG. This is based on the observations that: (i) arachidonic acid induces phosphorylation of a 40 kDa polypeptide with a concentration-dependence similar to the activation of protein kinase C in vitro; (ii) in situ phosphorylation of the 40 kDa protein by arachidonic acid is inhibited by protein kinase C inhibitors; (iii) phosphorylation of the 40 kDa protein induced by PMA or arachidonic acid shows identical proteolytic fragments; and, (iv) the phosphorylation of the 40 kDa protein and other cytosolic proteins by arachidonic acid is inhibited in cytosol from which protein kinase C has been depleted by immunoprecipitation with polyspecific antibody. These studies show that arachidonic acid is able to activate soluble protein kinase C resulting in the phosphorylation of a subset of protein kinase C substrates.

These studies have two important implications. First, the ability of arachidonic acid to induce phosphorylation of only a subset of phosphoproteins induced by PMA in platelet cytosol suggests that protein kinase C isoenzymes show specific substrate preferences under physiologic situations in response to arachidonic acid or DAG. Ongoing studies are aimed at determining the specific patterns of substrate phosphorylation induced by the different isoenzymes of protein kinase C in platelet cytosol. Second, these studies strongly point to a possible physiologic role for arachidonic acid in modulating protein kinase C activity. The free generated arachidonic acid in activated platelets may achieve intracellular levels in the μM range [26]. These levels appear to be sufficient to activate protein kinase C. Arachidonic acid may, therefore, play a role in modulating the activity of cytosolic protein kinase C. Further studies are required to delineate the precise physiologic function of arachidonic acid as a second messenger modulating protein kinase C activity.

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