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Characterization of bovine aortic protein kinase C with histone and platelet protein P47 as substrates

Karen R. DELL,* Michael P. WALSH† and David L. SEVERSON*‡

*Department of Pharmacology and Therapeutics and †Department of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada

A Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) was partially purified from the media of bovine aortas by chromatography on DEAE-Sephacel and phenyl-Sepharose. Enzyme activity was characterized with both histone and a 47 kDa platelet protein (P47) as substrates, because the properties of protein kinase C can be modified by the choice of substrate. Both phosphatidylserine and Ca^{2+} were required for kinase activity. With P47 as substrate, protein kinase C had a K_a for Ca^{2+} of 5 μM. Addition of diolein to the enzyme assay caused a marked stimulation of activity, especially at low Ca^{2+} concentrations, but the K_a for Ca^{2+} was shifted only slightly, to 2.5 μM. With histone as substrate, the enzyme had a very high K_a (> 50 μM) for Ca^{2+}, which was substantially decreased to 3 μM-Ca^{2+} by diolein. A Triton X-100 mixed-micelle preparation of lipids was also utilized to assay protein kinase C with histone as the substrate. Under these conditions kinase activity was almost totally dependent on the presence of diolein; again, diolein caused a large decrease in the K_a for Ca^{2+}, from > 100 μM to 2.5 μM. The increased sensitivity of protein kinase C to Ca^{2+} with P47 rather than histone, and the ability of diacylglycerol to activate protein kinase C without shifting the K_a for Ca^{2+}, when P47 is the substrate, illustrate that the mechanism of protein kinase C activation is influenced by the exogenous substrate used to assay the enzyme.

INTRODUCTION

A variety of signals such as some hormones and growth factors can activate phospholipase C in the membrane of target cells (Berridge, 1987). The resulting degradation of phosphatidylinositol 4,5-bisphosphate leads to the production of two second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DG). $InsP_3$ releases Ca^{2+} stored in the endoplasmic reticulum and DG activates the Ca^{2+}- and phospholipid-dependent protein kinase, usually referred to as protein kinase C (Nishizuka, 1986). The role of protein kinase C in cell regulation can be investigated by using cell-permeable DGs such as 1-oleoyl-2-acetylglycerol (OAG) or 1,2-dioctanoylglycerol, or with phorbol esters, which also activate the enzyme (Nishizuka, 1986).

In aortic smooth-muscle cells (SMC), angiotensin II stimulates phosphoinositide breakdown and the production of $InsP_3$, resulting in the increase in intracellular Ca^{2+} that is required for muscle contraction (Alexander *et al.*, 1985). Treatment of cultured aortic SMC with phorbol esters or OAG to activate protein kinase C prevented the subsequent angiotensin II-induced production of $InsP_3$, probably by modulating the coupling of the angiotensin II receptor to phospholipase C (Brock *et al.*, 1985). Activation of protein kinase C with phorbol esters also attenuated the response of aortic SMC to vasopressin and adrenergic agonists (Aiyar *et al.*, 1986). Interestingly, the treatment of aortic strips with phorbol esters alone caused a slow muscle contraction (Itoh & Lederis, 1987). Regulation of Ca^{2+} channels (Galizzi *et al.*, 1987) or stimulation of the Na^+/H^+ exchange system (Berk *et al.*, 1987) may be involved in the acute response of aortic SMC to activation of the protein kinase C pathway.

In addition to modulating aortic SMC contraction, protein kinase C may have a role in regulating proliferative and metabolic responses of aortic SMC to growth factors such as platelet-derived growth factor (PDGF) (Bowen-Pope *et al.*, 1985). PDGF has been reported to stimulate phosphoinositide turnover (Habenicht *et al.*, 1981), and, in aortic SMC, PDGF increased the intracellular Ca^{2+} concentration (Berk *et al.*, 1986) and stimulated Na^+ influx (Owen, 1984). Phorbol esters increased DNA synthesis in cultured rabbit aortic SMC (Kariya *et al.*, 1987), and so the protein kinase C pathway may also be involved in the proliferation of aortic SMC that is a characteristic feature of atherosclerotic lesions (Schwartz *et al.*, 1986).

In this study, we have partially purified protein kinase C from bovine aorta and characterized the enzyme using two substrates: lysine-rich histone and a 47000-Da protein from platelets (P47) which is known to be an endogenous substrate for the enzyme (Kaibuchi *et al.*, 1983).

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (> 10 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Frozen bovine aortas were purchased from Pel Freez Biologicals Inc. (Rogers, AR, U.S.A.) Outdated human platelets

Abbreviations used: $InsP_3$, inositol trisphosphate; DG, diacylglycerol; OAG, 1-oleoyl-2-acetylglycerol; SMC, smooth-muscle cells; PDGF, platelet-derived growth factor; P47, 47000 Da platelet protein; PS, phosphatidylserine; PMSF, phenylmethanesulphonyl fluoride.

‡ To whom reprint requests should be addressed.

were donated by the Foothills Hospital Blood Bank (Calgary, Alberta, Canada). Histone III-S was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphatidylserine (PS; bovine brain), 1,2-diolein, 1-stearoyl-2-arachidonoylglycerol, OAG, CDP-diacylglycerol (dioleoyl), 1,2-dioctanoylglycerol, dioleoylphosphatidic acid and arachidonic acid were all purchased from Serdary Research Laboratories (London, Ontario, Canada). DEAE-Sephacel and phenyl-Sepharose were purchased from Pharmacia (Mississauga, Ontario, Canada), hydroxyapatite was from Calbiochem (La Jolla, CA, U.S.A.) and gel-electrophoresis reagents and M_r markers were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Vinculin, α-actinin and filamin were purified from chicken gizzard by the method of Feramisco & Burridge (1980).

Purification of protein kinase C

Protein kinase C was partially purified from bovine aorta by modifications to the procedure described by Kikkawa *et al.* (1983). All procedures were performed at 4 °C or on ice. Frozen bovine aortas (30 g) were thawed, and the adventitia and intima were removed by dissection. The media fraction was homogenized in a Waring blender for 3 × 10 s, and then with a Brinkman Polytron at setting 6 for 30 s, in 200 ml of buffer A [0.25 M-sucrose/2 mM-EDTA/10 mM-EGTA/20 mM-Tris/HCl (pH 7.5)/leupeptin (1 mg/l)/pepstatin (1 mg/l)/soya-bean trypsin inhibitor (1 mg/l)/0.5 mM-phenylmethanesulphonyl fluoride (PMSF)]. After centrifugation at 1000 *g* for 15 min, the low-speed supernatant was removed and the pellet was resuspended in 100 ml of buffer A, homogenized and centrifuged again at 1000 *g* for 15 min. The low-speed supernatants were combined and centrifuged at 100000 *g* for 60 min. The resulting supernatant was loaded on a DEAE-Sephacel column (2.5 cm × 40 cm) equilibrated with buffer B [1 mM-dithiothreitol/2 mM-EDTA/5 mM-EGTA/20 mM-Tris/HCl (pH 7.5) and proteinase inhibitors as in buffer A]. The column was washed with buffer B at 75 ml/h until A_{280} returned to baseline (about 500 ml), followed by 200 ml of buffer C [1 mM-dithiothreitol/1 mM-EDTA/1 mM-EGTA/20 mM-Tris/HCl (pH 7.5) and proteinase inhibitors as in buffers A and B]. Protein kinase C was eluted from the column with a linear gradient made from 600 ml each of buffer C and buffer C containing 0.3 M-NaCl. Fractions with protein kinase C activity (measured by an increase in activity in the presence of Ca^{2+} and PS over activity with Ca^{2+} alone; see below) were pooled and concentrated by the addition of solid $(NH_4)_2SO_4$ to 70% saturation, followed by dialysis overnight against 2 × 10 l of buffer D [1 mM-dithiothreitol/5 mM-EGTA/20 mM-Tris/HCl (pH 7.5)]. Enzyme activity was stable at −80 °C in the presence of 10% (v/v) glycerol and soya-bean trypsin inhibitor (0.2 mg/ml) for up to 4 months.

As enzyme was required, a portion of the DEAE-Sephacel fraction (7–10 mg) was adjusted to 1 M-NaCl and loaded on a phenyl-Sepharose column (1 cm × 10 cm) previously equilibrated with buffer E [20 mM-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/1 mM-EGTA/1 M-NaCl]. The column was washed with 15 ml of buffer E, and proteins were eluted by a linear gradient (40 ml) of 1 M- to 0 M-NaCl in buffer E, followed by a 15 ml wash with buffer E with 0 M-NaCl. Column fractions with protein kinase C activity were pooled and stored for up to 2 weeks at −80 °C with 10% glycerol and soya-bean trypsin inhibitor (0.2 mg/ml).

Protein kinase C from rat brain was purified by DEAE-Sephacel and phenyl-Sepharose chromatography as described above.

Purification of platelet P47 protein

P47 was partially purified by a modification of the method of Imaoka *et al.* (1983). Platelet lysates were prepared as described by Lim *et al.* (1985). Briefly, human platelets (10–20 units; 800–1600 ml) were centrifuged at 600 *g* for 20 min at room temperature. The supernatant (platelet-rich plasma) was then centrifuged at 16000 *g* for 20 min. After removal of the plasma supernatants, the platelet pellet was gently resuspended in 500 ml of washing buffer [10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/5 mM-EDTA] at room temperature and centrifuged again at 16000 *g* for 10 min. The washing buffer was discarded and the platelet pellet was resuspended in lysis buffer [5 mM-Tris/HCl (pH 7.5)/5 mM-EDTA] at 0–4 °C and frozen. Combined lysates from about 50 units of platelets were thawed in a 25 °C water bath, PMSF was added (final concn. 0.1 mM), and the lysate was homogenized with a motor-driven Potter–Elvehjem homogenizer. Solid $(NH_4)_2SO_4$ was added to 58% saturation, and the solution was gently stirred for 20 min before centrifugation at 15000 *g* for 20 min (Imaoka *et al.*, 1983). The pellet was discarded, and $(NH_4)_2SO_4$ was added to the supernatant to saturation, followed by stirring for 20 min and centrifugation at 15000 *g* for 30 min. The supernatant was discarded, and the pellet was resuspended in a minimum volume of buffer F [20 mM-Tris/HCl (pH 7.5)/1 mM-EGTA/0.5 mM-dithiothreitol] and dialysed against 2 × 10 l of buffer F overnight. The dialysed sample was loaded on a DEAE-Sephacel column (2.5 cm × 40 cm) and the column was washed with buffer F at 50 ml/h until A_{280} returned to baseline. Proteins were eluted with a linear gradient made from 600 ml each of buffer F and buffer F containing 0.35 M-NaCl. Fractions containing P47 were identified by examination on SDS/polyacrylamide-gel electrophoresis and by their ability to act as substrates for aortic protein kinase C when substituted for histone in the assay mixture. Fractions were also assayed for endogenous protein kinase C activity with histone as the substrate. Appropriate fractions were pooled and stored at −80 °C.

The presence of endogenous protein kinase C activity in some P47 preparations required an additional purification step using hydroxyapatite (Imaoka *et al.*, 1983). The DEAE-Sephacel pool of P47 was dialysed against 2 × 10 l of buffer G [50 mM-potassium phosphate (pH 7.0)/0.5 mM-dithiothreitol/0.1 mM-PMSF], benzamidine was added (final concn. 1 mM), and the solution was loaded on a hydroxyapatite column (1.5 cm × 30 cm) equilibrated with buffer G containing 1 mM-benzamidine. After washing with buffer G (15 ml/h), proteins were eluted from the column by a linear potassium phosphate gradient (50–200 mM) in 200 ml of buffer G, followed by a final wash with 500 mM-potassium phosphate in buffer G. P47 was identified as described above, and appropriate fractions were pooled and stored at −80 °C.

Protein kinase C assays

The activity of protein kinase C was measured by two different assay methods. In some experiments, enzyme

activity was measured essentially by the method of Kikkawa *et al.* (1982). Briefly, the enzyme (2–3 μg of phenyl-Sepharose pool) was incubated with histone III-S (0.2 mg/ml)/20 mM-Pipes/HCl (pH 6.5)/5 mM-$MgCl_2$/PS (40 μg/ml)/0.5 mM-$CaCl_2$. 1,2-Diolein (3.2 μg/ml) was sometimes added with PS. Lipids were dried under N_2 and sonicated into water before addition to the assay, and so activity measured by this method is referred to as the liposomal assay. Reactions were started by adding [γ-^{32}P]ATP (100000 c.p.m./nmol) to a final concentration of 10 μM. The total reaction volume was 250 μl. After incubation for 10 min at 30 °C, the reaction was stopped with 25% (w/v) trichloroacetic acid/2% (w/v) $Na_4P_2O_7$, and protein-bound radioactivity was determined (Walsh *et al.*, 1983). As noted in the text, the Ca^{2+} concentration in the assay was sometimes varied from 0.1 to 100 μM (pCa 7–4) with the aid of Ca^{2+}/EGTA buffer systems, by using association constants for Ca^{2+} and H^+ binding to EGTA at pH 6.5 determined by Fabiato (1981). Protein kinase C activity was also measured in assays where the histone substrate was replaced by either P47 (0.15 mg/ml) or vinculin (0.2 mg/ml).

Protein kinase C activity was also determined with a mixed-micellar assay modified from the procedure described by Hannun *et al.* (1985), where lipids are present in a physically defined system. Appropriate quantities (determined as mol% of Triton X-100) of PS and diolein in chloroform were combined in a glass tube and the chloroform was evaporated under N_2. A 0.3% (w/v) Triton X-100 solution was added to the lipids, vortex-mixed briefly, and then sonicated at 75 W for 30 s. After incubation for 10 min at 27 °C, 25 μl of the mixed micelle solution was added to the assay volume of 250 μl; the final assay incubation contained 0.03% Triton X-100, PS (20 or 80 mol%; 73 or 290 μg/ml), 1,2-diolein (20 mol%; 57 μg/ml), and the other assay components listed above.

Protein kinase C activity is routinely expressed as nmol of P_i incorporated/min per mg of protein. Kinase activity was linear with respect to time (up to 15 min of incubation) and protein (up to 3.2 μg of enzyme protein per assay).

Other methods

Gel electrophoresis was performed in 10%- or 7.5–20%-polyacrylamide slab gels (1.5 mm thick), with a 5%-acrylamide stacking gel, in the presence of 0.1% (w/v) SDS at 36 mA, in the discontinuous buffer system of Laemmli (1970). Autoradiography was done with Kodak X-Omat AR diagnostic film in Kodak X-Omatic cassettes with intensifying screens.

Protein concentrations were determined by the Coomassie Blue spectrophotometric assay (Spector, 1978), with dye reagent purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.).

RESULTS AND DISCUSSION

Purification of protein kinase C

The medial fraction of bovine aortas was homogenized in a buffer containing EGTA and a variety of proteinase inhibitors in order to isolate protein kinase C from the soluble subcellular fraction in a form that was not modified by proteolysis (Kikkawa *et al.*, 1983). Treatment of particulate fractions with Triton X-100 and EGTA (Katoh & Kuo, 1982) did not solubilize significant amounts of protein kinase C activity (results not shown). Protein kinase C activity could not be determined reliably in the high-speed supernatant, probably owing to the presence of Ca^{2+}- and phospholipid-independent kinases, phosphatases and perhaps inhibitors (McDonald *et al.*, 1987), but was easily detected after elution from DEAE-Sephacel columns at a NaCl concentration of 0.1–0.15 M. Further chromatography on phenyl-Sepharose resulted in a 6–12-fold increase in specific activity; protein kinase C activity was eluted when the NaCl concentration was lowered to approx. 0.1 M. This partially purified preparation was utilized in subsequent characterization studies. The aortic protein kinase C preparation was not homogeneous when subjected to polyacrylamide gel electrophoresis in the presence of SDS, but the addition of cyclic AMP or calmodulin did not increase histone phosphorylation. Multiple isoenzymes of protein kinase C have been detected (Huang *et al.*, 1987), and the distribution of isoenzymes may be cell-specific. Further investigations will have to determine which isoenzyme(s) is(are) present in aorta. The presence of protein kinase C in bovine aorta is consistent with the previous report of protein kinase C activity in a crude extract of rat aortas (Kuo *et al.*, 1980).

Substrate specificity

Protein kinase C activity has been most frequently characterized in assays with histone as a convenient substrate; however, several other proteins have been identified as substrates *in vitro* (Nishizuka, 1986). For example, protein kinase C preparations have been reported to phosphorylate smooth-muscle proteins such as heavy meromyosin (Nishikawa *et al.*, 1984) and myosin light-chain kinase (Ikebe *et al.*, 1985), and phorbol ester treatment of bovine trachea resulted in the phosphorylation of desmin, synemin and caldesmon (Park & Rasmussen, 1986). Except in a few instances (e.g. Ahmad *et al.*, 1984), protein kinase C activity determined with non-histone substrates has not been fully characterized with respect to Ca^{2+}-dependency and activation by DG. Bazzi & Nelsestuen (1987) have divided protein kinase C substrates into three categories: (*a*) those requiring no cofactors (protamine); (*b*) those requiring only phospholipid (myelin basic protein, synthetic copolymers); and (*c*) those requiring both Ca^{2+} and phospholipid (histone, troponin I, myosin light chains). Interestingly, significant DG activation was observed only with category-(*c*) substrates (Bazzi & Nelsestuen, 1987).

Since the substrate can have such an influence on the apparent properties of protein kinase C, it was our objective to characterize the partially purified aortic protein kinase C with both histone and non-histone substrates. Vinculin is an actin-binding cytoskeletal protein that has been identified as a protein kinase C substrate both in experiments *in vitro* and in studies with intact cells (Werth *et al.*, 1983; Werth & Pastan, 1984). The phosphorylation of cytoskeletal proteins could be involved in the migration of aortic SMC from the media to the intima during atherogenesis (Schwartz *et al.*, 1986). As shown in Table 1, however, aortic protein kinase C activity determined with vinculin was only 12% of that measured with histone as substrate. These results are in contrast with experiments with brain protein

Table 1. Substrate specificity of aortic protein kinase C

Enzyme activity was measured in the presence of Ca^{2+} (0.5 mM) and in the presence or absence of phosphatidylserine (PS; 40 μg/ml), with histone (0.2 mg/ml), vinculin (0.2 mg/ml) or P47 (0.15 mg/ml) as the substrates. Results from two experiments with different enzyme preparations are shown.

Substrate	Protein kinase C activity (nmol of P_i incorporated/min per mg)	
	+Ca−PS	+Ca+PS
Histone III-S	0.11, 0.34	2.06, 1.74
Vinculin	0.05, 0.06	0.19, 0.29
P47	0.01, 0.02	2.02, 2.34

kinase C, where vinculin was a better substrate than histone (Werth *et al.*, 1983), but results from our laboratory with protein kinase C purified from rat brain indicated that vinculin was not as good a substrate as histone (K. Dell, unpublished work). The results with the aortic enzyme are also consistent with assays performed with extracts of BC3H-1 myocytes (Cooper *et al.*, 1987). The phosphorylation of α-actinin and filamin by aortic protein kinase C could not be detected (results not shown).

A 47000-Da protein (P47) is phosphorylated by protein kinase C in intact platelets stimulated by thrombin and OAG (Imaoka *et al.*, 1983; Kaibuchi *et al.*, 1983), but studies *in vitro* of the cofactor requirements for this phosphorylation have been restricted to autoradiographic experiments (Sano *et al.*, 1983). Aortic protein kinase C activity measured with P47 was equivalent to the activity determined with histone (Table 1). The platelet P47 preparation was not homogeneous, as shown by a Coomassie Blue-stained polyacrylamide gel (Fig. 1), but an autoradiogram indicated that a 47-kDa protein was the only phosphoprotein detected after incubation with aortic protein kinase C. The major low-molecular-mass protein (21.5 kDa) seen by Coomassie Blue staining (Fig. 1) was soya-bean trypsin inhibitor from the protein kinase C preparation.

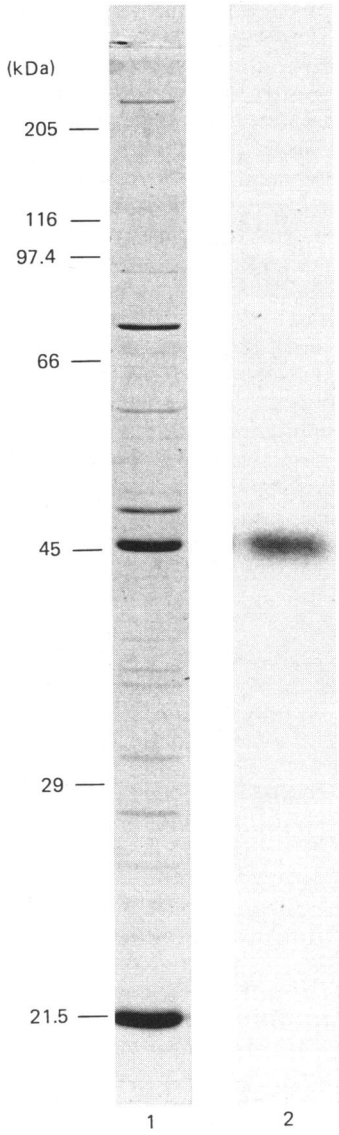

Fig. 1. Phosphorylation of P47 by protein kinase C

P47 purified by chromatography on DEAE-Sephacel (see the Materials and methods section), was phosphorylated by aortic protein kinase C in a standard assay and examined by 0.1%-SDS/10%-polyacrylamide-gel electrophoresis and autoradiography. Lane 1, Coomassie Blue-stained gel; lane 2, autoradiogram. The relative migrations of molecular-mass marker proteins are indicated (values in kilodaltons).

Characterization of protein kinase C

The phosphorylation of P47 was very dependent on the presence of both PS and Ca^{2+} (Table 1). The effect of different concentrations of PS on protein kinase C activity is shown in Fig. 2. PS (80 μg/ml) resulted in a 60-fold increase in kinase activity in assays with 10 μM-$CaCl_2$. The effect of PS on protein kinase C was much less at 0.1 μM-Ca^{2+}. Increasing the Ca^{2+} concentration from 0.1 μM to 10 μM had no effect on protein kinase C activity measured in the absence of PS (Fig. 2).

The effect of Ca^{2+} on aortic protein kinase C activity was determined with both histone and P47 as substrates (Fig. 3). In assays with histone, Ca^{2+} (0.1–100 μM) in the absence of PS also had no effect on protein kinase C activity (Fig. 3*a*). In the presence of PS, kinase activity was increased by concentrations of Ca^{2+} greater than 1 μM, but an optimum was not reached by 100 μM, and so a K_a value could not be determined accurately, but could be estimated to be > 50 μM. By comparison, the K_a for Ca^{2+} was 5 μM with P47 (Fig. 3*b*) and 4 μM with vinculin (results not shown).

DG was originally reported to activate protein kinase C by decreasing the requirement for Ca^{2+}, from a K_a value of 70 μM to 4–8 μM (Kishimoto *et al.*, 1980). Similar results were obtained with the aortic enzyme in assays with histone, where the presence of diolein (3.2 μg/ml) resulted in a decrease in the K_a for Ca^{2+} to approx. 3 μM (Fig. 3*a*). Diolein has been shown to decrease the K_a for Ca^{2+} to values of 5–10 μM in studies with many different protein kinase C preparations assayed with histone (e.g. Wise *et al.*, 1982; Noguchi *et al.*, 1985). Nevertheless,

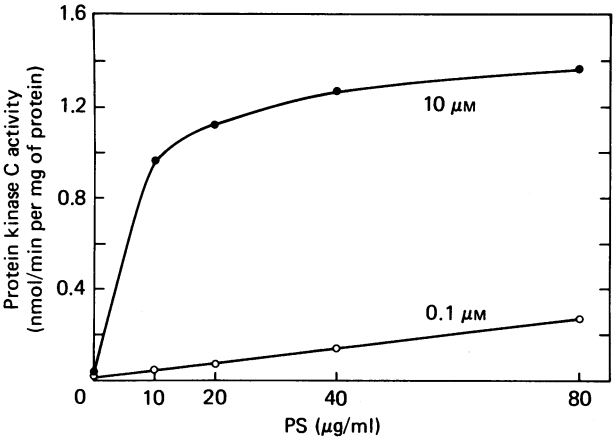

Fig. 2. Effect of PS concentration on protein kinase C activity

Protein kinase C activity was measured at the indicated concentrations of PS in liposomal assays with P47 as substrate and either 0.1 μM-Ca^{2+} (○) or 10 μM-Ca^{2+} (●). Results are the means of three experiments with different enzyme preparations.

there are several reports where DG has had little or no effect on protein kinase C activity (Schatzman *et al.*, 1983; Le Peuch *et al.*, 1983).

Diolein also increased aortic protein kinase C activity measured with P47 as substrate (Fig. 3*b*), consistent with effects of exogenous DG on intact platelets (Kaibuchi *et al.*, 1983). The activation by diolein was particularly marked at low concentrations of Ca^{2+}, but the K_a for Ca^{2+} was only modestly decreased to approx. 2.5 μM.

The effect of diolein on kinase C activity was concentration-dependent, with maximal activation at 3.2 μg/ml. The stimulation of aortic protein kinase C activity by diolein (3.2 μg/ml), determined with P47 as substrate, was 2.9-fold and 1.4-fold at 0.1 μM- and 10 μM-Ca^{2+} respectively. For assays with histone as substrate, the diolein activation was 4.1-fold and 2-fold at 0.1 μM- and 10 μM-Ca^{2+} respectively (mean of three experiments with different enzyme preparations).

Hannun *et al.* (1985, 1986) described a mixed-micellar assay in which protein kinase C activity was almost completely dependent on the presence of DG. With their experimental conditions, PS and diolein are dispersed into 3% Triton X-100, and assay incubations are performed at a final detergent concentration of 0.3%. When the aortic protein kinase C was assayed under these conditions, no activity was detected with either histone or P47 substrates. Therefore the influence of Triton X-100 on kinase activity was determined (Fig. 4). In assays with histone as substrate, the presence of low concentrations of Triton X-100 produced a profound inhibition of protein kinase C activity unless diolein was present (Fig. 4*a*). When lipids were dispersed into 0.3% Triton X-100 and kinase C activity measured at a final detergent concentration of 0.03% in the assay, diolein produced an 11-fold activation. For kinase assays with P47 as substrate, Triton X-100 inhibited activity determined in the absence of diolein (Fig. 4*b*). In the presence of diolein, the detergent resulted in an initial increase in activity that was followed by inhibition. Dispersion of lipids into a Triton X-100 concentration of less than 0.2% would result in a concentration in the final assay incubation that was less than the critical micellar concentration, and so further experiments with the micellar assay were performed only with histone as substrate.

Protein kinase C activity measured with 10 μM-Ca^{2+} and diolein (20 mol%; 57 μg/ml) had a K_a for PS of 13 mol% and was optimal at 80 mol% PS (290 μg/ml;

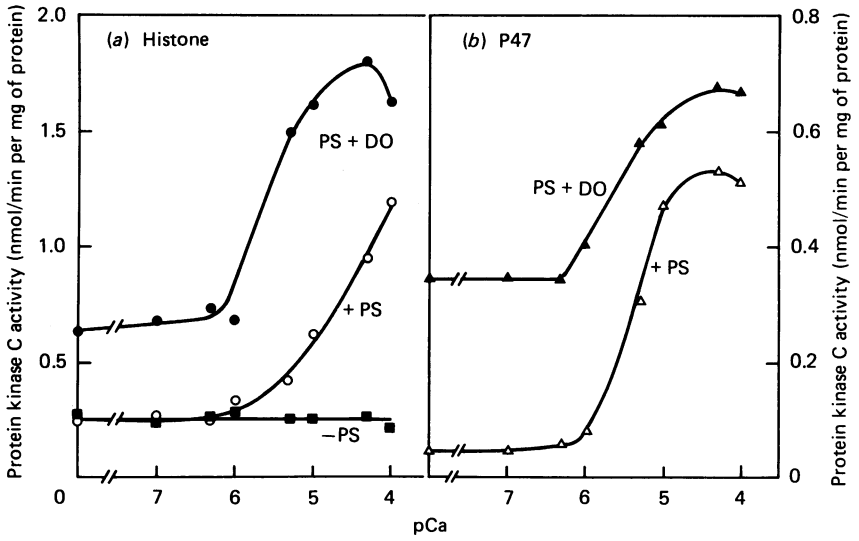

Fig. 3. Effect of Ca^{2+} on protein kinase C activity

Protein kinase C activity was measured in the presence of the indicated concentrations of Ca^{2+} under the following assay conditions: 40 μg of PS/ml plus 3.2 μg of diolein/ml (PS + DO; ●, ▲), 40 μg of PS/ml (PS; ○, △) or no lipids (■). Liposomal assays were performed with either histone (*a*) or P47 (*b*) as substrate (results are means of two experiments). Results were confirmed in experiments with at least two different enzyme preparations.

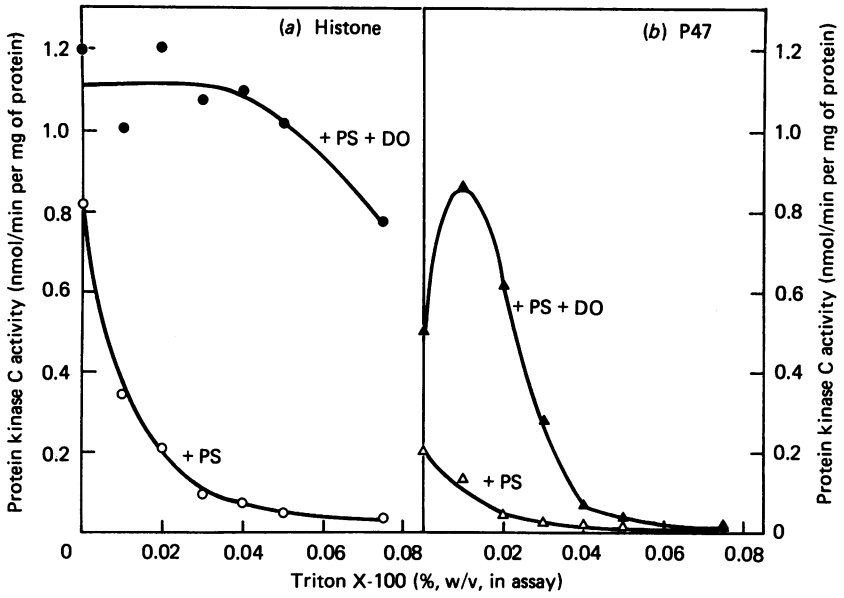

Fig. 4. Effect of Triton X-100 on protein kinase C activity measured with a micellar assay

Micelles containing a fixed concentration of PS were prepared in the absence (PS; ○, △) and in the presence of diolein (PS + DO; ●, ▲). The final assay concentrations of PS and DO were 290 μg/ml and 57 μg/ml respectively. Protein kinase C activity was determined in the presence of 10 μM-Ca^{2+} and the indicated final concentrations of Triton X-100 (%, w/v) in the assay.

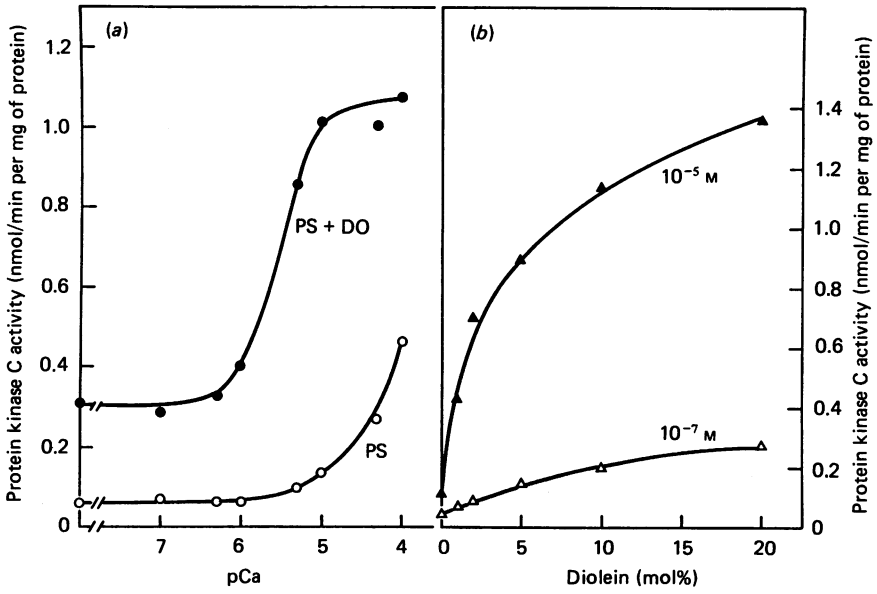

Fig. 5. Effect of Ca^{2+} and diolein on protein kinase C activity measured by the Triton X-100 micellar assay

In (*a*), protein kinase C activity was measured at the indicated Ca^{2+} concentrations with PS (80 mol%) and in the absence (○) and in the presence (●) of diolein (DO; 20 mol%). Results are means from two experiments. In (*b*) kinase activity was measured with PS (20 mol%) and either (△) 0.1 μM- or (▲) 10 μM-Ca^{2+} at the indicated concentrations of 1,2-diolein.

results not shown). In the presence of PS, protein kinase C activity was increased when Ca^{2+} concentrations were greater than 5 μM and, although no maximum was reached, a K_a of > 100 μM could be estimated (Fig. 5*a*). In the presence of diolein, protein kinase C activity was increased markedly, and the K_a for Ca^{2+} was decreased to 2.5 μM. These results are very similar to the Ca^{2+}-dependency observed with the liposomal assay (Fig. 3*a*), and to the results of Hannun *et al.* (1986) with brain protein kinase C. The effect of different concentrations of diolein is shown in Fig. 5(*b*); the diolein activation was much greater at 10 μM-Ca^{2+} (11-fold) than at 0.1 μM (6.5-fold).

Some DG analogues were examined for their ability to activate protein kinase C; assays were performed at 0.1 μM-Ca^{2+} with both histone and P47 substrates (Table

Table 2. Effect of diacylglycerol analogues and metabolites on protein kinase C activity

Protein kinase C activity was measured at 0.1 μM-Ca^{2+} in micellar assays with histone and PS (80 mol%) and in liposomal assays with PS (40 μg/ml) and either histone or P47 as substrates and with the indicated additions. DG analogues and metabolites were present at a concentration of 20 mol% or 3.2 μg/ml in the micellar and liposomal assays respectively. Results are means of two or three experiments.

Additions to assay	Protein kinase C activity (nmol of P_i incorporated/min per mg)		
	Histone (micellar)	Histone (liposomal)	P47 (liposomal)
None	0.05	0.17	0.06
1,2-Diolein	0.23	0.61	0.31
1-Stearoyl-2-arachidonoylglycerol	0.20	0.52	0.37
1,2-Dioctanoylglycerol	0.12	0.43	0.36
Phosphatidic acid	0.04	0.14	0.06
CDP-diacylglycerol	0.07	0.12	0.05
Arachidonic acid	0.04	0.13	0.07

2). The cell-permeable dioctanoylglycerol analogue and the naturally occurring 1-stearoyl-2-arachidonylglycerol were both as effective as 1,2-diolein in activating aortic protein kinase C; similar results have been reported in liposomal assays with histone as substrate (Go *et al.*, 1987). Ganong *et al.* (1986) have shown activation of protein kinase C by dioctanoylglycerol in a micellar assay; however, the naturally occurring DG was not tested. OAG also activated protein kinase C, but not to the same extent as the dioctanoyl analogue (results not shown). The metabolism of DG in aorta involves the formation of phosphatidic acid (kinase pathway) and CDP-diacylglycerol, or hydrolysis (lipase pathway) to release fatty acids (Severson & Hee-Cheong, 1986). These DG metabolites did not increase protein kinase C activity (Table 2). Increasing the phosphatidic acid and CDP-diacylglycerol concentrations by 10-fold had no effect on protein kinase C activity, and did not decrease the activation produced by the simultaneous presence of diolein (results not shown).

In summary, protein kinase C can be partially purified from bovine aortas. The activation of protein kinase C by DG, determined with histone as substrate in micellar and liposomal assays, was due to a decrease in the K_a for Ca^{2+} to 2–3 μM. In the absence of DG, the phosphorylation of P47 was more sensitive to Ca^{2+} (K_a 5 μM) than was the phosphorylation of histone. DG increased protein kinase C activity measured with P47, especially at low concentrations of Ca^{2+}, but the K_a for Ca^{2+} was not changed significantly. These results indicate the importance of characterizing protein kinase C with different exogenous substrates. It is not known if P47 is present in smooth muscle, but Majerus *et al.* (1986) have suggested that the platelet P47 protein is the $InsP_3$ phosphomonoesterase enzyme. If so, P47 may indeed be an endogenous substrate of protein kinase C in aortic SMC. Further experiments will be required to identify other important endogenous substrates for protein kinase C in smooth muscle.

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