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PHOSPHATIDIC ACID DIRECTLY ACTIVATES ENDOTHELIAL CELL PROTEIN KINASE C

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SUMMARY: The metabolism of phosphatidic acid (PA) yields diacylglycerol (DAG), a known activator of protein kinase C (PKC). To examine potential direct effects of PA on PKC activation, PKC purified from bovine pulmonary artery endothelial cells (BPAEC) was utilized in an *in vitro* assay examining γ-[^{32}P]ATP phosphorylation of H1 histone. In the presence of Ca^{2+} and phosphatidylserine (PS), DAG (80 μM) produced maximal PKC activity (6.4 pmol γ-[^{32}P]ATP incorporated/μg/min). Dioleoyl-PA (80 μM) and 1-stearoyl,2-arachidonyl-PA (80 μM) activated PKC in a concentration-dependent manner (maximal activity of 2.01 $\pm$ 0.1 pmol/μg/min). Unlike unlabelled phorbol esters or DAG, dioleoyl-PA did not significantly alter the binding of [^{3}H]-phorbol dibutyrate to PKC, suggesting that PA directly activates endothelial cell PKC in a manner distinct from DAG-mediated PKC activation.

A variety of agonists activate vascular endothelium through interaction with specific cell surface receptors, initiating signal tranducing events via the activation of a G-protein-coupled, PI-PLC, leading to the production of IP_3 and DAG. These second messengers result in increases in $[Ca^{2+}]_i$ and protein kinase C (PKC) activity. Many of the specific and important endothelial cellular responses elicited by bioactive agonists are regulated by PKC (1-6). Recent studies have also demonstrated the importance of agonist-induced activation of endothelial cell phospholipase D (PLD), which hydrolyzes membrane phosphatidylcholine, yielding PA (7,8). PA produced by PLD activation can be further metabolized to DAG via PA phosphatase (8,9), and may represent a major pathway for DAG production in vascular endothelium (7). Like DAG, PA is likely a short-lived species in signal transduction, and has been directly implicated in platelet aggregation (10), neutrophil oxidative burst (11), PDGF transcription (12), Ca^{2+} influx/release (9,13), insulin release (14), and regulation of DNA synthesis (12,13,15). The mechanism by which PA induces these responses, however, remains unknown. In the present study, we

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ABBREVIATIONS: BPAEC, bovine pulmonary artery endothelial cells; Ca^{2+}_i, intracellular calcium; DAG, diacylglycerol; DO-PA, dioleoylphosphatidic acid; DP-PA, dipalmitoylphosphatidic acid; IP_3, inositol-1,4,5-trisphosphate; MAG, monoacylglycerol; PO-PA, 1-palmitoyl,2-oleoylphosphatidic acid; PDBu, phorbol-12,13-dibutyrate; PMA, phorbol myristate acetate; PA, phosphatidic acid; PAPase, phosphatidic acid phosphohydrolase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; PC, phosphatidylcholine; PS, phosphatidylserine; PLD, phospholipase D; PKC, protein kinase C; SA-PA, 1-stearoyl,2-arachidonylphosphatidic acid.

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have demonstrated that PA directly activates endothelial cell PKC in vitro, via a mechanism which does not mimic PKC activation by DAG. These results suggest a distinct second messenger role for PA in endothelial cell signal transduction.

MATERIALS AND METHODS

Materials. DE-52 cellulose was obtained from Whatman (Maidstone, Kent, England). AH-Sepharose-4B was obtained from Pharmacia (Uppsala, Sweden). γ-[^{32}P]ATP and [^{32}P]orthophosphate were obtained from Dupont-New England Nuclear (Boston, MA). [^{3}H]Phorbol dibutyrate was obtained from Amersham (Arlington Heights, IL). Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Phorbol myristate acetate, phorbol dibutyrate, H1 histone (IIIS), and threonine were obtained from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce (Rockford, IL). Centricon-30 ultrafiltrators were obtained from Amicon (Danvers, MA).

Cell Culture. The well-characterized bovine pulmonary artery endothelial cell (BPAEC) line CCL-209 (6) was obtained from American Type Culture Collection (Rockville, MD) at passage 16 and subsequently used at passages 19-21. These cells were cultured in complete media consisting of M199 supplemented with 20% fetal calf serum and 20 μg/ml of endothelial cell growth supplement, and grown to confluency in 75-cm^2 tissue culture flasks in an atmosphere of 5% CO_2 at 37° C until confluent.

Lipid Purification. Lipids were purified by thin-layer chromatography (8), reconstituted in toluene:ethanol (1:1), and quantitated by phosphorus analysis (16). TLC-purified phosphatidic acids were further assessed for the presence of contaminating diacylglycerol, utilizing quantification of [^{32}P]PA by γ-[^{32}P]ATP and diacylglycerol kinase, followed by lipid extraction, TLC, and scintillation counting (17).

Purification of BPAEC Protein Kinase C. Bovine endothelial cell PKC was purified from confluent BPAEC monolayers (25-50 mg total cellular protein) by DEAE and threonine/sepharose chromatography, as we have previously described (6). Purified PKC fractions were pooled, desalted and concentrated by ultrafiltration (Centricon-30) and stored at -70° C after the addition of 0.01% Trition X-100.

Protein Kinase C Assay. Protein kinase C activity was assessed, as we have previously described (6). The reaction mixture (200 μL) consisted of 20 mM Tris/HCL pH 7.4, 1 mM $CaCl_2$, 10 mM Mg acetate, 20 μg H1 histone, 50 μM γ-[^{32}P]ATP (220 cpm/pmol), in the presence or absence of 125 μM PS and the specified concentrations of either DAG or PA. Aliquots of DAG, PA, and PS in toluene:ethanol (1:1) were evaporated under N_2, reconstituted in 20 mM Tris/HCL pH 7.4 and added to the reaction mixture. The reaction was initiated by addition of 50 μL (approximately 1 μg) threonine/sepharose-purified BPAEC PKC. The reaction was terminated after incubation at 25° C for 5 min, by the addition of 200 μL of 20 mM ATP at 4° C. Each sample was then filtered through 0.45 μm nitrocellulose (pre-wetted with 5% TCA). The filters were placed in a bath of 5% TCA with agitation x 30 min. Membranes were dried, dissolved in 10 mL scintillation fluid, and counted (Beckman model LS6000 IC liquid scintillation counter, Fullerton, CA). Specific kinase activity was calculated by subtracting cpm obtained in control samples, which did not contain lipids, from cpm obtained from experimental samples with lipids.

Phosphatidic Acid Phosphohydrolase Assay. Potential contaminating PAPase activity in our threonine/sepharose-purified BPAEC PKC preparations was examined using [^{3}H]-labeled PA which was prepared as follows: 1,2-diacyl-sn-[2-^{3}H]glycero-3-phosphate was prepared from the lipids of lima beans which had been germinated in the presence of 50 μCi [2-^{3}H]glycerol (8). The phosphatidylcholine fraction was isolated by TLC (8) and was subjected to hydrolysis by phospholipase D (Streptomyces chromofuscus) (18). [^{3}H]Phosphatidic acid was further purified by TLC using a chloroform:methanol:glacial acetic acid:water solvent system (65:15:3:2). The [^{3}H]-labelled PA was diluted with dioleoyl PA to achieve a specific activity of 22922 dpm/nmol. [^{3}H]-PA (80 μM) was

incubated with either threonine/sepharose-purified BPAEC PKC (2 μg) or BPAEC microsomes (8 μg) in the standard PKC assay reaction mixture (as described under "Protein Kinase C Assay") at 25° C for 5 min, followed by extraction of lipids in chloroform:methanol (2:1) and separation by thin-layer chromatography on layers of silica gel with hexane:ether:glacial acetic acid (50:50:1). TLC plates were scraped in 0.5 cm increments, dissolved in 10 mL scintillation fluid and counted. Formation of [^{3}H]-DAG from [^{3}H]-PA was taken as a measure of PAPase activity. Migration of extracted lipids was compared to migration of lipid standards in the same solvent system.

Protein Kinase C Binding Assay. Specific interaction of PKC activators with the diacylglycerol-binding site of PKC was assessed utilizing competitive binding of [^{3}H]-labeled PDBu, in a modification of the procedure described by Jaken (19). Aliquots of [^{3}H]PDBu in acetonitrile:water (40:60) were evaporated under nitrogen, and reconstituted at the desired concentration in 50 mM Tris/HCL pH 7.4, 22.5 mM Mg acetate, 1.5 mg/mL bovine serum albumin. Threonine/sepharose-purified BPAEC PKC (concentrated by ultrafiltration; 8 μg) was added to each reaction mixture (200 μL) consisting of 25 nM [^{3}H]PDBu (specific activity = 39160 dpm/pmol), 100 μM PS, 1 mM $CaCl_2$, and the specified concentration of "competing" PKC activator (PMA, PDBu, DAG, or DO-PA). Samples were incubated for 30 min at 25° C and filtered through 25 mm Gelman glass fiber filters which had been pre-soaked for 1 hour in 0.3% polyethylenimine, and rinsed for 30 min in a 0.3% polyethylenimine bath. Filter discs were air dried, dissolved in 10 mL scintillation fluid, and counted. Sample dpm were subtracted from control dpm (containing 5 mM excess unlabeled PDBu), and compared to samples devoid of PKC activators.

RESULTS

As expected (20), purified BPAEC PKC-mediated phosphorylation of H1 histone required the presence of Ca^{2+} and PS, and was dependent upon the concentration of DAG (Figure 1). Thus, DAG is a strict requirement for enzyme activation in vitro (20). We next assessed the ability of TLC-purified PA, demonstrated to be devoid of DAG contamination (as described in Materials and Methods), to activate BPAEC PKC. When DO-PA is substituted for DAG, there was a concentration-dependent phosphorylation of histone, with a specific PKC activity of 2.01 ± 0.1 pmol/μg/min at 80 μM DO-PA (Figure 1). While this significant PA-induced PKC activation was only 30% of the maximal DAG-mediated activation, PS alone at 100-500 μM produced only negligible activation (0.17 ± 0.07 pmol/μg/min). Similarly, PA without PS, did not activate BPAEC PKC (0.14 pmol/μg/min). Thus, both DAG- and PA-mediated PKC activation are dependent upon the presence of PS, and DO-PA can substitute for DAG in inducing in vitro PKC activation.

Further experiments were performed to examine PKC activation by naturally-occurring PA's with specific fatty acyl groups at the sn-1 and sn-2 positions. TLC-purified SA-PA, similar to DO-PA, resulted in dose-dependent PKC activation (80 μM SA-PA, 1.64 ± 0.06 pmol/μg/min) (Figure 2). In contrast to DO-PA, the highest concentration of PO-PA (80 μM) resulted in only minimal activation of BPAEC PKC (0.88 ± 0.09 pmol/μg/min, $p=0.03$ compared to PS alone), while DP-PA did not significantly activate PKC ($p>0.05$ compared to PS alone).

PA-mediated PKC activation might occur through the action of contaminating PAPase activity in the PKC preparation, resulting in the conversion of PA to DAG, thus "indirectly" activating PKC. Threonine/sepharose-purified BPAEC PKC was examined for the presence of PAPase activity by incubating [^{3}H]-PA with PKC under standard assay conditions, followed by quantification of [^{3}H]-DAG

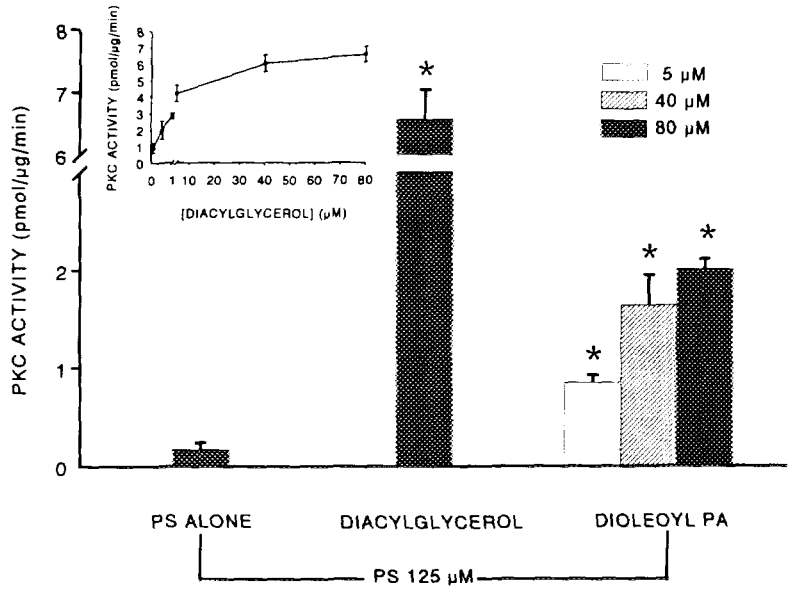

Fig. 1. Diacylglycerol- and dioleoylphosphatidic acid-mediated activation of BPAEC protein kinase C. Purified PKC (1 μg) obtained from BPAEC was added to an in vitro reaction mixture (200 μL) consisting of 20 mM Tris/HCL,pH 7.4, 1 mM $CaCl_2$, 10 mM Mg acetate, 20 μg H1 histone, 50 μM γ-[^{32}P]ATP, in the presence or absence of 125 μM PS and the specified concentration of DAG (inset) or with the substitution of DAG with 1,2-dioleoyl-PA at the specified concentration, or without DAG or PA ("PS alone"). Specific PKC activity was quantitated and expressed as pmol γ-[^{32}P]ATP incorporated/μg/min. Shown is pooled data from six separate experiments (n=3 for each experiment). DAG is demonstrated to produce concentration-dependent activation of BPAEC PKC (inset), and dioleoyl-PA is demonstrated to activate BPAEC PKC in a concentration-dependent fashion. * $p < 0.05$, compared to PS alone.

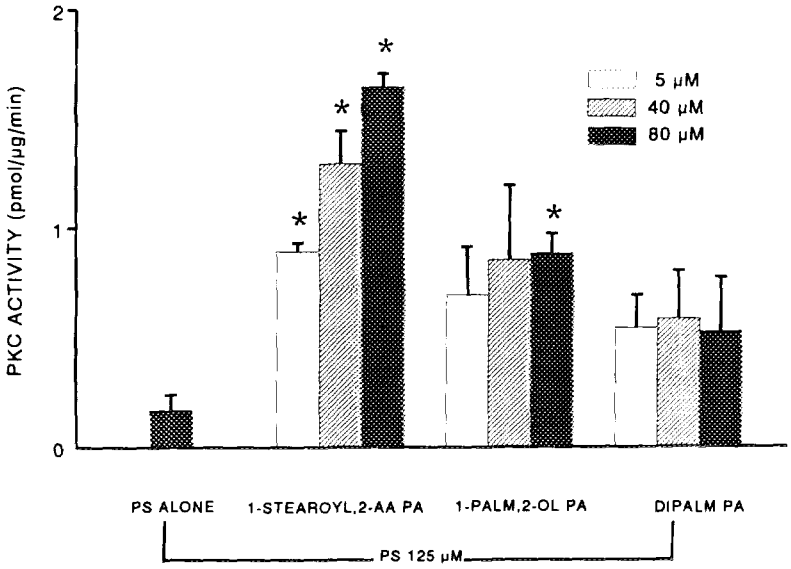

Fig. 2. Protein kinase C activation by other naturally occurring phosphatidic acids. Purified BPAEC PKC was added to an in vitro PKC reaction mixture, with substitution of DAG with either 1-stearoyl-2-arachidonyl-PA, 1-palmitoyl-2-oleoyl-PA, or 1,2-dipalmitoyl-PA, and subsequent quantitation of specific PKC activity. Shown is pooled data from three separate experiments (n=3 for each experiment). 1-Stearoyl-2-arachidonyl-PA (5, 40, and 80 μM) and 1-palmitoyl-2-oleoyl-PA (80 μM) significantly activate BPAEC PKC, similar to dioleoyl-PA. * $p < 0.05$, compared to PS alone.

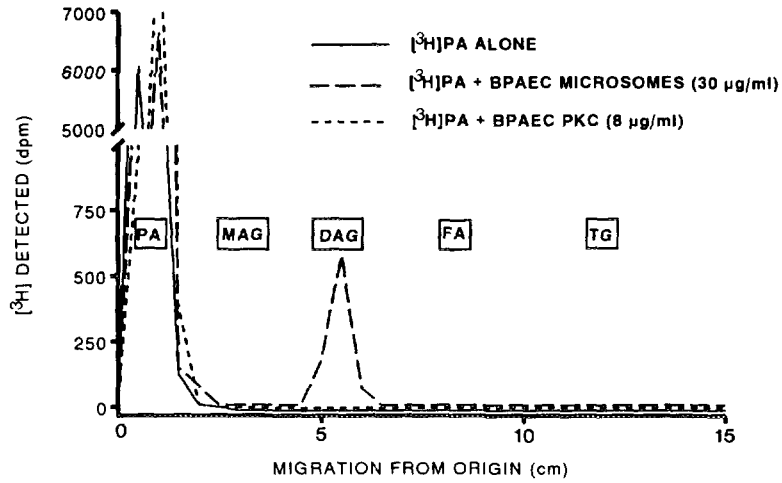

Fig. 3. Absence of phosphatidic acid phosphohydrolase activity in BPAEC protein kinase C. [^{3}H]-PA (80 μM) was incubated with either purified BPAEC PKC (2 μg) or BPAEC microsomes (8 μg) under standard PKC assay conditions (as described in Materials and Methods), followed by extraction of lipids in chloroform:methanol (2:1), separation by thin-layer chromatography, and quantitation of labelled lipids by scintillation counting. Migration of lipid standards from the origin on TLC plates is outlined by the enclosed boxes. PAPase activity (as demonstrated by production of [^{3}H]-DAG) is present in BPAEC microsomes, whereas purified BPAEC PKC or [^{3}H]-PA alone did not contain detectable PAPase activity.

and [^{3}H]-MAG production. [^{3}H]-PA incubated with BPAEC microsomes, resulted in the production of [^{3}H]-DAG (Figure 3), indicating the presence of PAPase activity. In contrast, incubation of BPAEC PKC with [^{3}H]-PA did not reveal detectable PAPase activity, as formation of either [^{3}H]-DAG or [^{3}H]-MAG was not observed (Figure 3).

Phorbol esters are known activators of PKC (21), and interact directly with the DAG-binding site of the enzyme (20). [^{3}H]PDBu binding to BPAEC PKC was examined in the presence or absence of various activators of PKC (Figure 4). The unlabelled phorbol esters, PDBu and PMA, inhibit [^{3}H]PDBu binding to PKC in a concentration-dependent manner (Figure 4), demonstrating competition for the DAG-binding site. In similar fashion, DAG competes with labelled phorbol for binding to BPAEC PKC, producing greater than 75% inhibition of [^{3}H]PDBu binding with 100 μM DAG (Figure 4). In contrast, neither DO-PA, nor PS by itself, inhibited [^{3}H]PDBu binding, even at concentrations of DO-PA up to 500 μM (Figure 5), suggesting that PA-mediated PKC activation does not involve direct interaction of PA with the DAG-binding site of PKC.

DISCUSSION

Receptor-mediated agonists, such as α-thrombin, are known activators of PI-PLC in vascular endothelium, resulting in the hydrolysis of membrane-bound PIP_2, yielding the second messengers, IP_3 and DAG (22), and activation of the serine-threonine kinase, PKC (20). In vascular endothelium, PKC-mediated phosphorylation has been demonstrated to have a negative feedback regulatory role on PI-PLC activity and $[Ca^{2+}]_i$ release (23,24), while augmenting agonist-induced phospholipase A_2 activation and prostacyclin synthesis (24), and is centrally involved in thrombin-induced barrier dysfunction (6).

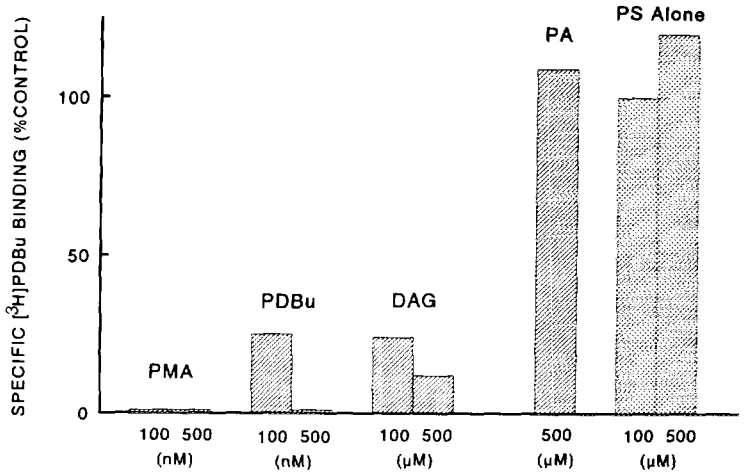

Fig. 4. Inhibition of [^{3}H]-phorbol dibutyrate binding to BPAEC PKC. Purified BPAEC PKC (8 μg) was added to a reaction mixture (250 μL) consisting of 10 mM Tris/HCL,pH 7.4, 25 nM [^{3}H]PDBu, 100 μM PS, 1 mM $CaCl_2$, 4.5 mM Mg acetate, 0.3 mg/mL bovine serum albumin, and the specified concentration of "competing" PKC activator (PMA, PDBu, DAG, or dioleoyl-PA) or PS alone, followed by quantitation of [^{3}H]PDBu binding (as described in Materials and Methods). Specific [^{3}H]PDBu binding to PKC is expressed as percent of control binding (100 μM PS alone without other activators). Addition of PMA, unlabelled PDBu, or DAG reduced [^{3}H]PDBu binding, indicating competition for binding to PKC, whereas [^{3}H]PDBu binding was unaltered by 500 μM dioleoyl-PA (109% of control binding).

Although sequential activation of PKC through agonist-induced, PI-PLC-mediated production of DAG has been demonstrated in a number of cell types (6,25-27), hydrolysis of the membrane precursor PIP_2 cannot singularly account for the levels of DAG produced in response to agonist stimulation (9,28,29). More recently, we and others have demonstrated both receptor-mediated and PKC-dependent activation of another membrane-bound endothelial cell phospholipase, PLD, whose primary substrate is membrane PC, which is hydrolyzed to form PA (8,9). PA produced in this manner is a substrate for DAG by the action of endothelial cell PAPase. Alternate production of DAG via the PLD pathway, therefore, is possibly a primary mechanism for PKC activation in endothelium (9). DAG produced via PLD activation could serve to either amplify, or possibly tightly regulate the cellular response to different agonists. Furthermore, evidence is accumulating that suggests PA itself may function as a distinct second messenger in signal transduction events (9-14,30) which promote insulin activity in pancreatic acinar cells (14) and activation of NADPH oxidase in human neutrophils (11). From recent studies, it appears that the vast majority of DAG in fluoride-stimulated neutrophils is actually derived from PA (31), as propranolol pretreatment, which inhibits PAPase-mediated conversion of PA to DAG, results in increased levels of PA and NADPH oxidase activity in neutrophils (11).

We have explored an additional potential mechanism for PA-mediated cellular responses, and the data reported here convincingly demonstrates in vitro PA activation of endothelial cell PKC. Interestingly, the extent of this activation varies with the species of PA used: DO-PA (di-18:1) > SA-PA (18:0, 20:4) > PO-PA (16:0, 18:1). This order of PA-mediated PKC activation suggests that the degree of fatty acid unsaturation at both the sn-1 and sn-2 positions is an important factor in PKC activation.

Consistent with this hypothesis is the observation that DP-PA (di-16:0), saturated at both positions, did not activate endothelial cell PKC. PA containing unsaturated fatty acyl groups has been shown to inhibit cAMP production and stimulate arachidonate release in 3T3 fibroblasts, whereas "saturated" PA was ineffective, and actually competitively inhibited these responses to "unsaturated" PA (30). DAG-mediated PKC activation is known to depend, at least in part, upon the particular fatty acyl moieties present in DAG, with unsaturation at the sn-2 position being a strict requirement for activity (20). One explanation for the effects of "unsaturated" PA, would be PAPase-mediated conversion of PA to DAG; therefore, PKC activation would be dependent upon the production of "unsaturated" DAG from "unsaturated" PA. Interconversion of PA to DAG, and DAG to PA, by the action of PAPase and DAG kinase respectively (32,33), has hampered delineation of the precise biochemical pathways involved in agonist-induced cellular responses. In the present study, PA-mediated PKC activation is demonstrated to be a direct effect of PA, since PAPase activity was absent under in vitro assay conditions (Figure 3). Furthermore, all species of PA had been purified and further analyzed by TLC, and shown to be devoid of DAG, as detected by DAG kinase assay in the presence of γ-[^{32}P]ATP.

In order to partially characterize the mechanism of direct PA-mediated PKC activation, specific [^{3}H]-phorbol binding to BPAEC PKC was examined in the presence or absence of DO-PA. Unlabelled phorbol esters (PDBu, PMA), or DAG, both competitively inhibited the avid [^{3}H]PDBu binding to PKC, whereas DO-PA, at concentrations demonstrated to activate PKC, did not affect binding. Thus, PA-mediated PKC activation appears to occur by a mechanism distinct from DAG-induced PKC activation, in that direct interaction of PA with the DAG-binding site appears unlikely. The observation that a number of PA-induced cellular responses parallel the effects of direct PKC activation by phorbol esters (11,12,14), suggests that direct PA-mediated BPAEC PKC activation may be an important and possibly physiologically-relevant mechanism in vivo (11). Recently, PA-dependent phosphorylation of cellular proteins from rat tissue extracts has been demonstrated in vitro (34). PA is also reported to activate PLC in platelets in a pertussis toxin-sensitive manner (35), suggesting the involvement of a guanine nucleotide regulatory protein, possibly linked to a distinct cell surface receptor for PA. While our studies strongly suggest that PA may participate in signal transduction events as a second messenger for direct PKC activation, further studies are required to determine the effect of PA on specific signal transduction pathways, as well as its role in PKC activation in intact vascular endothelial cells.

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