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Requirement for Diacylglycerol and Protein Kinase C in HeLa Cell-Substratum Adhesion and Their Feedback Amplification of Arachidonic Acid Production for Optimum Cell Spreading

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Release of arachidonic acid (AA) and subsequent formation of a lipoxygenase (LOX) metabolite(s) is an obligatory signal to induce spreading of HeLa cells on a gelatin substratum (Chun and Jacobson, 1992). This study characterizes signaling pathways that follow the LOX metabolite(s) formation. Levels of diacylglycerol (DG) increase upon attachment and before cell spreading on a gelatin substratum. DG production and cell spreading are insignificant when phospholipase A_2 (PLA_2) or LOX is blocked. In contrast, when cells in suspension where PLA_2 activity is not stimulated are treated with exogenous AA, DG production is turned on, and inhibition of LOX turns it off. This indicates that the formation of a LOX metabolite(s) from AA released during cell attachment induces the production of DG. Consistent with the DG production is the activation of protein kinase C (PKC) which, as with AA and DG, occurs upon attachment and before cell spreading. Inhibition of AA release and subsequent DG production blocks both PKC activation and cell spreading. Cell spreading is also blocked by the inhibition of PKC with calphostin C or sphingosine. The inhibition of cell spreading induced by blocking AA release is reversed by the direct activation of PKC with phorbol ester. However, the inhibition of cell spreading induced by PKC inhibition is not reversed by exogenously applied AA. In addition, inhibition of PKC does not block AA release and DG production. The data indicate that there is a sequence of events triggered by HeLa cell attachment to a gelatin substratum that leads to the initiation of cell spreading: AA release, a LOX metabolite(s) formation, DG production, and PKC activation. The data also provide evidence indicating that HeLa cell spreading is a cyclic feedback amplification process centered on the production of AA, which is the first messenger produced in the sequence of messengers initiating cell spreading. Both DG and PKC activity that are increased during HeLa cell attachment to a gelatin substratum appear to be involved. DG not only activates PKC, which is essential for cell spreading, but is also hydrolyzed to AA. PKC, which is initially activated as consequence of AA production, also increases more AA production by activating PLA_2.

INTRODUCTION

Arachidonic acid (AA)[1] and its metabolites or eicosanoids have been implicated in the regulation of a variety of physiological processes. The release of AA from phospholipids is the rate-limiting step for eicosanoids

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[1] Abbreviations used: AA, arachidonic acid; BPB, bromophenacyl bromide; DG, diacylglycerol; ECM, extracellular matrix; LOX, lipoxygenase; MG, monoacylglycerol; NDGA, nordihydroguaretic acid; OAG, 1-oleoy-2-acetyl glycerol; PDBu, phorbol 12, 13-dibutyrate; PDD, 4-α-12, 13-didecanoate; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PLA_2, phospholipase A_2; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

synthesis (Needleman *et al.*, 1986; Dennis, 1987). The primary mechanism for the release of AA is the direct hydrolytic removal of AA from phospholipids by phospholipase A_2 (PLA_2) (Chang *et al.*, 1987; Dennis *et al.*, 1991). AA can also be produced from diacylglycerol (DG), which is released by the action of phospholipase C (PLC) (Dennis *et al.*, 1991). The released DG is hydrolyzed by DG lipase resulting in the production of 2-monoacylglycerol (MG). MG lipase then produces glycerol and free fatty acids including AA (Ide *et al.*, 1990; Balsinde *et al.*, 1991; Dennis *et al.*, 1991). The availability of DG is the endogenous rate-limiting step for this pathway of AA production (Needleman *et al.*, 1986; Dennis, 1987).

Previous studies indicated that formation of a lipoxygenase (LOX) metabolite(s) of AA is a second messenger that induces spreading of HeLa cells on a gelatin substratum (Chun and Jacobson, 1992). AA appears to be released by the action of PLA_2 because the inhibition of PLA_2 blocks both AA release and cell spreading. However, it is not known how AA release initiates cell spreading. One possibility is that the released AA induces cell spreading by the modulation of protein kinases. It is known that AA and its metabolites stimulate guanylate cyclase that elevates the level of cellular cyclic guanosine 3,5'-monophosphate (cGMP) (Nishizuka, 1984). The cGMP formed can function as an intracellular messenger for various extracellular signals by activating a cGMP-dependent protein kinase (PKG) (Blackshear *et al.*, 1988). Another kinase, protein kinase C (PKC), is also activated by AA and its metabolites either in the presence or absence of Ca^{2+} or phospholipid (Blackshear *et al.*, 1988; Huang, 1989). The activation of PKC by AA can be either independent of or synergistic with DG, a well known activator of PKC (Kikkawa and Nishizuka, 1986; Shinomura *et al.*, 1991).

Although the coupling of AA production and PKG activation has been known, there is no evidence that PKG modulates adhesion of cells to an extracellular matrix (ECM). However, the involvement of PKC in cell-ECM adhesion has been suggested from the observations that activation of PKC with phorbol ester [e.g., 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)] or synthetic DG (Ashendel, 1985; Kikkawa and Nishizuka, 1986) enhances the ability of various cell types to adhere to an ECM. For example, fibroblasts treated with PKC activating phorbol ester show an enhanced adhesion to a fibronectin substratum (Danilov and Juliano, 1989). Adhesion of Lewis lung carcinoma cells to an ECM or to the surface of endothelial cells is also enhanced by the treatment of TPA or synthetic DG (Takenaga and Takahashi, 1986; Grossi *et al.*, 1989). Further, TPA treatment induces adhesion of mouse macrophages to a laminin substratum where cells can not adhere in the absence of stimulation (Mercurio and Shaw, 1988). However, there is no direct evidence indicating that endogenous PKC activity modulates attachment and spreading of cells to an ECM.

We have previously shown that there is an activation of PKC upon attachment and before spreading of HeLa cells on a gelatin substratum (Chun and Jacobson, 1992). It was suggested that the activation of PKC was not essential for cell spreading since staurosporine, a potent nonspecific inhibitor of PKC (Tamaoki *et al.*, 1986), did not block cell spreading. In this study, we further examined the role of PKC in HeLa cell spreading. Results indicate that PKC is activated as a consequence of AA release and DG production. Inhibition of PKC with specific inhibitors such as calphostin C and sphingosine blocks cell spreading. The blocking of cell spreading by PKC inhibition is abolished when cells are pretreated with KT 5720, a relatively specific inhibitor of cAMP-dependent protein kinase (PKA). The antagonistic effect of PKC by KT 5720 explains our earlier results where staurosporine, which inhibits both PKC and PKA, did not inhibit cell spreading (Chun and Jacobson, 1992). The data also provide evidence indicating that HeLa cell spreading is a cyclic feedback amplification process centered on the production of AA by the hydrolysis of DG and the activation of PLA_2 by PKC.

MATERIALS AND METHODS

Materials

[^{3}H]AA was purchased from New England Nuclear (Boston, MA). Inhibitors of protein kinases (Calphostin C, KT 5720, KT 5823, KT 5926, H7, K252b, and staurosporine) were obtained from CalBiochem (La Jolla, CA). RHC 80267 was from Biomol Research Laboratories (Plymouth, PA). All other chemicals were purchased from Sigma (St. Louis, MO).

Substratum Preparation, Cell Culture, and Spreading Assay

Type I gelatin from swine skin and bovine serum albumin (BSA) were covalently coupled to polystyrene culture dishes (Falcon, Oxanard, CA) as described previously (Chun and Jacobson, 1992). HeLa-S_3 (American Type Culture Collection, Rockville, MD) cells were grown in suspension to midlog phase (2-4 $\times$ 10^5 cells/ml) in RPMI-1640 medium (K.C. Biologicals, Lenexa, KS) supplemented with 5% calf serum (Gibco, Grand Island, NY), 0.3% $NaHCO_3$, 100 μg/ml dihydrostreptomycin, 60 μg/ml penicillin, and 0.002% butyl parahydroxybenzoate (Sigma). The cells used for attachment and spreading studies were harvested from suspension culture, washed twice, and resuspended in serum-free RPMI 1640 medium. Suspension cells were treated with various drugs or appropriate solvent at room temperature as indicated in each experiment and plated on substratum coated culture dishes. After incubating the cells for 30 min at 37°C in the presence of the treated drug unless otherwise indicated, cell spreading was assayed as previously described by Chun and Jacobson (1992) and Lu *et al.* (1992). Briefly, percent cells spread was calculated from the number of spread cells/total number of cells $\times$100 from 200–300 cells/culture dish in several microscopic fields of view by using phase contrast microscopy. A spread cell is defined as one that is at least two times the diameter of the nucleus.

Assays of Arachidonic Acid, Monoacylglycerol, and Diacylglycerol

HeLa cells were labeled with [^{3}H]AA (0.2 μCi/ml) before the spreading assays as described previously (Chun and Jacobson, 1992). Equal

numbers of the labeled cells were plated on substratum coated culture dishes (5×10^5 cells/dish). After incubation at 37°C for the indicated times, cells were scraped off from the culture dish with a rubber policeman and transferred to glass tubes containing 1 ml chloroform. The dishes were washed with 1 ml of methanol:HCl (1:0.01, vol/vol) and combined with the chloroform/RPMI medium. When cells were kept in suspension, lipids were extracted by addition of 2 ml of chloroform:methanol:HCl (1:1:0.01, by vol). After vortexing and separation of the phases by centrifugation, the lipid containing lower phase was concentrated under a stream of nitrogen gas. The lipids were separated on thin layer silica gel G plates with the use of heptane:diethyl ether: glacial acetic acid (60:40:2, by vol) as the chromatography solvent (Findlay, 1987). Individual lipids were visualized by iodine vapor and identified by comigration with standards. Areas of the silica gel corresponding to AA, MG, and DG were scraped into scintillation vials and counted.

Protein Kinase C Assay

Translocation of cytosolic PKC to the particulate membrane was assayed by partial purification of PKC (Kikkawa *et al.*, 1983) and in vitro measurement of PKC activity as described previously (Chun and Jacobson, 1992).

RESULTS

DG is Produced as a Result of AA Release Before HeLa Cell Spreading

When HeLa cells with phospholipids prelabeled with [^{3}H]AA are plated on a gelatin substratum, the levels of [^{3}H]-labeled DG increase upon attachment and before cell spreading (Figure 1A). DG production is relatively insignificant when cells are plated on BSA coated culture dishes where cells attach but do not spread (Figure 1A). The kinetics of DG production are similar to that of AA release (Chun and Jacobson, 1992). Both events occur upon attachment and before cell spreading suggesting that the increase is essential to initiate spreading. To examine the relationship between the release of AA and DG, cells were treated with bromophenacyl bromide (BPB) or nordihydroguaretic acid (NDGA) to respectively inhibit PLA_2 (Chang *et al.*, 1987) and LOX (Egan and Gale, 1985) before the spreading assay. Both inhibitors prevent attached HeLa cells from spreading (Chun and Jacobson, 1992). The increase in DG levels is not observed in the cells treated with either of the inhibitors (Figure 1B) suggesting that production of DG is induced by the formation of a LOX metabolite(s) from AA released during cell attachment to gelatin. To further examine the role of AA release in DG production, suspension cells with phospholipids prelabeled with tracer amounts of [^{3}H]AA were treated with exogenous cold AA. Exogenous AA increases the levels of [^{3}H]AA labeled DG (Figure 1C). AA-induced DG production is significantly blocked if the cells are treated with NDGA before the addition of AA (Figure 1C). This further supports the idea that a metabolite(s) of AA released upon cell attachment stimulates DG formation.

Membrane-Bound PKC Activity Increases Transiently upon Attachment of HeLa Cells

In HeLa cells plated on gelatin, PKC activity in the particulate membrane fraction is transiently increased in a time period that precedes cell spreading (Figure 2A). Membrane-bound PKC is markedly reduced by inhibition of PLA_2 with BPB (Figure 2B), which also inhibits cell spreading and the production of AA. Also, membrane-bound PKC activity is insignificant in cells either plated on a BSA coated culture dish or kept in suspension (Figure 2B). If the cells in suspension are treated

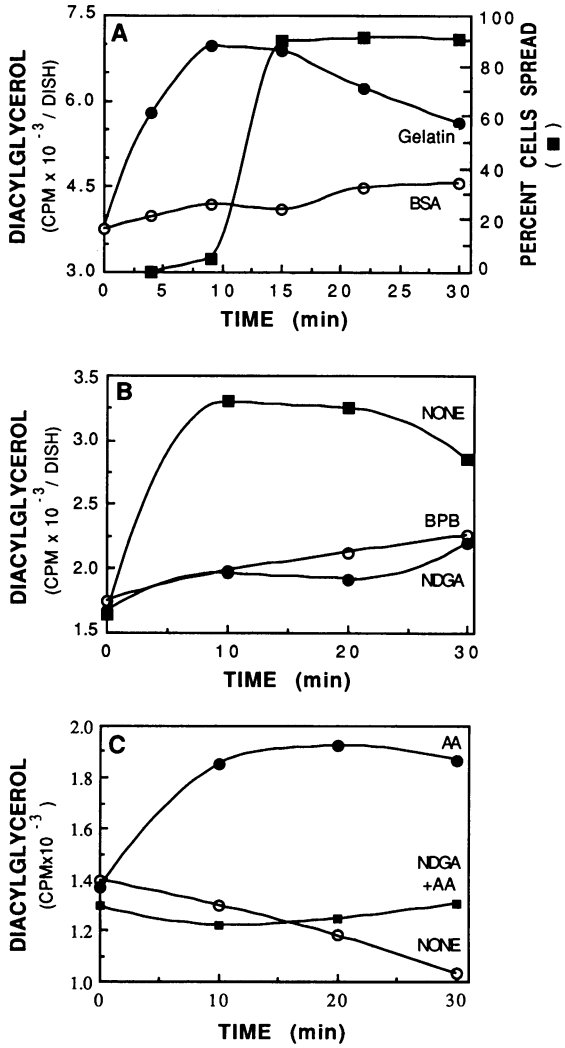

Figure 1. DG is produced as a result of AA release before HeLa cell spreading. (A) HeLa cells with phospholipids prelabeled with [^{3}H]AA were plated on gelatin (●) or BSA (○) coated culture dishes. At the indicated times, the amount of [^{3}H]AA containing DG was determined as described in MATERIALS AND METHODS. Percent cells spread (■) was scored from the cells plated on gelatin coated culture dishes. (B) Cells untreated (■) or treated with 12 μM BPB (○) or 30 μM NDGA (●) for 5 min in suspension were plated on gelatin coated culture dishes. The amount of [^{3}H]AA labeled DG was determined at the indicated times. (C) Suspension cells were treated with 15 μM AA (●) or 0.2% ethanol as a vehicle (○). NDGA (30 μM) and AA treated cells (●) were incubated with NDGA for 5 min before the incubation with AA. At the indicated times, the amount of [^{3}H]AA labeled DG was determined. The data represent a typical experiment conducted 12 times (A), 4 times (B), and 3 times (C) with comparable results.

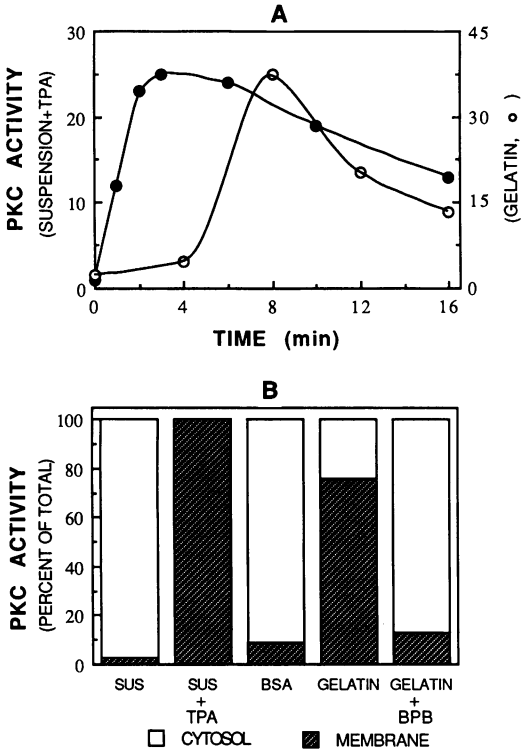

Figure 2. PKC is activated upon attachment and before spreading of HeLa cells. (A) HeLa cells were allowed to attach and spread on a gelatin substratum (○) or incubated with 0.4 μM TPA in suspension (●). At the indicated times, PKC activities were determined from both particulate membrane and cytosolic fractions, which were partially purified by DE-52 column. PKC activities in cells plated on gelatin or in suspension were measured independently and expressed as CPM $\times 10^{-3}$/mg protein. The data represent a typical experiment conducted 3 times with comparable results. (B) PKC activities were determined in HeLa cells kept in suspension in the absence (SUS) or presence of 0.4 μM TPA (SUS + TPA), plated onto BSA (BSA) or gelatin (GELATIN) for 10 min. Cells were treated with 12 μM BPB for 5 min in suspension before plating the cells on gelatin (BPB + GELATIN). The data represent average of at least 3 experiments.

with TPA, membrane-bound PKC activity is rapidly increased (Figure 2A) with no detectable cytosolic PKC activity (Figure 2B). Thus the events which occur upon cell attachment, i.e., AA release, DG production, and PKC activation is specific to a gelatin substratum where attached cells can spread.

Specific Inhibition of PKC Blocks HeLa Cell Spreading

When HeLa cells are treated with specific inhibitors of PKC such as calphostin C (Kobayashi *et al.*, 1989) and sphingosine (Hannun *et al.*, 1986) before the spreading assays, cell spreading is blocked in a dose-dependent manner (Figure 3). The dosage of calphostin C found to be optimal for inhibiting spreading is similar to that which inhibits PKC activation in HeLa cells. The observation that the concentration of calphostin C (1 μM) necessary for optimal inhibition of spreading and PKC activation in cells is higher than that needed to inhibit PKC in solution (concentration for 50% inhibition = 0.35–0.4 μM) is probably due to the decreased accessibility of PKC in intact cells. The inhibition of cell spreading with the inhibitors does not appear to be due to irreversible damage of the cells because the inhibitory effects of calphostin C and sphingosine can be reversed as is shown in Figure 4. The above results suggest that activation of PKC is required to initiate spreading of cells.

In contrast to the effect of specific inhibitors of PKC on cell spreading, treating cells with staurosporine, a potent nonspecific inhibitor of PKC, does not block cell spreading (Chun and Jacobson, 1992). This, in part, led us to previously conclude that PKC is not involved in cell spreading (Chun and Jacobson, 1992). HeLa cell spreading is also not blocked when the cells are treated with other nonspecific inhibitors of PKC such as H7 (Hidaka *et al.*, 1984) and K252b (Kase *et al.*, 1987) (Figure 4A). The inconsistency of the effects of calphostin C and sphingosine and that of nonspecific inhibitors on spreading may be due to either 1) the inhibition of spreading by calphostin C or sphingosine is due to nonspecific side effects in addition to their abilities to block PKC or 2) PKC inhibition by the broadly based inhibitors is antagonized by the concomitant inhibition of other protein kinases.

Inhibitors of Other Protein Kinases Antagonize the Inhibition of PKC

To examine whether other protein kinases that may antagonize the effect of PKC inhibition are involved in HeLa cell spreading, the cells were treated with relatively more specific inhibitors of the various protein kinases. The number of cells spread on gelatin is not af-

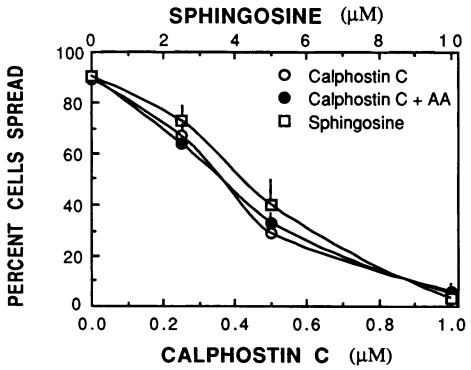

Figure 3. Inhibition of PKC blocks HeLa cell spreading. Suspension HeLa cells were treated with the indicated concentrations of calphostin C (○) or sphingosine (□) for 5 min, and plated on gelatin coated culture dishes. Calphostin C treated cells were also plated on a gelatin substratum in the presence of 15 μM arachidonic acid (●). Percent cells spread was scored following 30-min incubation at 37°C. The data represent the average of 5 experiments with duplicate samples and the standard deviation.

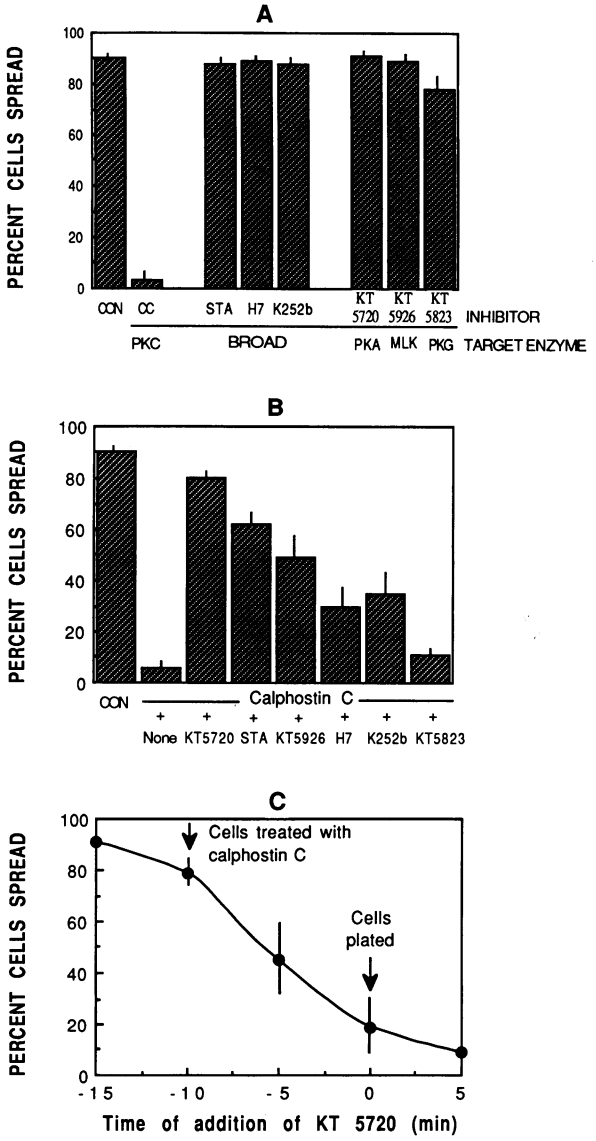

Figure 4. Inhibition of PKA antagonizes the effects of PKC inhibition on HeLa cell spreading. (A) Suspension HeLa cells were treated for 5 min with the following additions to the medium: 0.1% DMSO as the vehicle for the various inhibitors (CON), 1 μM of calphostin C (CC) or staurosporine (STA), 100 μM of H7, 5 μM of K252b, KT 5720, KT5823, or KT 5926. The cells were plated on gelatin for 30 min at 37°C and percent cells spread was scored. The protein kinases, which are preferentially blocked by the inhibitors, are indicated. PKC: protein kinase C, PKA: protein kinase A, PKG: protein kinase G, MLK: myosin light chain kinase, and Broad: all protein kinases. (B) HeLa cells were treated with 1 μM calphostin C alone or calphostin C and one of the following drugs: KT5720 (5 μM), staurosporine (STA: 1 μM), KT 5926 (5 μM), H7 (100 μM), K252b (5 μM), or KT 5823 (5 μM). The cells treated or untreated (CON) were plated on gelatin for 30 min and percent cells spread was scored. (C) Suspension HeLa cells were treated with both calphostin C (1 μM) 10 min before plating the cells on gelatin and KT5720 (5 μM) 15, 10, 5, or 0 min before plating the cells. Alternatively, cells treated with calphostin C for 10 min were plated on gelatin for 5 min before being exposed to KT5720. Percent cells spread on gelatin was scored following 30-min incubation at 37°C. The data (A, B, and C) represent average of five experiments with duplicate samples and standard deviation.

fected when the cells were pretreated with KT 5720 (an inhibitor of PKA), KT 5823 (an inhibitor of PKG), or KT 5926 (an inhibitor of myosin light chain kinase, MLK) (Kase *et al.*, 1987) at the concentrations indicated (Figure 4A). However, the inhibition of spreading induced by calphostin C is significantly reversed when the cells are simultaneously pretreated with KT 5720 (PKA inhibitor) and calphostin C (PKC inhibitor) (Figure 4B). Similarly, treating cells with KT 5720 simultaneously with sphingosine also significantly reverses the inhibition of cell spreading induced by 10 μM sphingosine (5 ± 3 to 81 ± 6 (SD)%). The effects of KT 5720 on calphostin C treatment are strictly dependent on the sequence of addition. Treating cells for 5 min before calphostin C completely blocks the calphostin C effect while addition of KT 5720 at increasing times following calphostin C addition has increasingly less effect on cell spreading (Figure 4C). This indicates that inhibition by the PKA inhibitor before PKC inhibition is required to antagonize the calphostin C effect. The recovery of spreading from the cells treated with calphostin C is also significant, albeit less than staurosporin, when the cells are simultaneously pretreated with calphostin C and the other broadly based kinase inhibitors, H7. or K252b (Figure 4B). Treating cells with KT 5926, an inhibitor of MLK, also significantly antagonizes the effect of calphostin C (Figure 4B) however, it is possible that at the concentration of KT 5926 necessary to get the effect might have inhibited PKA as well (Kase *et al.*, 1987). Taken together, the data suggest that inhibition of a protein kinase other than PKC abolishes the inhibitory effects of calphostin C on cell spreading and explains why broadly based protein kinase inhibitors such as staurosporin do not inhibit cell spreading (cf., Chun and Jacobson, 1992).

PKC is Activated as a Consequence of AA Release and DG Production

Increase in membrane-bound PKC activity is not observed when HeLa cells are treated with BPB to inhibit AA release and subsequent DG production (Figure 3B). Treating cells with BPB also decreases the percent cells spread in a dose-dependent manner (Figure 5A). Addition of exogenous AA overcomes the BPB inhibition of spreading (Chun and Jacobson, 1992). The inhibition of cell spreading by BPB is also reversed when the treated cells are plated on gelatin in the presence of TPA to activate PKC (Figure 5A). The ability of TPA to overcome BPB inhibition of cell spreading appears not to be mediated by production of AA since TPA treatment does not cause AA release in cells treated with BPB (see Figure 7). Direct activation of PKC with TPA also overcomes the inhibition of cell spreading induced by NDGA treatment (Figure 5B). This indicates that activation of PKC is sufficient to initiate cell spreading even in the absence of AA release and the subsequent

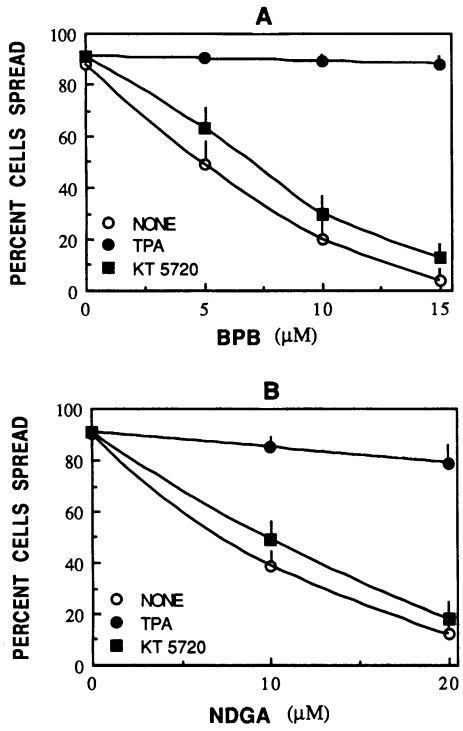

Figure 5. TPA treatment overcomes the inhibitory effects of BPB (A) and NDGA (B) on HeLa cell spreading. Suspension HeLa cells were treated with various concentrations of BPB (A) or NDGA (B) for 5 min and then plated on a gelatin substratum at 37°C in the absence (O) or presence of 0.4 μM TPA (●). The data represent the average of 4 experiments with duplicate samples and the standard deviations.

formation of a LOX metabolite(s), events which are required for cell spreading in the absence of TPA. The data fit the hypothesis that formation of a LOX metabolite(s) from cell attachment-induced release of AA on a gelatin substratum induces the activation of PKC, which in turn initiates cell spreading. Consistent with the hypothesis is that PKC inhibition does not block AA release and DG production (see Figure 7) although it does block cell spreading. In addition, exogenously applied AA does not reverse the inhibition of cell spreading induced by PKC inhibition (Figure 3).

The effect of KT 5720, which antagonizes the effects of PKC inhibition on cell spreading was examined to determine whether it also overcomes the inhibition of cell spreading induced by the PLA_2 inhibitor, BPB or the LOX inhibitor, NDGA. Unlike the TPA effects, addition of KT 5720 to the cells treated with BPB or NDGA is not effective at reversing the inhibition of spreading induced by BPB or NDGA (Figure 5, A and B). This indicates that while the PKA inhibitor reverses the effect of calphostin C inhibition of cell spreading it is not sufficient to induce cell spreading on its own in the absence of AA release, LOX metabolite(s) formation, and PKC activation.

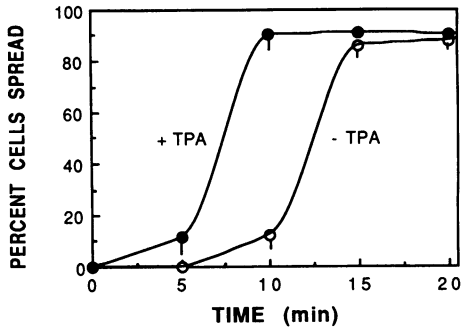

Figure 6. Activation of PKC enhances the rate of HeLa cell spreading. HeLa cells were allowed to spread on gelatin either in the absence (O) or presence of 0.4 μM TPA (●). Percent cells spread was scored at the indicated times. The data represent the average of 4 experiments with duplicate samples and the standard deviation.

TPA Enhances the Rate and the Extent of HeLa Cell Spreading

When HeLa cells are plated onto a gelatin substratum at 37°C, cells could be seen to be spread on 10-min incubation with the maximum number of cells spread (~90%) within 15 to 20 min (Figure 6). If the cells are allowed to spread in the presence of TPA, cells begin to spread as early as 5 min after plating the cells, and the maximum number of cells that spread is obtained within 10 min (Figure 6). In addition to the increase in the rate of cell spreading, the extent of spreading, i.e., the increase in the area of the substratum covered by a cell, is increased by PKC activation with TPA (Table 1). The increase in the rate and the extent of cell spreading is also observed when cells are treated with 0.4 μM of 4-β-phorbol dibutyrate (PKC activating phorbol ester, Ashendel, 1985), 0.4 μM of mezerein (a nonphorbol type PKC activator, Miyake *et al.*, 1984), or 50 μg/ml

Table 1. Activation of PKC enhances the extent of HeLa cell spreading

Cells	Relative cell area
Attached (n = 143)	1.00 ± 0.02
Attached with calphostin C (n = 52)	1.00 ± 0.02
Spread (n = 115)	3.97 ± 0.63
Spread with TPA (n = 92)	6.10 ± 1.06

HeLa cells were plated on gelatin coated culture dishes in the following conditions: *Attached*, cells were allowed to attach for 5 min; *Attached with calphostin C*, suspension cells were treated with 1 μM calphostin C for 5 min and plated on gelatin for 30 min; *Spread*, cells were allowed to attach and spread for 30 min; *Spread with TPA*, cells were plated for 30 min in the presence of 0.4 μM TPA. The cells were photographed by using phase contrast microscopy, and the relative cell area was determined by cutting and weighing the pictures of individual cells. The number of cells analyzed in each case is equal to n. Values are means ± SD.

of 1-oleoyl-2-acetyl-glycerol (synthetic DG, Kaibuchi *et al.*, 1983). However, cell spreading is not affected when cells are treated with 0.4 μM of 4-α-phorbol-12, 13-didecanoate (PDD) or the nonesterified form of phorbol, which do not activate PKC (Ashendel, 1985).

Amplification of AA Production by PKC

It was examined whether activation of PKC regulates AA production during HeLa cell adhesion to a gelatin substratum. Regulation of AA production by PKC has been suggested in many cell types (Emilsson *et al.*, 1986; Godson *et al.*, 1990; Zor *et al.*, 1990; Cybulsky, 1991; Weiss and Insel, 1991). When HeLa cells were treated with calphostin C to inhibit PKC before spreading assays, release of AA is not blocked in cells plated onto a gelatin substratum, whereas spreading is blocked (Figure 3). However, the amount of AA released is reduced when compared with that in untreated cells (Figure 7A). This suggests that substratum-induced PKC activation also increases AA release as a positive feedback mechanism. In contrast to the decrease in AA release by PKC inhibition, treating HeLa cells with TPA increases the amount of AA released and maintains the high levels of AA over a longer period of time (Figure 7A). This may indicate that TPA causes a persistent release of AA or reduces the re-incorporation of the released AA into phospholipids. TPA treatment also induces AA release in cells attached to a BSA substratum in which the substratum itself does not induce AA release or cell spreading (Figure 7B). This suggests that TPA-induced AA release does not require the interaction of the gelatin substratum with the collagen receptors on the surface of HeLa cells as is required for AA release and cell spreading in the absence of TPA (Chun and Jacobson, 1992). TPA-induced AA release in cells attached to BSA is insignificant when either PKC is inhibited with calphostin C or PLA_2 is blocked with BPB (Figure 7B) indicating that TPA induces AA release through the activation of PLA_2 by PKC. Unlike AA release, the amount of DG produced during cell spreading is not affected by activation of PKC (Figure 7C). Also, the amount of DG produced in cells plated on BSA is not increased when the cells are treated with 0.4 μM TPA or 1 μM calphostin C, however, TPA sustains the release of DG relative to the control during cell spreading on a gelatin substratum (Figure 7C). The data are consistent with the view that activation of PLA_2 is the mechanism responsible for the PKC-induced AA release and that the increase in DG production during cell attachment and its hydrolysis to AA is separate from the PKC-induced increase in AA release.

Amplification of AA Production by DG Hydrolysis

It is known that DG not only has the ability to activate PKC but it can also be hydrolyzed by the sequential action of DG and MG lipases to produce AA (Ide *et al.*,

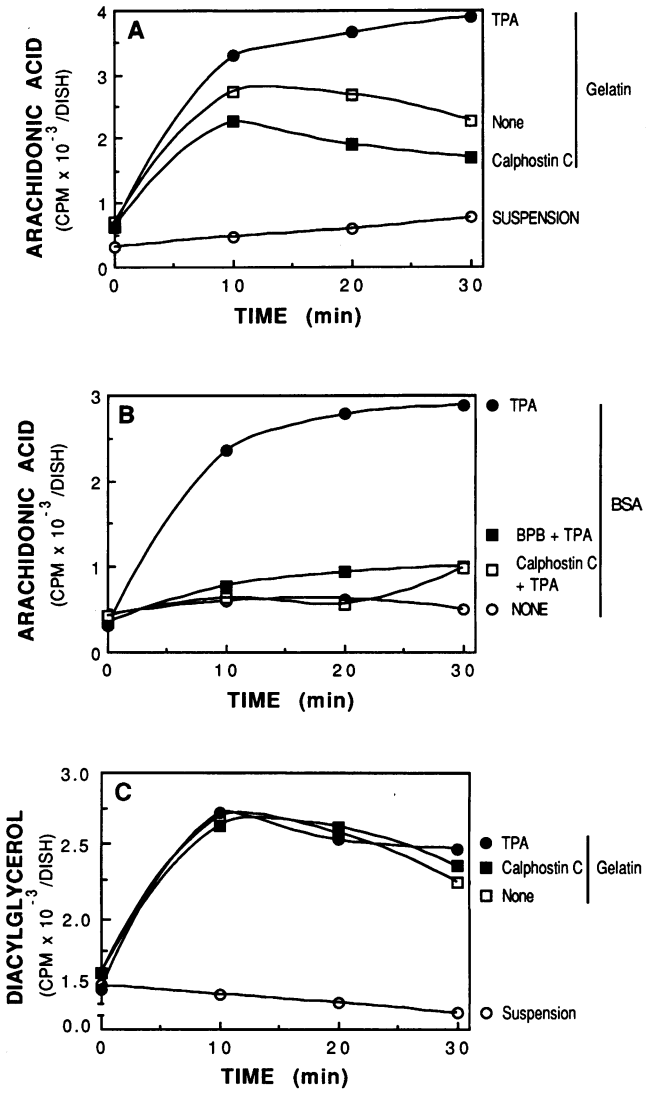

Figure 7. PKC activation amplifies AA production. (A) PKC modulates AA production in cells plated on a gelatin substratum. HeLa cells with phospholipids prelabeled with [^{3}H]AA were plated onto gelatin in the presence of 0.1% DMSO as a vehicle (□) or 0.4 μM TPA (●). Alternatively, cells were treated with 1 μM calphostin C (■) for 5 min and plated onto a gelatin substratum. As a control, untreated cells were kept in suspension in the presence of 0.1% DMSO (○). The amounts of AA released were determined at the indicated times. (B) Inhibition of PKC or PLA_2 blocks TPA-induced AA release in cells plated on BSA. Cells were allowed to attach to a BSA substratum in the absence (○) or presence of 0.4 μM TPA (●). Alternatively, cells were treated with 1 μM calphostin C (□) or 12 μM BPB (■) for 5 min and plated onto BSA in presence of 0.4 μM TPA. At the indicated times, the amount of AA released was determined. (C) PKC does not affect DG production in cells plated on a gelatin substratum. HeLa cells with phospholipids prelabeled with [^{3}H]AA were plated onto a gelatin substratum in the presence of 0.1% DMSO as a control (□) or 0.4 μM TPA (●). Suspension cells were treated with 1 μM calphostin C for 5 min and plated on gelatin (■). As a control, untreated cells were allowed to attach to a BSA substratum in the absence of TPA (○). The amount of DG was determined at the indicated times as described in MATERIALS AND METHODS. The data represent a typical experiment conducted 5 times (A and B) or 4 times (C) with comparable results.

1990; Balsinde *et al.*, 1991; Dennis *et al.*, 1991). To address the role of DG hydrolysis in AA production, formation of MG, which contains [^{3}H]AA, was assayed. As shown in Figure 8A, levels of MG increase in HeLa cells during spreading on a gelatin substratum indicating that the DG produced is effectively hydrolyzed by DG lipase. When cells are treated with RHC 80267, a specific inhibitor of DG lipase (Sutherland and Amin, 1982), the levels of MG decrease below those found in suspension cells, i.e., at time zero (Figure 8A). This suggests that there is a constant turnover of DG facilitated by DG lipase. Treatment of cells with RHC 80267 also reduces the normally higher level of AA observed upon attachment of cells to gelatin (Figure 8B). However, the initial rate of AA release is not affected by RHC 80267 treatment. This suggests that AA must first be released before it gives rise to DG which in turn produces more AA by the sequential action of DG and MG lipases. Treating cells with RHC 80267 also partially reduces the number of cells that spread in a dose dependent manner (Figure 8C). The inhibitory effect of RHC 80267 on cell spreading is overcome if the cells are exposed to exogenous AA or PKC activating phorbol ester, TPA (Figure 8C). The data indicate that hydrolysis of DG is responsible for maintaining elevated levels of AA that are required for the maximum number of cells to spread.

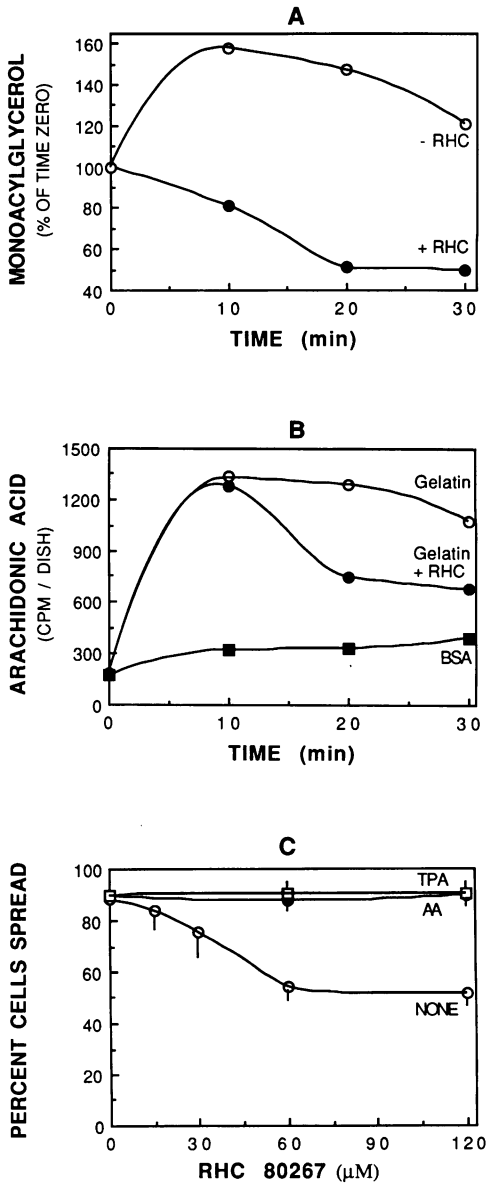

Figure 8. Hydrolysis of DG amplifies AA production. (A) Production of MG is blocked by the inhibition of DG lipase. HeLa cells with phospholipids prelabeled with [^{3}H]AA were plated on gelatin coated culture dishes in the absence (○) or presence of (●) of 60 μM RHC 80267. At the indicated times, the amounts of MG were determined as described in MATERIALS AND METHODS. (B). Inhibition of DG lipase partially blocks AA production. HeLa cells with phospholipids prelabeled with [^{3}H]AA were plated on BSA (■) or on gelatin in the absence (○) or presence (●) of 60 μM RHC 80267. The amounts of [^{3}H]AA released by the cells were determined at the indicated times. (C). Inhibition of DG lipase partially blocks HeLa cell spreading. Cells were treated with the indicated concentrations of RHC 80267 for 5 min in suspension and plated on gelatin coated culture dishes (○). Percent cells spread was scored following 30-min incubation at 37°C. The cells plated on gelatin for 30 min in the presence of RHC 80267 were exposed to either 15 μM AA (●) or 0.4 μM TPA (■). Percent cells spread was scored following 15-min incubation. The data represent a typical experiment conducted 6 times (A and B) and the average values of four experiments with duplicate samples and the standard deviations (C).

DISCUSSION

HeLa Cell Spreading Requires a Sequence of Events

Previous studies indicated that clustering of HeLa cell surface collagen receptors, upon attachment of cells to a gelatin substratum, induces release of AA by the action of PLA_2 and that AA is subsequently oxidized by LOX to produce a metabolite(s), which initiates cell spreading (Chun and Jacobson, 1992). In this study it was demonstrated that there is an additional sequence of reactions after the formation of a LOX metabolite(s) that is needed to initiate cell spreading: the production of DG and the activation of PKC. Figure 9 depicts a working hypothesis that is consistent with the data presented here and in a previous publication (Chun and Jacobson, 1992). The figure is not meant to be the final word but mainly a guide to assist the reader in pulling together the data.

The production of DG most likely occurs as a result of PLC activation by a LOX metabolite(s) formed from released AA. This is supported by the observations that 1) the inhibition of either AA release or LOX metabolite formation blocks cell spreading and DG production in HeLa cells attached to a gelatin substratum and 2) while treating cells in suspension with exogenous AA induces DG production, the induction is blocked by the LOX inhibitor, NDGA (Figure 1). The coupling of PLA_2 with PLC leading to a cellular function has been suggested for many systems such as the stimulation of platelets by certain agonists (Banga *et al.*, 1986; Sweatt *et al.*,

1986) and mitogenic stimulation of T lymphocytes (Mire-Sluis *et al.*, 1989).

Evidence indicates that PKC is activated as a result of the production of DG one of the best known physiological activators of PKC (Nishizuka, 1986). Activation of PKC occurs upon attachment and before cell spreading (Figure 2). Furthermore, inhibition of AA release and DG production blocks both PKC activation and cell spreading (Figure 2B), and the inhibition of cell spreading induced by blocking AA release is reversed by the activation of PKC with phorbol esters (Figure 5). Though it is likely that PKC is activated directly by the DG produced during cell attachment to initiate cell spreading, it is possible that PKC is also activated directly by AA or lysophospholipids, which are produced by cell attachment-activation of PLA_2. Others have shown that AA and lysophospholipids activate PKC (Blackshear *et al.*, 1988; Huang, 1989). Although we do not currently know whether lysophospholipids released during cell attachment stimulate PKC, we do know that AA itself does not since exogenously supplied AA in the presence of NDGA to inhibit LOX does not induced cell spreading (Chun and Jacobson, 1992).

Activation of PKC appears to be essential for cell spreading because inhibition of PKC with calphostin C or sphingosine blocks spreading (Figure 3). Consistent with this conclusion is that the activation of PKC with extracellular stimulators, e.g., phorbol ester, enhances the rate and the extent of cell spreading but not the percent of cells spread at 37° (Figure 6 and Table 1). In addition, direct activation of PKC with TPA is sufficient to induce cell spreading even in the absence of AA release and subsequent DG production (Figure 5). The involvement of PKC in cell-ECM adhesion in other cell types has also been suggested as described in the INTRODUCTION. Interestingly, whereas specific inhibition of PKC with calphostin C or sphingosine blocks spreading of cells, nonspecific inhibitors of PKC, i.e., staurosporine, H7, and K252b, have no effect on spreading of cells (Figure 4A). However, the data shown in figures 4, A and B suggest that the concomitant inhibition of other protein kinases in addition to PKC by nonspecific inhibitors antagonizes the effects of PKC inhibition on cell spreading. It is possible that the protein kinase responsible for antagonizing the effect of PKC inhibition could be PKA since a relatively specific inhibitor of PKA, KT 5720, is the most potent antagonist of calphostin C (Figure 4B). This does not rule out that other protein kinases could be involved in HeLa cell spreading.

Amplification of AA Production by DG Hydrolysis and PKC Activation

The data presented here indicate that HeLa cell spreading is an amplification process centered on AA production possibly by two pathways as portrayed in Figure 9. The first amplification of AA production comes from DG and its sequential hydrolysis to MG then to AA and glycerol (Figure 8). The existence of a functional DG lipase that produces AA in HeLa cells was demonstrated from the results that 1) the levels of MG increase in cells plated on gelatin and the increase in MG levels is blocked with the DG lipase inhibitor, RHC 80267 (Figure 8) and 2) specific inhibition of DG lipase reduces the sustained AA release induced by cell attachment to gelatin (Figure 8). The net result of DG release and its hydrolysis is to increase the pool of available AA, which is known to act as a rate-limiting step for eicosanoid synthesis (Needleman *et al.*, 1986; Dennis, 1987). The hydrolysis of DG is an important source for AA production in many cellular responses (Banga *et al.*, 1986; Grillone *et al.*, 1988; Bhagyalakshmi and Frangos, 1989; Ide *et al.*, 1990; Pandol *et al.*, 1991; Rapuano and Bockman, 1991). However, DG hydrolysis to AA does not appear to be an absolute requirement for HeLa cell spreading although it appears to be a facilitator of spreading (Figure 7).

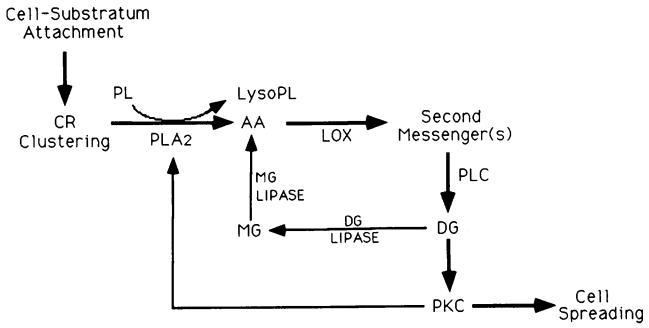

Figure 9. Working hypothesis for signaling pathway during HeLa cell adhesion to a gelatin substratum based on the results presented here and that in a previous paper (Chun and Jacobson, 1992). CR, collagen receptor; PL, phospholipids; LysoPL, lysophospholipids; PLA_2, phospholipase A_2; LOX, lipoxygenase; PLC, phospholipase C; DG, diacylglycerol; MG, monoacylglycerol; PKC, protein kinase C.

Because the activation of PKC with TPA enhances spreading of HeLa cells, it is reasonable to postulate that the inhibition of DG hydrolysis enhances cell spreading by activating PKC for a longer time period. However, levels of cellular DG are known to be controlled not only by DG hydrolysis but also by several different pathways such as conversion to phosphatidic acid by DG kinase (Kanoh *et al.*, 1990). This may explain why PKC activation upon attachment of HeLa cells to gelatin is transient reaching an optimum level 8 min after cell-substratum attachment before declining (Chun and Jacobson, 1992). Consistent with the rapid metabolism of DG is the sustained activation of PKC upon TPA treatment in HeLa cells. TPA is not readily metabolized by cells as is DG (Ashendel, 1985). Indeed, TPA causes a more rapid translocation of cytosolic PKC to the membrane (within 3 min) and the membrane-

bound PKC activity is maintained longer (≤15 min) than untreated cells (Figure 2). This is consistent with the extended activation of PKC by TPA compared with untreated cells where PKC activation depends upon DG production.

AA production also appears to be amplified by PKC activation. This is supported by the observations that inhibition of PKC with calphostin C reduces the amount of AA released, whereas TPA treatment enhances AA release in cells attached and spread on gelatin. TPA-induced AA release is PKC-dependent because calphostin C blocks TPA-stimulated AA release in cells attached to BSA (Figure 7). The ability of TPA to induce AA release appears to be mediated by its ability to activate PLA_2 because BPB inhibition of PLA_2 blocks TPA-induced AA release (Figure 7). Regulation of AA production by PKC activity has been shown in many cell types. For example, PKC activators such as TPA activate PLA_2, which releases AA in various cell types (Emilsson *et al.*, 1986; Godson *et al.*, 1990; Zor *et al.*, 1990; Cybulsky, 1991; Weiss and Insel, 1991). It is doubtful that the PKC induced amplification of AA greatly enhances cell spreading by increasing the levels of DG because the levels are not markedly increased by TPA treatment. However, it is possible that the feedback amplification of AA production by PKC enhances cell adhesion by sustaining cell spreading because TPA reduces the decline in DG seen in cells spreading on a gelatin substratum without TPA (Figure 7C). It is also possible that a lysophosphopholipid, the other product of PLA_2 activity, increases cell spreading by directly increasing PKC activity. Lysophosphatidylcholine is known to directly activate PKC (Blackshear *et al.*, 1988; Huang, 1989). This is currently being investigated.

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