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β_1 Integrins Signal Lipid Second Messengers Required during Cell Adhesion

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Clustering of integrin receptors during cell adhesion stimulates signal transduction across the cell membrane. Second messengers are generated, activating cytosolic proteins and causing cytoskeletal assembly and rearrangement. HeLa cell adhesion to a collagen substrate has been shown to initiate an arachidonic acid-mediated signaling pathway, leading to the activation of protein kinase C (PKC) and cell spreading. To determine the role of integrin receptors in triggering this signaling pathway, monoclonal antibodies to β_1 integrins were used to either cluster integrins on the cell surface or to provide an integrin-dependent substrate for cell adhesion. Using this approach, we have defined a pathway required for cell spreading that can be initiated by the ligation of integrins and leads to the activation of PKC. Specifically, our results indicate that clustering β_1 integrins results in the activation of phospholipase A_2 leading to the production of arachidonic acid and the activation of PKC.

INTRODUCTION

Integrins are $\alpha\beta$ heterodimers involved in cell-extracellular matrix adhesion (Akiyama *et al.*, 1990; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991; Hynes, 1992). They are transmembrane adhesion proteins that link the extracellular matrix (ECM) on the outside of the cell to cytoskeletal and signaling systems on the inside. As cells attach and spread on a specific ECM protein, focal adhesions form connecting ECM, integrins, and cytoskeletal proteins such as talin, vinculin, and actin, as well as regulatory proteins including focal adhesion kinase, protein kinase C (PKC), src, paxillin and tensin (Burridge *et al.*, 1988; Davison and Critchley, 1988; Turner *et al.*, 1990; Davis *et al.*, 1991; Woods and Couchman, 1992; Sastry and Horwitz, 1993).

Integrin receptors, occupied by extracellular matrix ligands, are able to trigger many intracellular signals during cell adhesion (Damsky and Werb, 1992; Hynes, 1992; Schwartz, 1992; Juliano and Haskill, 1993; Chen *et al.*, 1994a,b). Known signal transduction pathways involving integrins include the activation of platelet degranulation (Shattil and Brugge, 1991; Jans, 1992; Raghow, 1994), the Na^+/H^+ antiporter (Schwartz *et al.*, 1991a,b; Schwartz, 1993), changes in intracellular levels of Ca^{2+} (Ng-Sikorski *et al.*, 1991), changes in the levels of phosphoinositide and inositol phosphates (Breuer and Wagner, 1989; McNamee *et al.*, 1993; Chong *et al.*, 1994), tyrosine phosphorylation (Guan *et al.*, 1991; Kornberg *et al.*, 1991, 1992; Burridge *et al.*, 1992; Juliano and Haskill, 1993), activation of mitogen-activated protein kinases (Schlaepfer *et al.*, 1994; Chen *et al.*, 1994a), and the release of cytokines that ultimately cause T cell proliferation (Matsuyama *et al.*, 1989; Nojima *et al.*, 1990; Shimizu *et al.*, 1990; Fine and Kruisbeek, 1991; van Seventer *et al.*, 1991). Although much has been learned regarding the role of integrins in cell adhesion and signal transduction, the specific signal transduction pathways that lead to cell adhesion still remain elusive.

Previous studies from this laboratory have demonstrated that HeLa cell adhesion to a gelatin substrate activates lipid second messenger systems (Chun and Jacobson, 1992, 1993). This pathway involves the activation of phospholipase A_2 (PLA_2), which leads to the formation of arachidonic acid (AA). Generation of AA is required for the activation of PKC, which in turn is

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required for cell spreading. The importance of this signal transduction pathway in cell adhesion in other cell types has also been demonstrated (Chun and Jacobson, 1992). The purpose of the present study was to determine if β_1 integrins can signal the activation of this lipid second messenger pathway. Using monoclonal antibodies (mAb) against the β_1 integrin subunit to cluster integrin receptors or as a substrate for cell adhesion, we defined a role for β_1 integrins in activating lipid second messengers required for cell spreading. By this approach, we have demonstrated that the following signaling pathway can be regulated by β_1 integrins: β_1 integrin ligation → PLA_2 activation → AA formation → oxidation of AA by lipoxygenase (LOX) → activation of PKC → cell spreading.

METHODS AND MATERIALS

Materials

[^{3}H]AA was purchased from ICN (Cleveland, OH). Rhodamine-conjugated goat anti-mouse IgG, goat anti-rat, and fluorescein-conjugated anti-goat antibodies were obtained from Pierce (Rockland, IL). Rat anti-human β_1 integrin mAb 13 (Akiyama *et al.*, 1989) was a generous gift of Dr. Kenneth Yamada, National Institutes of Health, Bethesda, MD. mAb K20 was purchased from Immuntech (Westbrook, ME). Plastic polystyrene sheets, 0.8 mm in thickness, were supplied by KAMA (Hazleton, PA).

Cell Culture

HeLa-S3 (American Type Culture Collection, Rockville, MD) cells were grown in suspension to mid-log phase ($2–4 \times 10^5$) at 37°C in a humidified 5% CO_2 in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 5% calf serum (Life Technologies, Grand Island, NY) 0.3% $NaHCO_3$, 100 mg/ml dihydrostreptomycin, 60 mg/ml penicillin, and 0.0002% butyl parahydroxybenzoate (Sigma).

Substratum Preparation

Polystyrene culture dishes were coated with either gelatin or mAb 13 to the β_1 integrin subunit. Swine skin type I gelatin at 1 mg/ml was covalently coupled to 35-mm polystyrene cultured dishes (Falcon, Oxanard, CA) as described previously (Lu *et al.*, 1989, 1992; Beacham *et al.*, 1990). In some experiments, 96-well culture dishes were coated with either 50, 100, or 200 μg/ml mAb 13 for 1 h at 37°C.

In Vitro Assay for HeLa Cell Spreading

HeLa cells used for adhesion assays were grown in suspension culture to a density of $2–4 \times 10^5$ cells/ml, washed twice in serum-free medium, centrifuged at $300 \times g$ for 5 min, and then resuspended in serum-free medium. The cells were treated with various inhibitors or activators in suspension at ambient temperature. Solvents for these agents were always at less than 0.1% of final volume. The cells were then plated on substrate-coated culture dishes at either 10^4 cells/well in 96-well tissue culture plates or 10^5 cells/well in 35-mm tissue culture dishes for 30 min at 37°C. Cell spreading was assayed as previously described (Burke *et al.*, 1983; Chun and Jacobson, 1992; Lu *et al.*, 1992). The percentage of cells spread was calculated by determining the number of spread cells divided by the total number of cells × 100. Cell numbers ranged from 150–200 cells per culture dish and at least three fields of view were counted using phase contrast microscopy. A fully spread cell was defined as one that was at least two times the diameter of the nucleus.

Detection of Integrin Clustering Using Indirect Immunofluorescence Microscopy

HeLa cells were attached to sulfonated polystyrene plastic sheets as previously described (Lu *et al.*, 1989; Beacham *et al.*, 1990), and were treated with either fixative (3.7% paraformaldehyde in phosphate-buffered saline [PBS], pH 7.4), rat anti-human β_1 integrin mAb 13 (PBS containing 1 mM $MgCl_2$ and 5% fetal calf serum), or fluorescein-conjugated goat anti-rat IgG (PBS containing 1 mM $MgCl_2$ and 5% fetal calf serum) as indicated in Figure 4. The fixed cells were washed (five times) in PBS/5.0% fetal calf serum solution between incubations.

AA Assay

HeLa cells were labeled with [^{3}H]AA (0.2 μCi/ml) for 18–22 h at 37°C in suspension culture as described by Chun and Jacobson (1992). Labeled cells were collected and incubated with RPMI-medium on ice for 30 min. Equal numbers of the cells were washed and resuspended in serum-free RPMI medium and were allowed to attach and spread on substrate-coated culture dishes (5×10^5 cells/ml). Once the cells were added to the culture dishes at 37°C, starting at time = 0 min, aliquots of medium were taken in triplicate (3×50 μl) at indicated times. Aliquots were immediately microfuged for 2 min to remove any cells that had not yet attached to the substrate. The amount of [^{3}H]AA released at these times was determined for each aliquot of medium using a scintillation counter (Beckman Instruments, Palo Alto, CA).

RESULTS

β_1 Integrin Involvement in Spreading of HeLa Cells on a Gelatin Substrate

HeLa cells express the β_1 integrins ($\alpha_2\beta_1$ and $\alpha_3\beta_1$) that mediate adhesion to a collagen substrate (Roberts *et al.*, 1977; Beacham *et al.*, 1990; Hynes, 1992). To determine whether β_1 integrins mediate HeLa cell adhesion to gelatin, the ability of anti-functional antibodies to integrin β_1 to inhibit cell spreading on a gelatin substrate was examined. HeLa cells were treated in suspension with increasing concentrations of mAb 13 to the β_1 integrin and plated on a gelatin substrate. MAb 13 inhibited cell spreading and this inhibition was dependent upon the concentration of antibody (Figure 1A), consistent with published reports that mAb 13 inhibits cell adhesion to a collagen substrate (Yamada *et al.*, 1990). Furthermore, this inhibition was specific because antibodies against other cell surface proteins, such as the major histocompatibility class I (MHC I) that are not involved in cell adhesion, did not inhibit HeLa cell spreading on gelatin (Figure 1A). β_1 integrins function in HeLa cell adhesion and thus may initiate signaling pathways required for this process.

Release of AA during Cell Spreading on MAb 13 Requires PLA_2

Because lipid second messengers had previously been demonstrated to be required for HeLa cell spreading, we first tested whether β_1 integrins could initiate this signal transduction process. Two experimental approaches were employed as follows: the first approach

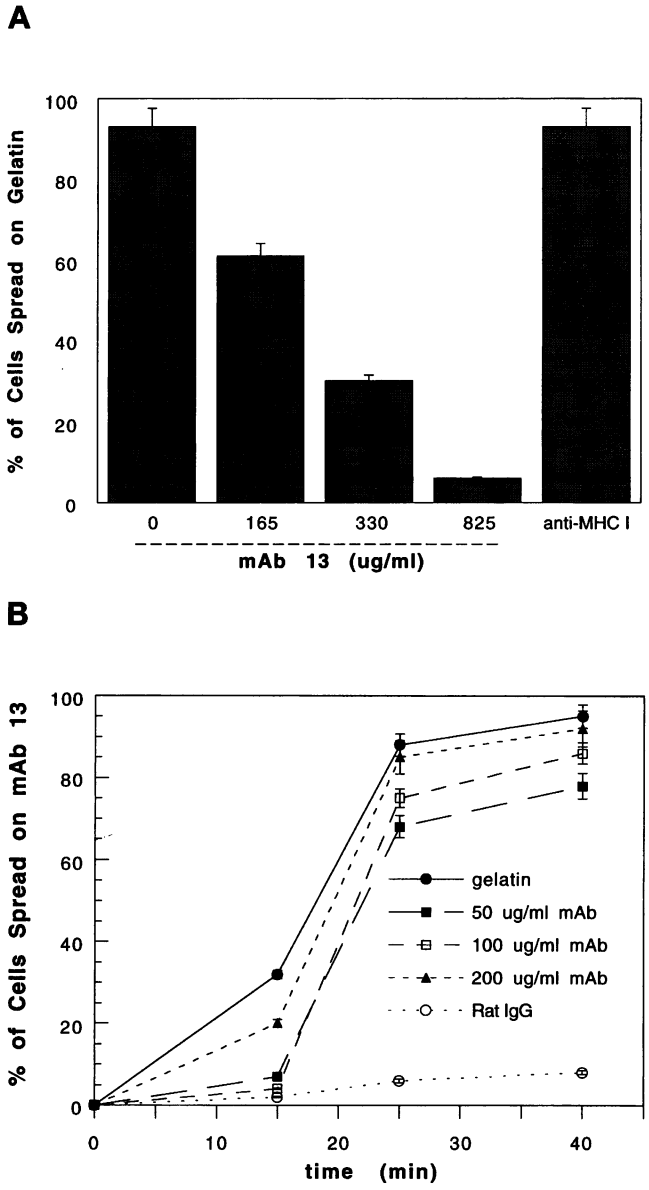

Figure 1. Effects of HeLa spreading in the presence of the β_1 integrin mAb. (A) HeLa cells were resuspended in RPMI with either 0, 165, 330, or 825 μg/ml of β_1 integrin mAb 13, or with polyclonal antibody to the MHC I surface receptor. The cells were incubated on ice for 15 min and then plated on gelatinized tissue culture dishes for 30 min at 37°C. HeLa cells that were incubated with anti-MHC I were resuspended in fresh DMEM to a concentration of either a 1:1, 1:2, 1:10, or 1:100 dilution of anti-MHC I (BB7.7 cell supernatant) in 3 ml of DMEM. The cell spreading represented on the graph for the highest concentration of anti-MHC I was representative of all concentrations of anti-MHC I. (B) HeLa cells were plated on 96-well tissue culture dishes coated with either 50, 100, or 200 μg/ml of β_1 integrin mAb 13 and incubated for 30 min at 37°C. Cell spreading was scored at 15, 25, and 40 min. The control cells were spread on gelatinized 35-mm tissue culture plates. The data represent the average value of three experiments performed in triplicate with standard deviations.

used antibodies to the β_1 integrin as a substrate for cell spreading and the second approach used antibodies to cluster β_1 integrins on the cell surface. Both of these approaches have proven useful in identifying roles of integrins in signal transduction (Guan *et al.*, 1991; Kornberg *et al.*, 1991, 1992; Ng-Sikorski *et al.*, 1991; Schwartz *et al.*, 1991a; Burridge *et al.*, 1992; Juliano and Haskill, 1993).

Initial experiments examined the concentration of mAb 13 on the substrate required to support HeLa cell spreading. To determine the concentrations that allowed maximal cell spreading, 96-well plates were coated with increasing concentrations of mAb 13 and the percentage of cells spread was observed (Figure 1B). Maximal cell spreading (80–90%) occurred at coating concentrations between 100–200 μg/ml. These concentrations are similar to those found to promote cell spreading of other cell types (Akiyama *et al.*, 1989; Duband *et al.*, 1991).

The release of AA has been shown to activate HeLa cell spreading on a gelatin substrate by stimulating the activity of protein kinase C (Chun and Jacobson, 1992, 1993). Because the release of AA is an important step in the pathway leading to cell spreading, we tested whether ligation of β_1 integrins could trigger the release of AA. HeLa cells were plated on a mAb 13 substrate and the release of [3H]AA in the culture media was determined at various times after plating (Figure 2). Levels of AA released during cell adhesion on the mAb 13 substrate were twofold greater when compared with cells that were inhibited from spreading in the presence of a phospholipase A_2 inhibitor on the mAb 13 substrate (Figure 2). Furthermore the release of AA occurred early in the adhesion process, before cell spreading, consistent with previous observations (Chun and Jacobson, 1992).

AA can be released from membrane phospholipids by many types of phospholipases (Dennis, 1987; Dennis *et al.*, 1991). Previous experiments indicate that PLA_2 is required for AA release during cell spreading. To examine the role of PLA_2 in the β_1 integrin-triggered release of AA, we tested the ability of bromophenacyl bromide (BPB), a specific inhibitor of PLA_2 (Roberts *et al.*, 1977; Dennis, 1987; Glaser *et al.*, 1993; Gelb *et al.*, 1994), to inhibit β_1 integrin-triggered release of AA. The addition of BPB was found to inhibit the release of AA (Figure 2) and, as expected, the inhibition by PLA_2 also inhibited cell spreading on the mAb 13 substrate (Figure 2 and Table 1). Results indicate that although BPB inhibited the formation of AA and further downstream second messengers needed for HeLa cell spreading, the addition of exogenous AA bypassed the inhibition and HeLa cells were able to spread on the mAb 13 substrate (Table 1). The interaction of HeLa cells with an antibody to the β_1 integrin as substrate was sufficient to activate PLA_2 leading to the release of AA and cell spreading.

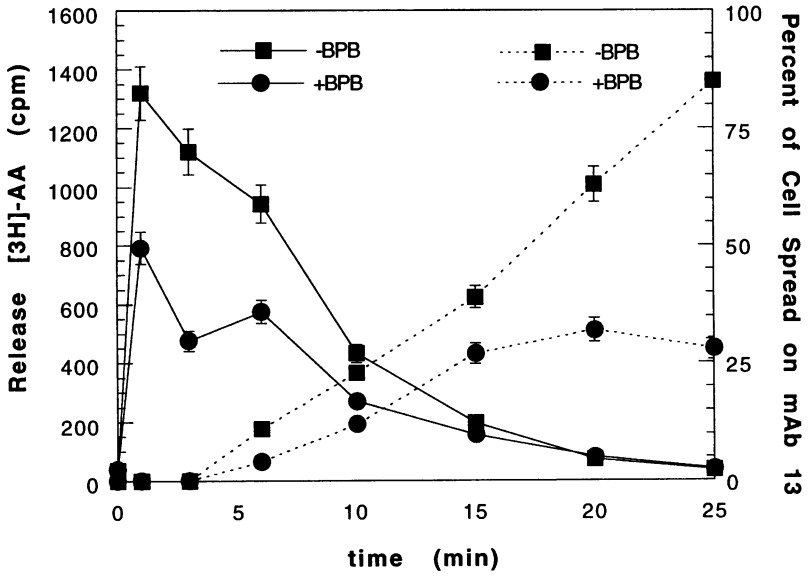

Figure 2. Release of AA in the presence of a PLA_2 inhibitor BPB during HeLa cell spreading on a β_1 integrin mAb substrate. Cells were treated with 6 μM BPB (circles) or without BPB (squares) in suspension and then were plated on a β_1 integrin mAb 13 (100 μg/ml on a 96-well culture dish) substrate for 40 min. Aliquots were taken at 0, 1, 3, 6, 10, 15, 20, and 25 min, and [3H]AA was counted (solid lines). The percent of cells spread on the β_1 integrin mAb 13 substrate were also counted at the same times (hashed lines). The data represents averages of three experiments with triplicate samples.

AA Is Released upon β_1 Integrin Receptor Clustering

Because integrin clustering can signal other second messenger pathways, we next tested whether clustering of β_1 integrin receptors could signal a release of AA. Integrin clustering was obtained by treating cells in suspension with mAb 13 alone or together with polyvalent secondary antibody. Treatment with both mAb 13 and secondary antibody resulted in the formation of distinct integrin antibody clusters on the cell surface (Figure 3). We found that although clustering the β_1 integrin with primary antibody alone was sufficient to induce release of AA at levels significantly above control, clustering β_1 integrins with both primary and secondary antibodies resulted in the release of even higher levels of AA, levels similar to those caused by adhesion on a gelatin substrate (Figure 4). As a control we tested the effects of the mAb K20 on the extracellular portion of the β_1 integrin and found that AA was released during receptor clustering. Therefore, clustering integrins on the cell surface signals the release of AA and the higher the degree of receptor clustering, the higher the levels of AA released. Furthermore, the clustering-dependent release of AA could be inhibited by the addition of BPB, indicating the requirement for the activation of PLA_2 (Figure 2 and Table 1). Clustering of β_1 integrins, therefore, triggers AA release by signaling the activation of PLA_2.

Table 1. Effects of various AA metabolism inhibitors on HeLa cell spreading on β_1 integrin mAb-coated culture dishes

Treatment	Percentage of cells spread
Untreated	91±3
BPB (6 μM, 5 min)	25±8
BPB, added AA (0.4 μM)	93±1
BPB, added TPA (1.2 μM)	74±4
NDGA (25 μM, 5 min)	47±6
NDGA, added AA (0.4 μM)	83±5
NDGA, added TPA (1.2 μM)	86±4
Indomethacin (100 μM, 5 min)	88±6
Metyrapone (1μM, 5 min)	80±3

HeLa cells in suspension were treated with various inhibitors for the indicated period of time and concentration. The cells were then plated on β_1 integrin mAb 13 (100 μg/ml)-coated tissue culture dishes and incubated at 37°C at which time the percentage of cell spreading was determined. BPB is an inhibitor of PLA_2. NDGA is a competitive inhibitor of LOX, while indomethacin and metyrapone are inhibitors of COX and EOX, respectively. To look at the recovery of the inhibition of cell spreading by BPB and NGDA, either exogenous AA or TPA, a PKC activator, were added 3 min after the addition of the inhibitors. The data represent the average of at least three experiments in triplicate with standard deviations.

AA Is Metabolized by Lipoxygenase

Various enzymes catalyze the metabolism of AA. To determine whether AA metabolism is required for cell spreading on the mAb 13 substrate, we tested the ability of inhibitors of cyclooxygenase (COX), LOX, and cytochrome p-450 to inhibit this spreading (Table 1). The inhibitors indomethacin and metyrapone, of the cyclooxygenase and cytochrome p-450–dependent pathways, respectively, were examined. Although these agents slightly inhibited cell spreading, they were not strong inhibitors (Table 1), indicating that these pathways may serve as possible feedback mechanisms for producing small amounts of AA. We next

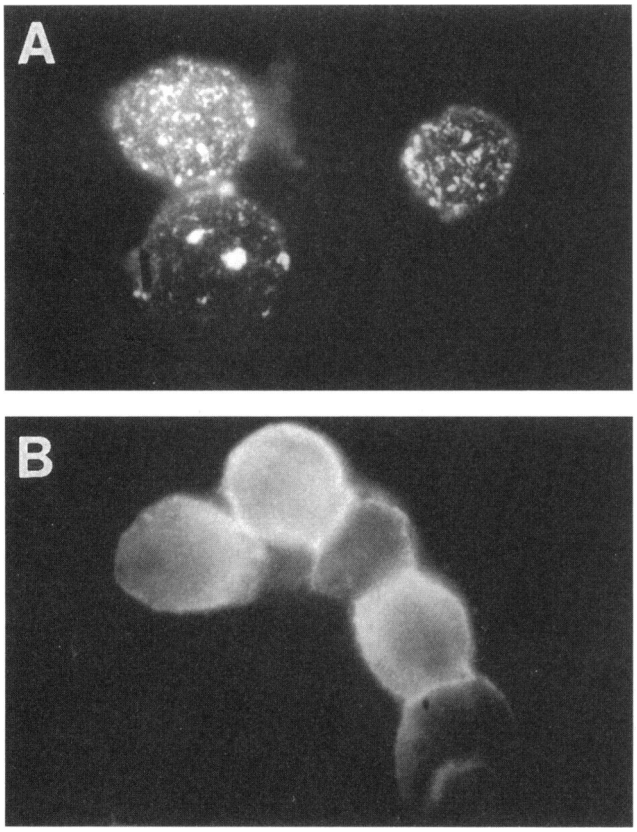

Figure 3. The β_1 integrin mAb (mAb 13) induces β_1 integrin receptors to cluster. (A) To cluster β_1 integrins, HeLa cells were incubated with mAb 13 (200 μg/ml) for 20 min at 4°C, washed, incubated in fluorescein-conjugated goat anti-rat IgG for 10 min at 37°C and then fixed. (B) No clustering was observed when cells were first fixed and then incubated with β_1 integrin mAb (200 μg/ml) for 20 min at 4°C, then incubated with fluorescein-conjugated goat anti-rat IgG for 10 min at 37°C. Cells treated with RPMI and secondary antibody alone gave no detectable fluorescence.

looked at the requirement for LOX metabolism of AA in cell spreading using NDGA (Yoshimoto *et al.*, 1982), a competitive inhibitor of the LOX pathway. NDGA was a strong inhibitor of cell spreading on the mAb substrate (Table 1). These results strongly suggest that the formation of LOX (Capdevila *et al.*, 1981) metabolites are essential for cell spreading on the mAb 13 substrate as was found on a gelatin substrate (Chun and Jacobson, 1992).

To verify the sequence of second messenger events during β_1 integrin-activated cell adhesion, PLA_2-mediated release of AA, and subsequent formation of LOX metabolites, cells were treated with inhibitors of the PLA_2 and LOX pathways in the presence of exogenous AA (Table 1). HeLa cells treated with a PLA_2 inhibitor before the addition of exogenous AA are able to regain their ability to spread on a β_1 integrin mAb substrate (Table 1). HeLa cells treated with a competitive inhibitor to LOX followed by exogenous AA also allow cell spreading on the β_1 integrin mAb substrate (Table 1). In summary, the inhibition of cell spreading by PLA_2 and LOX inhibitors indicates that a metabolite(s) released from AA that was catalyzed by LOX acts as a signal molecule(s) required for β_1 integrin-mediated HeLa cell spreading on the mAb substrate.

Activation of Protein Kinase C Is Required for HeLa Cell Spreading on a β1 Integrin mAb Substrate

As previously reported, during adhesion of HeLa cells on a gelatin substrate, AA metabolism by way of LOX produces an unknown second messenger that turns on the production of DG that ultimately activates PKC (Chun and Jacobson, 1993). To determine whether PKC activation was required for HeLa cell spreading on a β_1 integrin mAb substrate, a specific inhibitor for PKC (Kobayashi *et al.*, 1989), calphostin C, was tested. HeLa cells were treated in suspension with calphostin C and then plated on a β_1 integrin mAb substrate for 30 min. The results demonstrate that calphostin C inhibited cell spreading on the antibody substrate in a dose-dependent manner with maximal inhibition (80%) occurring at 1.0 μm of calphostin C (Figure 5), indicating that PKC is required for integrin-dependent cell spreading. Results also indicate that treatment of fibroblast cells with calphostin C inhibited cell spreading on a fibronectin substrate, while others have shown that treatment of Chinese hamster ovary cells with a PKC inhibitor also inhibited cell spreading (Vuori and Ruoslahti, 1993). To confirm that the requirement of PKC activation is downstream of PLA_2 and LOX, the ability of the PKC activator, phorbol ester (TPA), to bypass inhibition of PLA2 and LOX was examined. Both the addition of BPB to inhibit PLA_2 and the addition of NDGA to inhibit LOX was overcome by the addition of the PKC activator, TPA (Table 1). These results indicate that ligation of β_1 integrins initiates a signaling pathway that ultimately leads to the activation of PKC.

DISCUSSION

Cell adhesion involves the interaction of the cell surface with the extracellular matrix, a remodeling of the plasma membrane, and the assembly of the cytoskeleton. Roles have been defined for specific cell surface receptors, lipid second messengers, kinases, and cytoskeletal proteins. In previous studies from our laboratory, a lipid second messenger pathway was defined that was required for the activation of cell spreading (Chun and Jacobson, 1992; Lu *et al.*, 1992). Because integrins are the major class of cell surface receptors involved in the interaction of cells with their extracellular matrix and are likely to initiate signaling pathways for cell adhesion and spreading, we studied

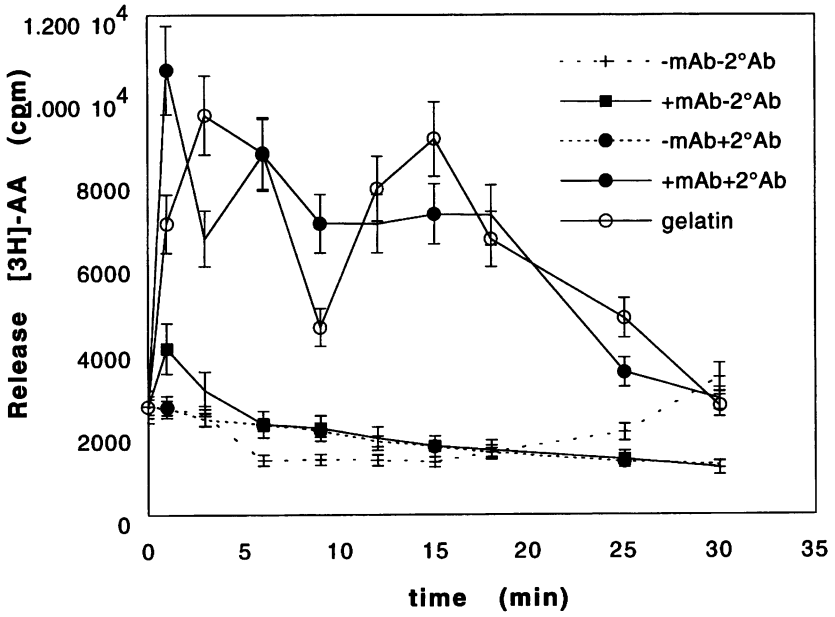

Figure 4. AA release during receptor clustering in the presence of β_1 integrin mAb. HeLa cells were labeled with [^{3}H]AA overnight, attached to sulfonated culture dishes for 15 min at 37°C, and washed with RPMI to remove any unbound cells. The cells bound to the culture dishes were either incubated with 200 μg/ml mAb 13 and goat anti-rat IgG (secondary Ab) at 37°C, or with mAb 13 alone or control cells without mAb 13 or secondary Ab. For comparison, AA release was also measured when cells were plated on gelatin-coated tissue culture dishes. Aliquots were taken at 0, 1, 3, 6, 9, 12, 15, 18, 25, and 30 min and counted. The data represents averages of three experiments with samples in triplicate and standard deviations.

the ability of β_1 integrins to activate this lipid second messenger pathway.

Using specific inhibitors for PLA_2, LOX and PKC, we have defined a role for β_1 integrins in activating cell spreading. AA is a second messenger that is generated early during cell spreading and is formed by phospholipid hydrolysis in the cell membrane. The phospholipid from which AA is hydrolyzed during cell spreading is not known, although the phospholipase responsible for its hydrolysis was found to be PLA_2. The inhibition of PLA_2 was found to inhibit cell spreading on an anti-β_1 integrin mAb substrate, while the addition of exogenous AA provided excess substrate for the generation of required AA metabolite(s) by the LOX enzyme to restore spreading. NDGA, a competitive inhibitor of LOX, inhibited AA metabolism and cell spreading. This interaction was reversed by the addition of exogenous AA. In the presence of the LOX inhibitor, we were also able to restore cell spreading by activating PKC. The activation of PKC bypasses the need for an AA metabolite to activate a downstream second messenger. These results indicate that an AA metabolite was somehow necessary for the activation of PKC required for β_1 integrin-dependent cell spreading. Studies from other laboratories suggest that this metabolite may be 12(S)-HETE (Liu *et al.*, 1991; Tang *et al.*, 1993), because this eicosanoid alters the organization of the cytoskeletal elements by enhancing PKC-dependent phosphorylation (Tang *et al.*, 1992). Our results show that integrin-mediated cell spreading was also dependent on PKC by the addition of a PKC inhibitor, calphostin C, which was able to inhibit cell spreading. Our studies agree with other researchers who have also shown that in some cell systems cell adhesion is a PKC-dependent event (Woods and Couchman, 1992; Chun and Jacobson, 1993; Herbert, 1993; Vuori and Ruoslahti, 1993). The activation of PKC during HeLa cell spreading on the purified mAb 13 substrate was integrin dependent, whereas it appears that other surface membrane proteins such as syndecans are not needed for PKC activation during cell spreading.

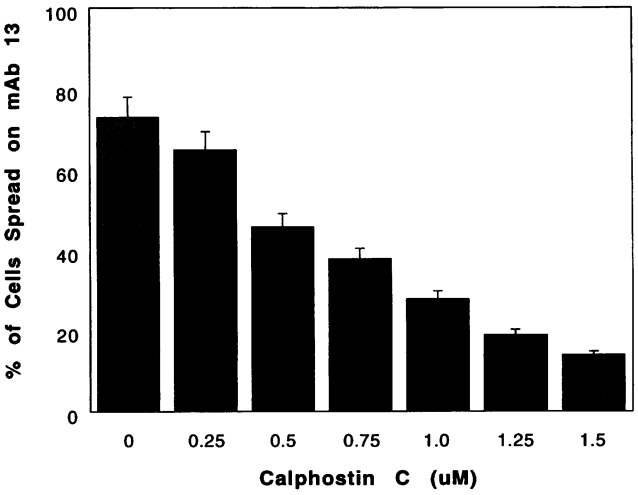

Figure 5. Inhibition of PKC blocks HeLa cell spreading on a β_1 integrin mAb substrate in a dose-dependent manner. HeLa cells were treated with indicated concentrations of calphostin C for 5 min and plated on β_1 integrin mAb 13 (100 μg/ml)-coated 96-well culture dishes. Percentage of cells spread were scored following 30 min of incubation at 37°C. The data represent the average of two experiments with triplicate samples and standard deviations.

The regulation of PKC was found to be necessary for integrin-mediated HeLa cell spreading on an anti-β_1 integrin mAb substrate. Although we are unclear exactly how AA or a LOX metabolite regulates PKC activation, a likely suspect would be the activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-phosphate (PIP_2) to form DG and inositol triphosphate (IP_3) during integrin-mediated adhesion (Rana and Hokin, 1990). McNamee *et al.* describe a different system in which C3H 10t1/2 fibroblasts spread on fibronectin in the presence of platelet-derived growth factor that activates PLC to produce DG and IP_3 (McNamee *et al.*, 1993). As shown in previous studies, the levels of DG and PKC activation increased during HeLa cell attachment before cell spreading on a gelatin substrate (Chun and Jacobson, 1993). Other work shows that the production of DG by PIP_2 hydrolysis stimulated actin polymerization (Shariff and Luna, 1992), a vital component of cell adhesion. Therefore the activation of PKC by DG could be a signal that also activates cytoskeleton assembly and other intracellular interactions during cell adhesion. Recent data suggests that activation of PKC elicits an increase in filamentous actin in HeLa cells spread on gelatin (Chun, Auer, and Jacobson, unpublished results). Others have shown that PKC activation is required for cell motility, cell adhesion, and focal contact formation, processes that require reorganization of the cytoskeleton (Haverstick, 1992; Woods and Couchman, 1992; Herbert, 1993; Vuori and Ruoslahti, 1993). Because ligation of β_1 integrins results in the activation of PKC, PKC may be an important component in the crosstalk between the cell membrane and the cell's cytoskeletal assembly machinery.

The importance of integrins in cell signaling is becoming increasingly clear. Integrins are no longer being thought of as only structural receptors to assist the cell during adhesion; integrins are now believed to play a central role in signaling cytoskeleton rearrangement as well as other intracellular responses (Kornberg *et al.*, 1991; Hynes, 1992; Schwartz, 1992; Juliano and Haskill, 1993) during cell-cell and cell-substrate adhesion. In this paper we demonstrated that clustering of β_1 integrins can signal the hydrolysis of a phospholipid to release AA, which becomes metabolized by LOX to form a second messenger that leads to the activation of PKC, resulting in cell spreading (Figure 6). These results provide one pathway of crosstalk between the ECM to the cytoskeleton, however, questions concerning the identity of the phospholipids that release AA and the necessary LOX metabolites produced as well as the mechanism for the activation of PKC remain to be addressed and are a part of ongoing research.

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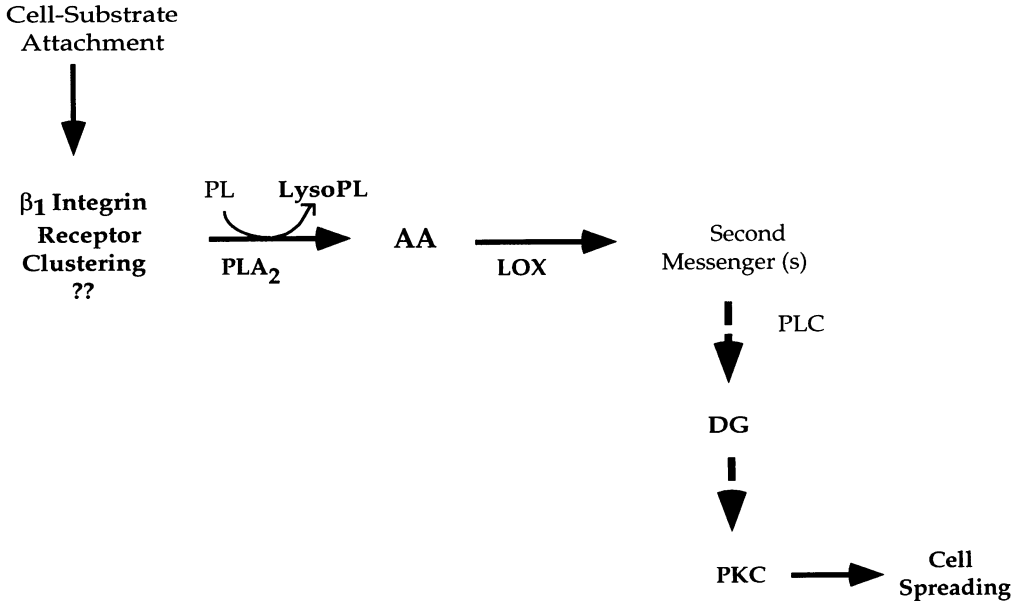

Figure 6. Proposed role of β_1 integrin receptors during HeLa cell adhesion on a gelatin substrate. HeLa cell attachment to a gelatin substrate induces phospholipase A_2 (PLA_2) activation to produce arachidonic acid (AA) and lysophospholipids (LysoPL) from phospholipids (PL). Lipoxygenase (LOX) oxidizes AA to a metabolite that activates protein kinase C (PKC). The solid lines represent confirmed second messengers whereas the hatched lines represent second messengers undetermined in the pathway. PLC, phospholipase C; DG, diacylglycerol.

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