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Hydrolysis of Phosphatidylcholine to Initiate HeLa Cell Adhesion to a Gelatin Substratum

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HeLa cells, a transformed human epithelial cell line, attach to various substrata but subsequent spreading is specific to collagen or gelatin. The spreading is initiated by the activation of phospholipase A_2 (PLA_2) which produces arachidonic acid (AA) as a consequence of cell surface collagen receptor clustering. This study examines the mechanism of PLA_2 activation and which phospholipids are hydrolyzed by PLA_2 to release AA in response to HeLa cell adhesion to a gelatin substratum. The levels of phosphatidylcholine decreases, among various phospholipids, during attachment and spreading of HeLa cells. Lysophosphatidylcholine is the only lysophospholipids formed during HeLa cell adhesion indicating that clustered collagen receptors activate PLA_2 to hydrolyze posphatidylcholine to AA and lysophosphatidylcholine. Among various molecular entities which are known to regulate PLA_2 activation, we have previously shown that PLA_2 activation is not mediated by either changes in Ca^{2+} levels, alkalinization of cytoplasmic pH, or activation of protein kinase C. It is also likely that PLA_2 activation is not mediated by either pertussis or cholera toxin-sensitive G proteins as those toxins do not affect both AA release and cell spreading.

KEY WORDS: Cell Adhesion, Arachidonic Acid, Phosphatidylcholine, Phospholipase A_2, G Proteins

Interaction of cells with extracellular matrix (ECM) is an important regulatory step in a number of biological processes such as growth, proliferation, and differentiation of cells. The adhesion of cells to a specific ECM is mediated by cell surface receptors. Integrins consisting of α and β subunits are a family of receptors that mediate cell-ECM adhesion (Albelda and Buck, 1990; Hynes, 1992). Integrins interact with cytoskeletal proteins on the cytoplasmic side providing a linkage between the ECM and the cytoskeleton (Burridge *et al.*, 1988). Increasing evidences indicate that integrins also give rise to biochemical signals within the cells such as activation of tyrosine kinase (Konberg *et al.*, 1992), protein kinase C (Chun and Jacobson, 1993; Vuori and Ruoslahti, 1993), and mitogen-activated protein kinases (Chen *et al.*, 1994; Zhu and Assoian, 1995). These signaling molecules are believed to mediate cellular responses of ECM signal such as gene expression (Juliano and Haskill, 1993).

HeLa cells, a transformed human epithelial cell line, attach to a variety of substrata but they

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spread only on collagen or gelatin substratum (Fairman and Jacobson, 1983). Spreading of HeLa cells on a collagen substratum is induced by the clustering of cell surface collagen receptors which initiates transmembrane signaling (Lu *et al.*, 1992). The clustered receptors activate phospholipase A_2 (PLA_2) which produces arachidonic acid (AA). Released AA is further metabolized via lipoxygenase (LOX) and a LOX metabolite(s) induces production of diacylglycerol (DG) which is correlated with the activation of protein kinase C (PKC) (Chun and Jacobson, 1992, 1993). Membrane-bound PKC activity is transiently increased upon attachment and prior to cell spreading. Inhibition of PKC blocks cell spreading while activation of PKC enhances cell spreading (Chun *et al.*, 1995).

The release of AA is essential signal to initiate spreading of HeLa cell on a collagen or gelatin substratum (Chun and Jacobson, 1992). AA is almost exclusively found in the *sn*-2 position of phospholipids and can be released by the activity of various lipases (Denis *et al.*, 1991; Liskovitch and Cantley, 1993). The primary mechanisms for the liberation of AA from phospholipid include the hydrolytic removal of AA by PLA_2 and sequential action of phospholipase C (PLC) to produce diacylglycerol (DG) and DG lipase to release AA. In HeLa cells, release of AA upon attachment to a gelatin substratum appears to be mediated by the action of PLA_2 since PLA_2 inhibition blocks both AA release and cell spreading (Chun and Jacobson, 1992).

This study examines which phospholipids are hydrolyzed by PLA_2 to release AA in response to HeLa cell adhesion to a gelatin substratum. The evidences obtained indicate that clustered collagen receptors upon interaction with immobilized gelatin activate PLA_2 which hydrolyzes phosphatidylcholine to AA and lyso-phosphatidylcholine. The activation of PLA_2 was not affected by pertussis or cholera toxin indicating that the toxin sensitive G proteins are not involved in PLA_2 activation.

Materials and Methods

Cell culture, substratum preparation, and spreading assay.

Type I gelatin from swine skin or bovine serum albumin (BSA) was covalently coupled to 35 mm polystyrene culture dishes as described previously (Chun and Jacobson, 1992). HeLa-S_3 cells were grown in suspension to mid-log phase ($2\text{-}5 \times 10^5$ cell/ml) in RPMI-1640 medium (K. C. Biologicals, Lenexa, KS, USA) supplemented with 5% calf serum (Gibco BRL, Grand Island, NY, USA). The cells used for spreading assay were harvested from suspension, washed twice, and resuspended in serum-free medium. The cells were plated on substratum coated culture dishes (5×10^5 cell/ml/dish) and incubated for 30 min at 37 °C Cell spreading was assayed as described previously (Chun and Jacobson, 1993; Chun *et al.*, 1995).

Assay of arachidonic acid and neutral lipids.

HeLa cells were labeled overnight in suspension culture with 0.2 μCi/ml of [^{3}H]AA (New England Nuclear, Boston, MA, USA), and used for spreading assay as described above. After incubation at 37 °C for the indicated period, cells were scraped off from the culture dishes and transferred to glass tubes. The dishes were washed with 1 ml of methanol containing 0.01% HCl and combined with the cells. Lipids were extracted by the addition of 1 ml of chloroform and vortexing. After separation of phase by brief centrifugation, the lipid containing organic phase was collected, washed with an equal volume of methanol:water (1:1, v/v), and concentrated under a stream of N_2 gas. The lipids were separated on thin layer silica gel G plates using heptane:diethyl ether:glacial acetic acid (60:40:2, v/v) as the chromatography solvent (Findlay, 1987). The separated lipids were visualized by spraying the plates with EN^{3}HANCE (New England Nuclear, Boston, MA, USA) and exposing the plates to Kodak XAR X-ray film at −80°C. Individual lipids were identified by comigration with standards. Areas of the silica gel corresponding to individual lipids were scraped into scintillation vials and counted to quantitate the relative amount.

Assay of phospholipids.

HeLa cells labeled with [^{3}H]AA were extracted with chloroform:methanol:HCl as described above. Phospholipids were separated on thin layer silica gel plates using chloroform:methanol:acetic acid:water (60:50:1:4, v/v) as the chromatography solvent (Findlay, 1987). This system resolves major classes of phospholipids including phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SPH). Lysophospholipids remain close to the origin and are not completely separated from each other. Individual phospholipids were visualized by autoradiography. The relative abundance of [^{3}H] AA containing phospholipids was determined by scraping the areas of silica gel corresponding to individual phospholipids and counting the radioactivity.

In order to separate individual lysophospholipid, cells were labeled with 10 μCi/ml of [^{14}C]glycerol (New England Nuclear, Boston, MA, USA) for overnight in suspension culture. The labeled cells were collected, washed twice, and plated on gelatin for the indicated period. Lipids were extracted as described above and separated on thin layer silica gel plates by using a two-dimensional solvent system; chloroform:methanol: acetic acid:water (60:35:1:5, v/v) in the first dimension and chloroform:methanol:40% aqueous methylamine:water (13:7:1:1, v/v) in the second dimension (Findlay, 1987). Lipids standards were applied in both dimensions. The plates were exposed to Kodak XAR X-ray film at −80°C and individual lysophospholipids were identified by comigration with standards.

Assay of lipid phosphate.

Total lipids in HeLa cells (1.5×10^7 cells) were extracted and phospholipids were separated on silica gel plates as described above. After identification of phospholipids by iodine vapor, spots for individual phospholipids were scraped into glass tubes. The lipids were eluted with chloroform:methanol (1:1, v/v), and assayed for inorganic phosphate as described by Ames (1985) to determine the relative amounts of individual phospholipids.

Results and Discussion

Requirement of AA release to initiate HeLa cell spreading on a gelatin substratum.

Spreading of HeLa cells upon attachment to a collagen or gelatin substratum requires a cascade of events that are initiated by AA release. Consistent with the previous result (Chun and Jacobson, 1992), levels of cellular AA was increased upon attachment and prior to the spreading of cells (Fig. 1A). Inhibition of PLA_2 with bromophenacyl bromide (BPB) or 7,7-dimethyleicosaenoic acid (DMEA) blocked cell spreading (Fig. 1B) indicating that hydrolysis of phospholipids by PLA_2 is an obligatory signal to induce cell spreading.

Distribution of [^{3}H]AA in HeLa cells.

Intracellular distribution of [^{3}H]AA in various lipids was analyzed prior to determine which phospholipids are hydrolyzed to release AA. When HeLa cells were labeled with [^{3}H]AA overnight in suspension, more than 90% of [^{3}H]AA was incorporated into cells. Distribution of the incorporated [^{3}H]AA in individual lipids was determined by the separation of extracted lipids on thin layer silica gel plates as shown in Fig. 2. The relative abundance of [^{3}H]AA in individual lipids were determined by scraping the areas of silica gel corresponding to each lipids and by counting. As shown in Table I, only a trace amount (0.2%) of the incorporated [^{3}H]AA was found as intracellular free [^{3}H]AA while 4.7% of the [^{3}H]AA is esterified into neutral lipids such as mono-, di-, and triacylglycerol. The majority of [^{3}H]AA (95%) was eaterified into phospholipids. Among the phospholipids, [^{3}H]AA was found in the following order: PC (33.1%), PE (31.1%), PI (19.1%), and PS (9.7%). Table I also lists the relative prevalence of individual phospholipids in HeLa cells as measured by lipid phosphate content. Over half of the measured lipid phosphate was from PC (53.6%), and most of the remaining lipid phosphate was found in PE (34%). The ratio of AA uptake to lipid phosphate was determined to measure the amount of AA taken up relative to the phospholipid pool size. Based on this ratio, AA was more efficiently esterified into

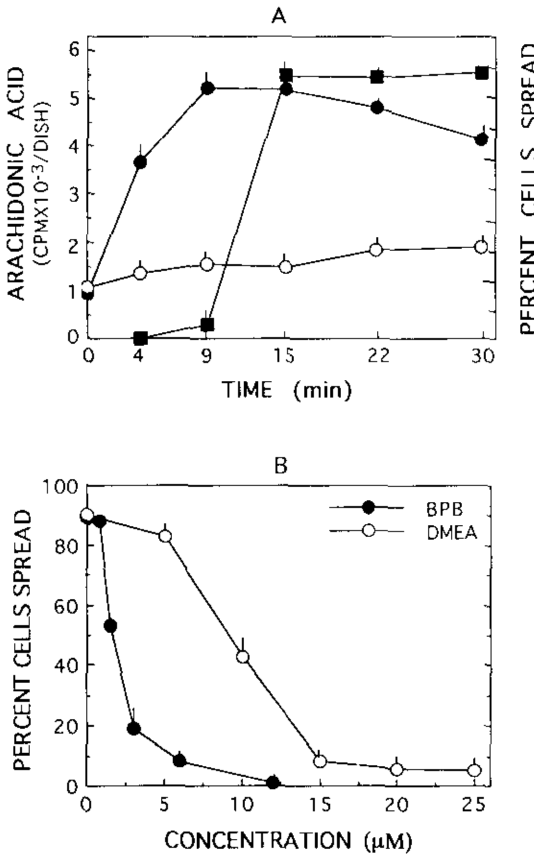

Fig. 1. Release of AA is required for HeLa cell spreading on a gelatin substratum. (A) [^{3}H]AA labeled cells were plated on a gelatin substratum where cells attach and spread (closed circle) or on a BSA where cells attach but do not spread (open circle). At the indicated times, the amount of cellular [^{3}H]AA was determined. Percent cells spread (closed square) was determined from the cells plated on gelatin. The data represent the average value of triplicate samples with the standard deviation from a typical experiment conducted more than 10 times. (B) HeLa cells were treated with BPB (closed circle) or DMEA (open circle) at the indicated concentration in suspension for five minutes. The cells were plated on gelatin in the presence of the treated drugs for 30 min and the percent cells spread was scored. The data represent the average value of five experiments with the standard deviation.

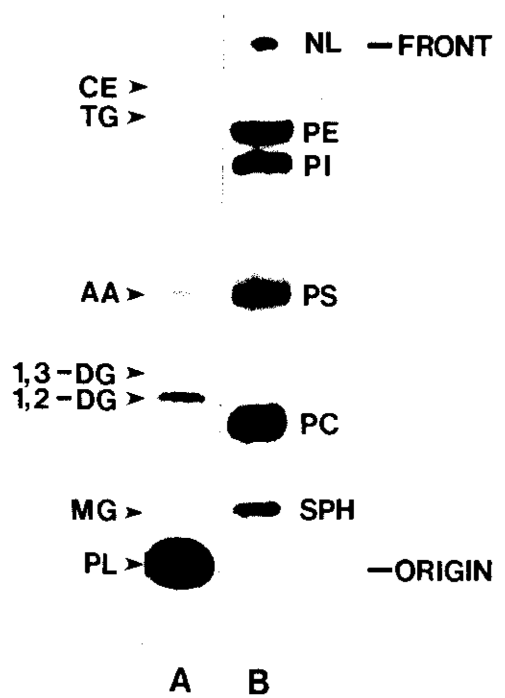

Fig. 2. Separation of lipids by thin layer chromatography. Total lipids were extracted with chloroform:methanol:HCl (1:1:0.01, v/v) from HeLa cells labeled with [^{3}H]AA and separated on silica gel G plates. The plates were sprayed with EN^{3}HANCE and exposed to Kodak XAR X-ray film at -80°C. (A) Neutral lipids were separated using heptane:diethyl ether:acetate (60;40;2, v/v). (B) Phospholipids were separated using chloroform:methanol:acetate:water (60:50:1:4, v/v). Individual lipids were identified by comigration with standards. CE: cholesterol ester, TG: triacylglycerol, DG: diacylglycerol, MG: monoacylglycerol, PL: phospholipid, PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, PC: phosphtidylcholine, SPH: sphingomyelin, NL: neutral lipid.

PS and PI although the pool size of PS and PI is relatively small (Table I).

Phosphatidylcholine is hydrolyzed to release AA.

To determine which phospholipids are hydrolyzed to release AA in response to HeLa cell adhesion to gelatin, individual phospholipids containing [^{3}H]AA were separated on thin layer silica gel G plates, and the level of each phospholipids was determined during cell adhesion. There was no significant changes in the levels of PS and PE. However, the level of PC decreased in spreading cells suggesting that PC is hydrolyzed during cell adhesion (Fig. 3). The possibility of PC hydrolysis by PLA_2 to release AA

Table 1. Distribution of [^{3}H]AA and comparison to phospholipid pool size.

Lipids	Percent uptake (a)	Percent of lipid phosphate (b)	a/b
Cholesterol ester	0.2±0.1		
Triacylglycerol	2.0±0.1		
Diacylglycerol	2.2±0.2		
Monoacylglycerol	0.3±0.3		
Arachidonic acid	0.2±0.2		
Phospholipids	95.1±0.6		
Phophatidylcholine	33.1±5.3	53.6±3.2	0.62
Phosphatidylethanolamine	31.1±5.1	34.0±2.1	0.92
Phosphatidylinositol	19.3±4.5	9.7±1.2	1.99
Phosphatidylserine	9.7±4.1	2.7±0.5	3.60
Other	2.6±1.5	–	–

Total lipids were extracted from HeLa cells labeled with [^{3}H]AA by using chloroform:methanol:HCl (1:1:0.01, v/v). Individual lipids were separated on thin layer silica gel G plates as described in Materials and Methods. Relative amount of [^{3}H]AA in individual lipids were determined by scraping the areas of silica gel corresponding to each lipid and by counting. Amounts of individual phospholipids were determined by assaying inorganic phosphate. The data represent the average value of four experiments with standard deviation.

was directly examined by assaying the formation of individual lysophospholipids by using two dimensional thin layer chromatography. Lysophosphatidylcholine was the only detectable lysophospholipids produced in spreading HeLa cells (Fig. 4) indicating that PC is the substrate for PLA_2.

Interestingly, the levels of phospholipids containing an inositol moiety increased during attachment and spreading of HeLa cells (Fig. 3). Hydrolysis of phosphatidylinositol-bisphosphate (PIP_2) produces intracellular second messengers, inositol triphosphate (IP_3) and diacylglycerol (Berridge, 1987). Increased turnover of phophatidylinositol was observed in spreading BHK-21 cells (Breuer and Wagener, 1989). In HeLa cells, however, there was no detectable changes in IP_3 levels during cell adhesion (data not shown) suggesting that hydrolysis of PIP_2 by phospholipase C is not involved in spreading of HeLa cells. In addition to the generation of signaling molecules, the formation of PIP_2 is known to serve as a signal for certain cellular responses such as F-actin polymerization (Apgar, 1995; Forscher, 1989). It is, therefore, possible that increased levels of inositol containing phospholipids cause a change in the organization

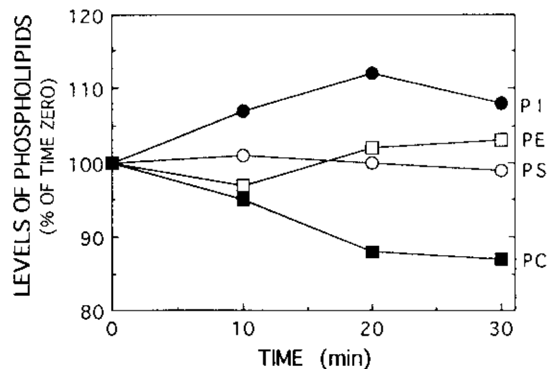

Fig. 3. Changes in the levels of [^{3}H]AA containing phospholipids during HeLa cell adhesion. HeLa cells labeled with [^{3}H]AA were plated on gelatin coated culture dishes. At the indicated times, total lipids were extracted and phospholipids were separated on thin layer silica plates. Relative amount of individual phospholipids was determined as described in Materials and Methods. PE: phophatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, PC: phosphatidylcholine.

of actin filaments during cell spreading. F-actin polymerization is essential for HeLa cells to spread on a gelatin substratum (Lu *et al.*, 1992).

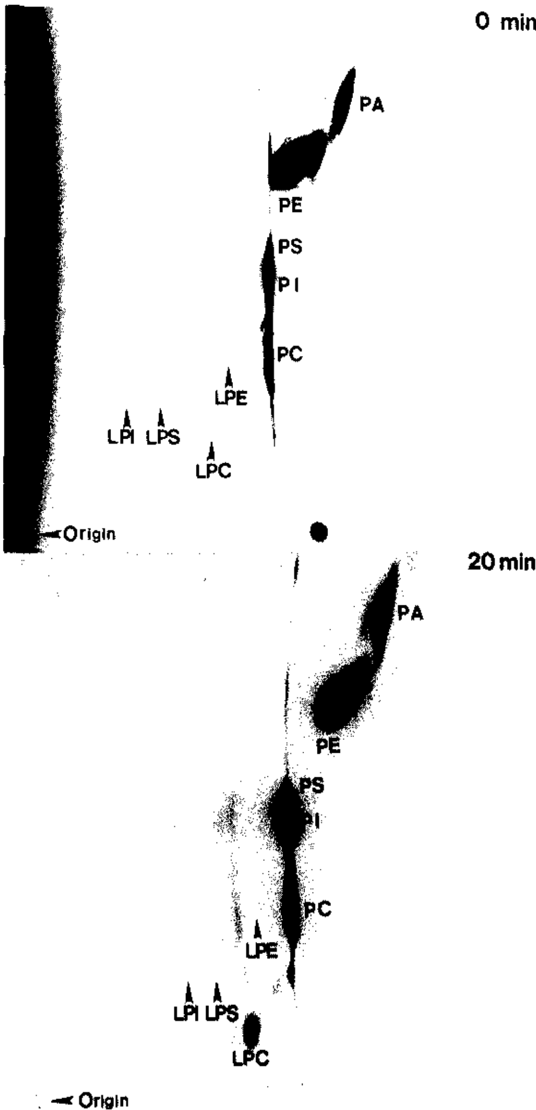

Fig. 4. Formation of lysophosphatidylcholine during HeLa cell attachment and spreading. HeLa cells labeled with [^{14}C]glycerol were either kept in suspension (A) or plated on gelatin for 20 min where cells attach and spread (B). The lipids were extracted and separated by two dimensional thin layer chromatography. The plates were exposed to X-ray film and individual phospholipids were identified by comigration with standards. PE; phosphatidylethanolamine. PI: phosphatidylinositol, PS: phosphatidylserine. PC: phosphatidylcholine, LPE: lysophosphatidylethanolamine, LPI: lysophosphatidylinositol, LPS: lysophosphatidylserine. LPC: lysophosphatidylcholine, SPH: sphingomyelin, PA: phosphatidic acid.

Mechanism of PLA_2 activation.

Regulation of PLA_2 activity is mediated by a variety of molecular entities which are involved in transmembrane signaling. These include G proteins, mobilization of cytoplasmic free Ca^{2+}, Na^+/H^+ antiporter-mediated alkalinization of cytoplasmic pH, and protein kinase C (Birnbaumer *et al.*, 1990; Chang *et al.*, 1987). To examine whether PLA_2 is linked to G proteins, HeLa cells were treated with pertussis toxin, which inhibits Gi and Go, or cholera toxin, which stimulates Gs (Reisine, 1990), prior to spreading assay. As shown in Table 2, these toxins did not affect both AA release and cell spreading. Thus, it is likely that the toxin-sensitive G proteins are not involved in coupling of receptor stimulation to PLA_2 activation during HeLa cell adhesion to a gelatin substratum. In addition, there is no detectable changes in Ca^{2+} levels during cell spreading (Chun and Jacobson, 1992) suggesting that an increase in Ca^{2+} levels is not a cause of PLA_2 activation. We have also shown that alkalinization of cytoplasmic pH via Na^+/H^+ antiport is not involved in AA release (Chun, 1995). Also, activation of PKC occurs as a result of AA release (Chun and Jacobon, 1993; Chun *et al.*, 1995).

Redistribution of cell surface receptors such as clustering, patching, and capping is closely associated with several enzymatic activities and activation of cytoskeleton (Bourguignon and Bourguignon, 1984). Schwartz *et al.* (1991) reported that clustering of integrins activates Na^+/H^+ antiport which induces alkalinization of cytoplasmic pH in fibroblasts. Clustering of $\beta 1$ integrin also enhances activity of tyrosine kinase such as focal adhesion kinase (Kornberg *et al.*, 1991). It is, therefore, possible in HeLa cells that clustered collagen receptors either directly interact with PLA_2 to cause AA release or alters the microenvironment of membrane phopholipid so that PLA_2 can more easily access to the phospholipids.

Acknowledgements

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Table 2. Pertussis or cholera toxin does not affect AA release and cell spreading.

Substratum/ Treatment	AA release (Percent of time zero)	Percent cells spread
BSA	250±74	None
Gelatin	1013±231	90±2
Gelatin + Pertussis toxin	933±257	86±4
Gelatin + Cholera toxin	1051±193	90±2

HeLa cells were treated with 0.5 μg/ml of pertussis or 5 μg/ml of cholera toxin for 6 hours during the [^{3}H]AA labeling period. Cells either treated or untreated with the toxins were plated on the indicated substratum. The amount of AA released was assayed as described in Materials and Methods. The percent increase of AA release was calculated from a peak value of the released AA against time zero in each condition. The data represent the average value of four experiments with standard deviation.

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Phosphatidylcholine의 분해에 의한 HeLa 세포와 Gelatin 기질과의 상호작용의 유도

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인간의 상피세포로부터 유래된 암세포의 일종인 HeLa 세포는 거의 모든 기질분자에 부착하지만 spreading은 오직 collagen 혹은 gelatin에서만 일어난다. HeLa 세포의 spreading은 세포막 collagen 수용체의 clustering 결과 활성화된 phospholipase A_2 (PLA_2)에 의한 arachidonic acid(AA)의 형성으로 유도된다. PLA_2에 의해 분해되는 인지질을 확인하기 위해 각종 인지질의 농도변화를 spreading 과정에서 측정한 결과 단지 phosphatidylcoline 만이 감소하였으며, 또한 다양한 lysophospholipids 중 lysophosphatidylcholine 만이 spreading 과정에 생성되는 것으로 보아 phosphatidylcoline이 PLA_2에 의해 분해되어 AA가 형성되는 것으로 보인다. PLA_2의 활성화는 세포질 Ca^{2+}의 농도변화 및 세포질 pH의 알칼리화에 기인하지 않으며, 또한 pertussis 혹은 cholera toxin-sensitive G protein 역시 PLA_2 활성화와는 무관한 것으로 나타났다.