Activation of Actin Polymerization by Phosphatidic Acid Derived from Phosphatidylcholine in IIC9 Fibroblasts

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Abstract. α-Thrombin induced a change in the cell morphology of IIC9 fibroblasts from a semiround to an elongated form, accompanied by an increase in stress fibers. Incubation of the cells with phospholipase D (PLD) from *Streptomyces chromofuscus* and exogenous phosphatidic acid (PA) caused similar morphological changes, whereas platelet-derived growth factor (PDGF) and phorbol 12-myristate 13-acetate (PMA) induced different changes, e.g., disruption of stress fibers and cell rounding. α-Thrombin, PDGF, and exogenous PLD increased PA by 20–40%, and PMA produced a smaller increase. α-Thrombin and exogenous PLD produced rapid increases in the amount of filamentous actin (F-actin) that were sustained for at least 60 min. However, PDGF produced a transient increase of F-actin at 1 min and PMA caused no significant change. Dioctanoylglycerol was ineffective except at 50 μg/ml. Phospholipase C from *Bacillus cereus*, which increased diacylglycerol (DAG) but not PA, did not change F-actin content. Down-regulation of protein kinase C (PKC) did not block actin polymerization induced by α-thrombin. H-7 was also ineffective. Exogenous PA activated actin polymerization with a significant effect at 0.01 μg/ml and a maximal increase at 1 μg/ml. No other phospholipids tested, including polyphosphoinositides, significantly activated actin polymerization. PDGF partially inhibited PA-induced actin polymerization after an initial increase at 1 min. PMA completely or largely blocked actin polymerization induced by PA or PLD. These results show that PC-derived PA, but not DAG or PKC, activates actin polymerization in IIC9 fibroblasts, and indicate that PDGF and PMA have inhibitory effects on PA-induced actin polymerization.

Actin is a globular protein which is polymerized into filamentous actin (F-actin).[1] The mechanism of actin polymerization is a topic of interest since actin polymerization is known to be important in cell motility, changes in cell morphology, cell division, and intracellular movements (6, 13, 31). There have been several reports on the regulation of actin polymerization, but the mechanism is not clear. Phorbol 12-myristate 13-acetate (PMA) activates actin polymerization in rat basophilic leukemia cells (1) and neutrophils (9), but does not cause activation in human fibroblasts (21). In plasma membranes purified from *Dictyostelium discoideum*, dioctanoylglycerol (DOG) stimulates actin nucleation, but PMA does not (29). Tyrosine phosphorylation has been also proposed to activate actin assembly in cells such as B lymphocytes (20), platelets (25), and *Dictyostelium* (14).

Recently, polyphosphoinositides have been reported to be important in the regulation of actin polymerization (4, 6, 11, 16, 31, 37). Polyphosphoinositides have been proposed to bind to actin-binding proteins such as gelsolin (7, 16, 37), profilin (4), and villin (11), resulting in an increase in actin monomers or nucleation sites and a stimulation of actin polymerization. However, there has been one study reporting that the changes in the levels of polyphosphoinositides induced in A431 cells by several agents did not correlate with those of actin-gelsolin complexes (8).

The hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) produces phosphatidic acid (PA) in various cell types stimulated with hormones or growth factors (10). PA derived from PC may then be converted into diacylglycerol (DAG) by PA phosphohydrolase (17), leading to activation of certain isozymes of protein kinase C (PKC) (12). PA has also been reported to cause Ca^{2+} influx (26, 28), induction of mRNA for c-*fos* and c-*myc*, stimulation of DNA synthesis (18, 22), and increased protein phosphorylation (3). However, the physiological function of PA is still obscure.

In this report, we present novel results that PA derived from PC but not DAG or PKC, activates actin polymerization

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1. *Abbreviations used in this paper*: DAG, diacylglycerol; DOG, dioctanoylglycerol; F-actin, filamentious-actine; H-7, (+)-1-(5-Isoquinolinesulfonyl)-2-methylpiperazine; LPA, lysophosphatidic acid; NBD-phallacidin, N-(7)-nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin; PA, phosphatidic acid; PC, phosphatidylcholine; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

in IIC9 fibroblasts. These results provide evidence for a possible role of PA in the regulation of actin polymerization.

Materials and Methods

Materials

Culture medium components and polyclonal antibodies raised against synthetic peptides corresponding to specific sequences in PKC isozymes were from GIBCO-BRL (Gaithersburg, MD). α-Thrombin (~3,000 U/mg protein), cytochalasin D, Triton X-100, PMA, *Streptomyces chromofuscus* PLD (500–3,000 U/mg solid), and phosphatidylinositol 4-monophosphate (PIP) were from Sigma Immunochemicals (St. Louis, MO). PDGF B/B (human recombinant), phosphatidylinositol 4,5-bisphosphate (PIP_2), and *Bacillus cereus* PC-specific phospholipase C (PLC) were from Boehringer Mannheim Corp. (Indianapolis, IN). Phospholipids were from Avanti Polar Lipids Co. (Birmingham, AL). Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) was kindly given by Dr. John R. Falck (University of Texas Southwestern Medical Center, Dallas, TX). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD-phallacidin) and (+)-1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) were purchased from Molecular Probes Inc. (Eugene, OR) and LC Laboratories (Woburn, MA), respectively. [9,10-^{3}H]-Myristic acid (33.5 Ci/mmol) was purchased from DuPont-New England Nuclear (Braunschweig, Germany). Immobilon-P was from Millipore Corp. Waters Chromatography (Milford, MA) and Vectastain alkaline phosphate ABC kit was from Vector Labs. Inc. (Burlingame, CA).

Cell Culture

IIC9 cells, a subclone of Chinese hamster embryo fibroblasts (kindly given by Dr. Daniel M. Raben, Johns Hopkins University School of Medicine, Baltimore, MD), were grown and maintained according to the methods of Ha and Exton (12). Briefly, cells were grown on 100-mm culture dishes or 6-well plates for 2 d in serum-containing medium and subconfluent cultures were incubated for 2 d in serum-free medium.

PKC Down-regulation

Cells were grown on 100-mm culture dishes, serum starved for 1 d, and then treated with 0.8 μM PMA in serum-free medium for 1 d. Cells were scraped, fractionated into the cytosolic and particulate fractions, and then the extracted proteins were separated by 10% SDS-PAGE according to the procedures of Ha and Exton (12). PKC isozymes α, ε, and ζ were identified by Western blotting with polyclonal antibodies raised against isozyme-specific peptides, and the relative density of each PKC isozyme band was measured by laser densitometry (2202 Ultrascan; LKB Instruments Inc., Broma, Sweden).

Filamentous Actin (F-actin) Measurement

Serum-starved cells on 6-well plates were washed twice with serum-free medium, stabilized in the same medium for 1 h, and then treated with 500 ng/ml α-thrombin, 50 ng/ml PDGF, 5 U/ml *S. chromofuscus* PLD, micelles of different phospholipids, or various amounts of *B. cereus* PLC. To study inhibitory effects of PDGF and PMA on actin polymerization, the stabilized cells were preincubated with 50 ng/ml PDGF or 160 nM PMA for 15 min, or 1 μM cytochalasin D for 30 min, and then incubated with 1 μg/ml PA or 5 U/ml *S. chromofuscus* PLD for 15 min. Phospholipid micelles were made by sonicating the dried phospholipids in serum-free medium. F-actin content was measured by a modification of the procedures developed by Howard and Oresajo (15). The treated cells were quickly rinsed with ice-cold PBS and fixed with 3.7% formaldehyde in PBS for 30 min on ice. Following permeabilization with 0.2% Triton X-100 in PBS for 30 min, the cells were stained with 600 μl/well of 0.165 μM NBD-phallacidin in PBS for 1 h, washed three times with PBS, and then the bound NBD-phallacidin was extracted with 1.5 ml/well of methanol for 1 h. The fluorescence intensity was measured using a SPEX Fluorolog (1681 0.22 m spectrometer; SPEX Inds. Inc., Edison, NJ) with an excitation wavelength of 465 nm and an emission wavelength of 535 nm. The results were expressed as the relative fluorescence index calculated from the ratio of the fluorescence intensity of stimulated cells to that of unstimulated cells.

Measurement of Phosphatidic Acid (PA) Content

The amount of PA was measured according to the procedures of Ha and Exton (12). Briefly, cells were labeled with 10 μCi/100-mm dish of [^{3}H]-myristic acid for 2 d in serum-free medium and stimulated with various agonists. After quickly washing with ice-cold PBS, cells were scraped into ice-cold methanol and lipids were extracted by the method of Bligh and Dyer (2). PA was separated by a thin layer chromatography system using the solvent system of ethyl acetate/iso-octane/acetic acid/H_2O (130:20:30:100, vol/vol). The PA band was identified, scraped, and counted using a scintillation counter.

Fluorescence Microscopy

Cells were grown on glass coverslips for 2 d, serum starved for 2 d, and then treated with agonists for 15 min in a 6-well plate. The cells were fixed with 3.5% paraformaldehyde on ice, permeabilized, and stained with NBD-phallacidin as explained above for F-actin measurement. Following washing twice with PBS, the coverslip was mounted on a slide and then observed with a fluorescence microscope (Leitz, E. Inc., Rockleigh, NJ).

Results

Morphological Changes

Incubation of IIC9 fibroblasts with α-thrombin caused a change in the appearance of the cells from a semiround to an elongated form. The shape change was accompanied by an increase in the number of intracellular stress fibers and an elongation of these fibers (Fig. 1 *b*). These changes were evident at 1–2 min, maximal at 15–30 min, and apparent for at least 2–3 h (data not shown). Treatment of the cells with PLD from *S. chromofuscus* or exogenous PA produced similar morphological changes (Fig. 1, *c* and *d*). However, PDGF and PMA induced different morphological changes, e.g., cell rounding and a decrease in stress fibers (Fig. 1, *e* and *f*).

Changes in F-actin

In order to understand the biochemical basis for the morphological changes observed with α-thrombin, changes in the cell content of F-actin were quantified fluorimetrically using NBD-phallacidin. Fig. 2 *a* shows that α-thrombin rapidly increased F-actin, with a significant effect at 1 min and a maximal increase at 15 min. The level then decreased slightly over 60 min. *S. chromofuscus* PLD produced a similar rapid effect which was sustained for 60 min (Fig. 2 *b*). In contrast, PDGF produced an increase which peaked at 1 min and then declined to control at 15 min (Fig. 2 *c*).

Changes in Phosphatidic Acid

Since the preceding results suggested a role for PA, the product of PLD action, in the F-actin changes induced by α-thrombin, the effects of agents on the level of this lipid were quantified. It had previously been shown that α-thrombin and PDGF elevated DAG in IIC9 cells (12), but the levels of PA were not measured. Fig. 3 shows that both agonists increased [^{3}H]PA in cells prelabeled with [^{3}H]myristic acid, with α-thrombin being more effective than PDGF, as noted previously for DAG (12). As expected, exogenous PLD elevated PA, whereas PMA produced a smaller change. The elevation of PA with PLD is rapid, paralleling that in F-actin (data not shown).

Lack of Effect of Diacylglycerol

Because of the evidence that PMA and DOG promote actin polymerization in some cells (1, 9), the possibility that the effects of α-thrombin and PLD on F-actin were secondary to

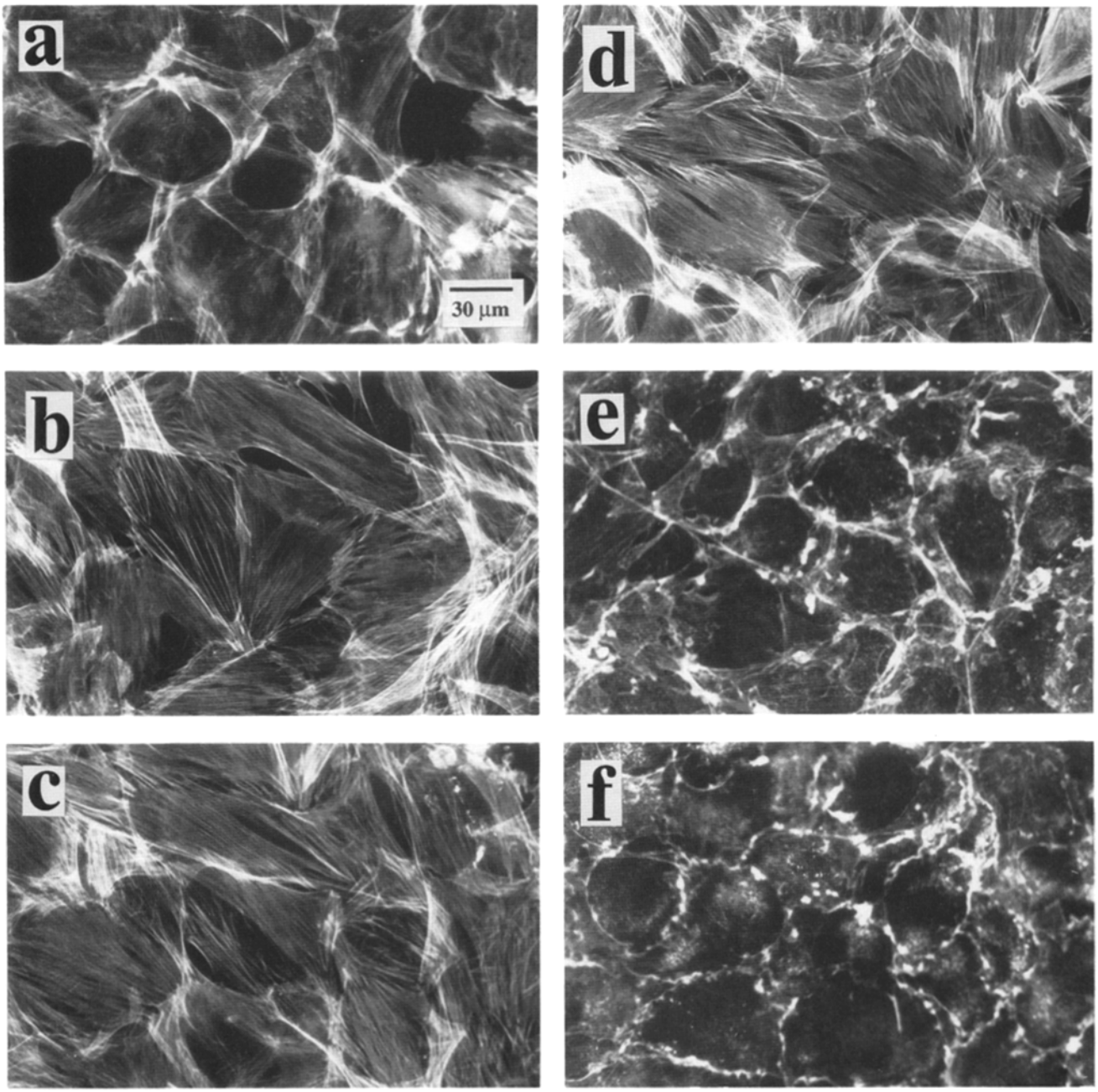

Figure 1. Changes in F-actin organization of IIC9 fibroblasts. Cells were grown on coverslips, serum starved, and then treated with no agonist (*a*), 500 ng/ml α-thrombin (*b*), 5 U/ml *S. chromofuscus* PLD (*c*), 1 μg/ml PA (*d*), 50 ng/ml PDGF (*e*), or 160 nM PMA (*f*) for 15 min. The cells were fixed, permeabilized, stained with NBD–phallacidin, and photographed as described in Materials and Methods. The changes are representative of those seen in three separate studies.

the production of DAG from PA was explored. First, F-actin content was measured after incubating cells with PMA or DOG to test for possible activation of actin polymerization. However, PMA had no significant effect (data not shown), and DOG had no effect except at 50 μg/ml (Fig. 4 *a*). In addition, neither PMA nor DOG induced cell elongation or stress fiber formation in morphological studies (Fig. 1 *f* for PMA and data not shown for DOG). These results suggest that DAG and PKC do not activate actin polymerization or that actin polymerization requires other factors in addition to PKC in IIC9 fibroblasts.

To test whether endogenously generated DAG is also without effect on actin polymerization, cells were incubated for 15 min with *B. cereus* PC-specific PLC. This enzyme increases DAG (12) with no significant change in PA (Fig. 3) and activates PKC ε translocation (12). It did not significantly change the F-actin amount at any concentration from 0.5 to 10 U/ml (Fig. 4 *b*).

Lack of Effects of Protein Kinase C Inhibitor and of Down-regulation

Lack of activation of actin polymerization by PKC was further supported by studies employing PKC down-regulation

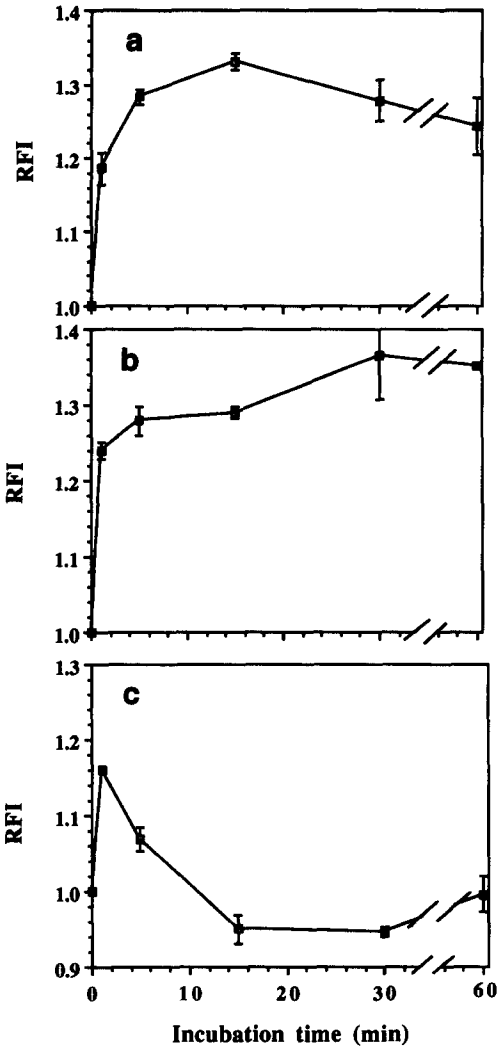

Figure 2. Time course of changes in F-actin content. IIC9 cells, grown and serum starved on 6-well plates, were treated with 500 ng/ml α-thrombin (*a*), 5 U/ml *S. chromofuscus* PLD (*b*), or 50 ng/ml PDGF (*c*) for the times indicated. F-actin content was measured as described in Materials and Methods. Data are means ± SE from three independent experiments.

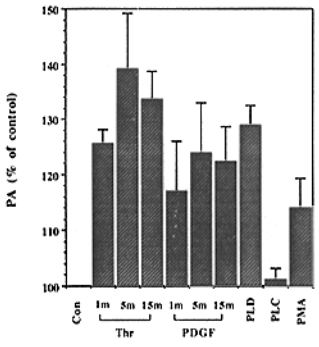

Figure 3. Changes of PA content in response to various agonists. IIC9 cells were grown on 100-mm culture dishes and labeled with 10 μCi/dish [^{3}H]myristic acid for 2 d in serum-free medium. The cells were then treated with 500 ng/ml α-thrombin (*Thr*) or 50 ng/ml PDGF for 1, 5, or 15 min. Cells were also treated with 5 U/ml *S. chromofuscus PLD* (PLD), 4 U/ml *B. cereus* PC-PLC (PLC), or 160 nM PMA for 15 min. [^{3}H]PA was determined in Materials and Methods. Values are expressed as a percentage of values obtained from control cells. Data are means ± SE from three independent experiments.

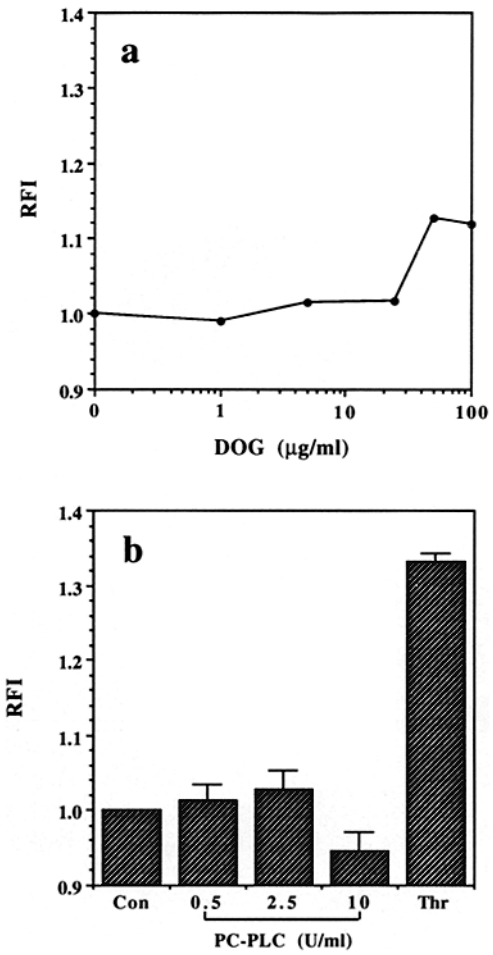

Figure 4. Lack of effect of DOG (*a*) and PC-PLC (*b*) on F-actin content. IIC9 cells, grown and serum starved on 6-well plates, were treated with various concentrations of DOG (*a*) or PC-PLC (*b*), or 500 ng/ml α-thrombin (*Thr*) for 15 min. F-actin content was measured as described in Materials and Methods. Data are means of two independent experiments for (*a*) and means ± SE of three independent experiments for (*b*).

and a protein kinase inhibitor, H-7 (Fig. 5, *a* and *b*). PKC was down-regulated by incubating cells with 0.8 μM PMA for 24 h. This treatment caused loss of almost all PKC α and about 90% of PKC ε (unpublished data). The prolonged PMA treatment increased the basal level of F-actin (Fig. 5 *a*) and caused a small PA increase (data not shown). More importantly, it did not inhibit actin polymerization activated by α-thrombin (Fig. 5 *a*). A protein kinase inhibitor, H-7 also did not affect α-thrombin–induced actin polymerization, except for a small inhibition at 25 μM (Fig. 5 *b*).

Effects of Phosphatidic Acid and Other Phospholipids

Because the experiments shown in Figs. 4 and 5 indicated that DAG and PKC play little, if any, role in actin polymerization induced by α-thrombin in IIC9 cells, the possibility that PA, the first product of PC–PLD action, has an important function was explored. To test for PA activation of actin polymerization, cells were incubated with various concentrations of PA micelles. Fig. 6 shows that actin polymerization was activated by PA, with a significant effect at 0.01

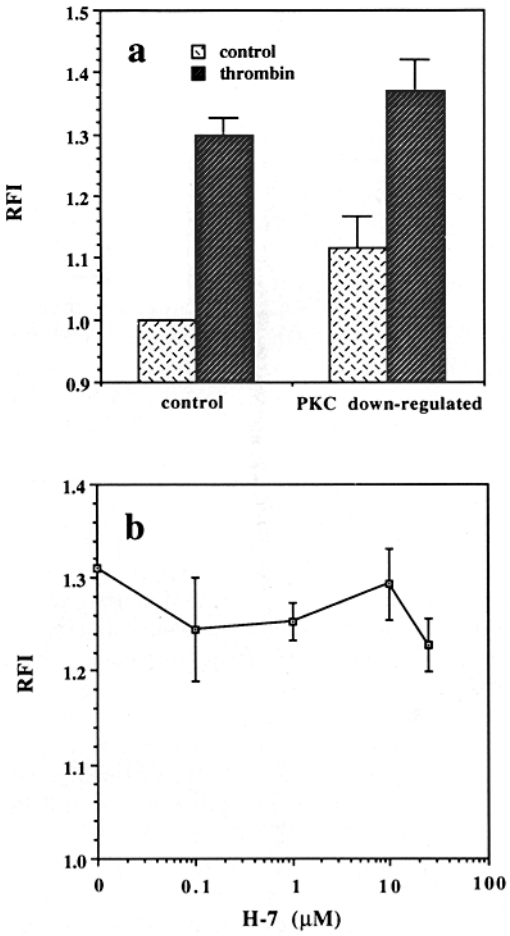

Figure 5. Lack of effect of PKC down-regulation (*a*) and H-7 (*b*) on the α-thrombin induced increase in F-actin content. Cells were grown on 6-well plates and serum starved for 1 d. The cells were treated with 0.8 μM PMA (PKC down-regulated) for 1 d (*a*) or with various concentrations of H-7 for 30 min (*b*), and then incubated with 500 ng/ml α-thrombin for 15 min. Data are means ± SE from three independent experiments.

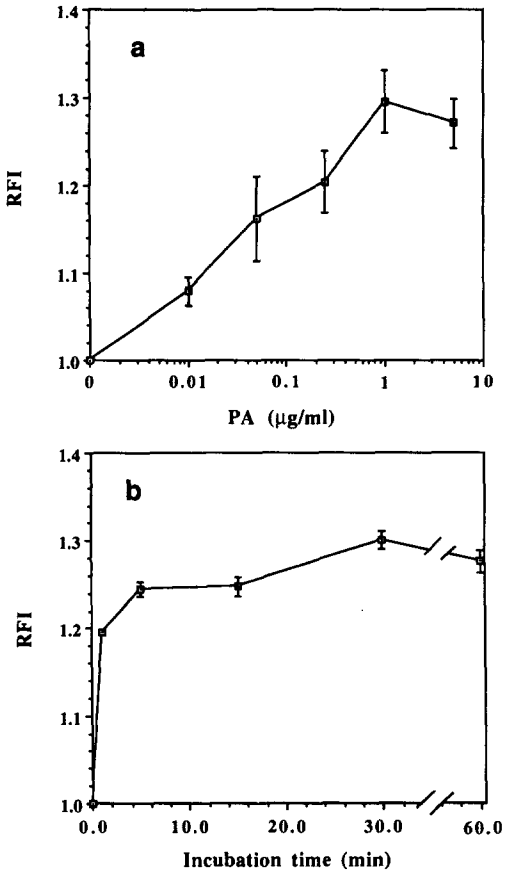

Figure 6. Effect of PA on F-actin content. Cells, grown and serum starved, were incubated with various concentrations of PA for 15 min (*a*), or incubated with 1 μg/ml of PA for the times indicated (*b*). Data are means ± SE from three independent experiments.

μg/ml and a maximal increase at 1 μg/ml (Fig. 6 *a*). Fig. 6 *b* shows the time course with 1 μg/ml PA. There was a very rapid increase within the first 5 min, and the maximal increase was similar to that induced by α-thrombin or *B. cereus* PLD (compare Fig. 6 *b* with Fig. 2). To test for specificity, IIC9 cells were also incubated with 1 μg/ml of different phospholipid micelles (Fig. 7). None of the other tested phospholipids, including polyphosphoinositides, significantly activated actin polymerization.

Inhibitory Effects of PMA and PDGF

Fig. 3 shows that PMA caused a rise in PA. However, it did not induce stress fiber formation (Fig. 1 *f*) or activate actin polymerization (data not shown). To test for a possible inhibitory effect of PMA on actin polymerization, cells were incubated with PA or PLD after preincubation with PMA. Fig. 8 shows that PMA completely blocked actin polymerization induced by PA and largely inhibited that induced by PLD. Cytochalasin D, a known inhibitor of F-actin formation (5) had similar effects. These results indicate that PMA has an inhibitory effect on actin polymerization induced by PA.

A possible delayed inhibitory effect of PDGF on actin polymerization was also studied by incubating cells with PDGF for 15 min and then with various concentrations of PA. PDGF completely blocked actin polymerization induced by 0.01 μg/ml PA, and partly inhibited that produced by PA concentrations from 0.05 to 1 μg/ml (data not shown).

Discussion

The present results show that in IIC9 fibroblasts, α-thrombin elevates PA (Fig. 3), induces a morphological change from

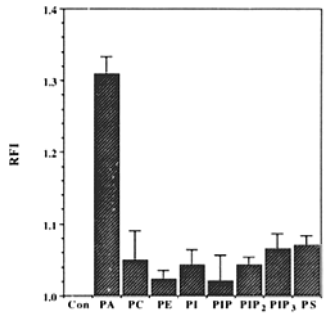

Figure 7. Effect of different phospholipids on F-actin contents. Cells, grown and serum starved, were incubated with 1 μg/ml of the indicated phospholipids for 15 min. Data are means ± SE from three independent experiments.

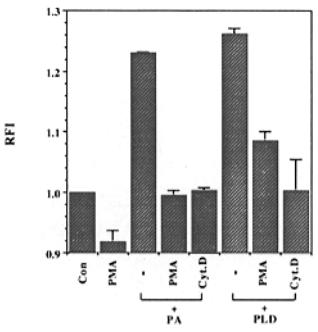

Figure 8. Effects of PMA and cytochalasin D on actin polymerization induced by PA and PLD. Cells, grown and serum starved, were treated with 160 nM PMA for 15 min or 1 μM cytochalasin D (*Cyt. D*) for 30 min and then incubated with 1 μg/ml PA or 5 U/ml *S. chromofuscus* PLD for 15 min. Data are means ± SE from three independent experiments.

a semiround to an elongated form with enhanced appearance of stress fibers (Fig. 1), and increases actin polymerization (Fig. 2). Since *S. chromofuscus* PLD induces similar effects (Figs. 1–3), these observations suggest that activation of PC-PLD is responsible for the change in cell morphology and increase in actin polymerization. PC hydrolysis induced by α-thrombin in IIC9 cells increases PA and DAG, which in turn activates PKC ϵ (12). Thus, these candidates, PA, DAG, and PKC ϵ, could potentially play a role in the activation mechanism of actin polymerization.

A role for PKC ϵ activation in actin polymerization was excluded by four different experiments, and all the results indicated that this PKC isozyme was ineffective. PMA and DOG, which induce PKC ϵ translocation in IIC9 cells (12), caused little or no actin polymerization (Fig. 4 *a*). PC-specific PLC from *B. cereus* which increases DAG and causes PKC ϵ translocation (12), did not increase F-actin (Fig. 4 *b*). Furthermore, PKC down-regulation caused by incubation with PMA for 24 h did not block the increase of actin polymerization caused by α-thrombin (Fig. 5 *a*), and H-7, a PKC inhibitor, slightly inhibited actin polymerization only at a high concentration (Fig. 5 *b*).

There have been divergent results regarding the activation of actin polymerization by PKC in different cell types. In rat basophilic leukemia cells, PMA and DOG activated actin polymerization, and antigen-stimulated actin polymerization was blocked by the PKC inhibitors sphingosine and staurosporine (1). In neutrophils, PMA and DOG activated actin polymerization, but PMA-induced actin polymerization was not inhibited by PKC inhibitors such as H-7, calphostin C, sphingosine, and staurosporine (9). However, in human fibroblasts, PMA was ineffective in inducing membrane ruffling and also inhibited PDGF-induced membrane ruffling (21), suggesting that PKC is an inhibitor rather than an activator of actin polymerization in these cells. In *Dictyostelium discoideum*, DOG activated actin polymerization by inducing de novo synthesis of actin nucleation sites in the absence of PKC (29), but the increase in actin polymerization was not blocked by staurosporine or mimicked by PMA.

In the present studies with IIC9 cells, actin polymerization was not activated by DOG (Fig. 4 *a*), and although *B. cereus* PC-specific PLC produced a large increase in DAG (12), it did not activate actin polymerization at any concentration tested (Fig. 4 *b*). The negative result with the PLC was not due to its failure to increase DAG in the inner leaflet of the membrane since it induced membrane translocation of PKCϵ within 30 s in IIC9 cells (unpublished findings). Furthermore, other studies have shown that DAG, produced by exogenous PC-specific PLC, readily crosses the plasma membrane in Chinese hamster V79 fibroblasts (23), indicating a very rapid transbilayer movement of DAG.

The lack of effects of DAG and PKC on actin polymerization in IIC9 cells led us to examine if PA could be an activator by incubating cells with different phospholipid micelles. Phospholipids have been shown to cross the lipid bilayer membrane (reviewed in 36) and there is evidence that exogenous PA is rapidly incorporated into the outer leaflet of lipid bilayer and internalized into cells (24, 32, 36). In IIC9 cells, exogenous PLD rapidly translocates PKC ϵ and increases DAG (data not shown). These data are consistent with the conclusion that PA produced by PLD acting on the outer leaflet of the plasma membrane moves into the inner leaflet and is then hydrolyzed to DAG which activates PKC ϵ translocation.

In IIC9 cells, PA induced an activation of actin polymerization that was specific for this phospholipid (Fig. 6), although it must be recognized that some of the other phospholipids may not have produced significant increases in their plasma membrane concentrations due to slow incorporation. The activation induced by PA was dose dependent and rapid, with increases being detectable at 0.01 μg/ml and at 1 min (Fig. 6 *a*). A recent report has also indirectly implicated PA in actin polymerization in platelets because of the correlated increases of PA and F-actin induced by pervanadate, a protein tyrosine phosphatase inhibitor (25). In these cells the origin of the PA was apparently DAG generated from PIP_2 by PLC. This is different from the situation in IIC9 cells where PA is mainly derived from PC-hydrolysis by PLD (for review see reference 12 and Fig. 3). There is another recent indication to support PA activation of actin polymerization. Lysophosphatidic acid (LPA) increased PA by activating PC-hydrolyzing PLD in Rat-1 fibroblasts (35), and activated the formation of focal adhesion and stress fibers in Swiss 3T3 cells (27).

There is much evidence to support the idea that LPA functions as an agonist in transmembrane signaling. This includes the existence of a putative receptor of LPA, activation of a pertussis toxin-insensitive G-protein with PIP_2 hydrolysis and Ca^{2+} mobilization, activation of a PC-hydrolyzing PLD, and activation of actin polymerization (27, 30, 33–35). These findings raise the possibility that PA might activate actin polymerization by a similar mechanism. However, PA can rapidly cross the lipid bilayer (24, 32, 36), and it is difficult to distinguish a direct effect of PA after crossing the bilayer from one involving a putative receptor.

Interestingly, PDGF induced a rapid, but transient increase of F-actin content (Fig. 2 *c*), which was accompanied by cell rounding and a loss of stress fibers (Fig. 1). Since there was an increase of PA that was maximal at 5 min (Fig. 3), it is clear that other changes induced by the growth factor caused a reversal of the effects of PA. In *Dictyostelium* mutants in which the gene for the phosphotyrosine phosphatase was disrupted, tyrosine phosphorylation of actin was rapid and prolonged, and the cells became round (14), suggesting that tyrosine phosphorylation was involved in the cell shape change. In the present study, PDGF was found to inhibit actin polymerization induced by PA. These findings support the idea that tyrosine phosphorylation is responsible for the differences in morphological and actin responses produced by the growth factor in IIC9 cells compared with those induced by α-thrombin.

PMA did not increase F-actin by itself, even though it increased PA (Fig. 3). The discrepancy can be explained by the findings that PMA inhibited PA-induced actin polymerization (Fig. 8) and caused the disappearance of stress fibers and cell rounding (Fig. 1 *f*). Interestingly, the changes in cell morphology and stress fibers induced by PMA were similar to those induced by PDGF (Fig. 1 *e*) or cytochalasin D (not shown). PDGF and PMA may utilize a common mechanism to inhibit actin polymerization induced by PA.

PA has been reported to increase cytosolic Ca^{2+} in some cells by activating Ca^{2+} uptake (26, 28) or Ca^{2+} release from intracellular stores (22). Thus, it is possible that PA might activate actin polymerization by regulating Ca^{2+}-dependent actin binding proteins such as gelsolin (16, 37) or villin (11). In IIC9 cells, the activation of actin polymerization induced by exogenous PLD or α-thrombin was largely or completely blocked by preincubating cells with EGTA or *bis* (2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate acetoxymethyl ester to deplete or buffer intracellular Ca^{2+} (data not shown). However, ionomycin induced a large Ca^{2+} increase, but did not activate actin polymerization (data not shown), in agreement with findings in other cells (1, 21, 29). These results suggest that PA does not activate actin polymerization by increasing cytosolic Ca^{2+} over basal. However, a basal level of Ca^{2+} is required for actin polymerization.

What are the possible mechanisms by which PA activates actin polymerization? The first mechanism is that PA might activate a PA-dependent protein kinase(s) (3), which might affect actin polymerization or regulate actin-binding proteins (6). A second possible mechanism is that PA is deacylated into LPA by phospholipase A_2, and LPA activates actin polymerization. There is a report that LPA induces stress fiber formation, perhaps by regulating the activity of the small molecular weight GTP-binding protein rho (27). In IIC9 cells, LPA also induced actin polymerization, but there was no significant increase of LPA in cells incubated with α-thrombin or PLD (unpublished data). Furthermore, although pertussis toxin markedly inhibited the effect of LPA on actin polymerization, it did not alter the effect of PLD and had a minimal effect on the action of PA (unpublished data). Thus, the possibility that PA activates actin polymerization by LPA may not be the case in IIC9 cells. A third possible mechanism is that PA interacts directly with actin-binding proteins as has been proposed for polyphosphoinositides (6). These phospholipids have been proposed to regulate actin polymerization through their ability to bind to actin-binding proteins such as profilin, villin and gelsolin (4, 6, 16). The binding of the phosphoinositides has been proposed to release actin monomers or free barbed ends of actin filaments from actin-binding proteins–actin complexes or actin-binding proteins–actin filament complexes. According to this model, the hydrolysis of polyphosphoinositides by agonists would decrease F-actin content, and their subsequent rise due to resynthesis would allow actin polymerization. However, in IIC9 cells, none of polyphosphoinositides (PIP, PIP_2, and PIP_3) significantly activated actin polymerization (Fig. 8), and there is no evidence that growth factors increase PIP_2 or PIP above basal levels in any cell. Furthermore, in a detailed study using A431 cells incubated with EGF, bradykinin, and cholera toxin, discrepancies between the phosphoinositide changes and the levels of gelsolin-actin complex were observed (8). Thus, the present findings suggest that PA might be an alternative to phosphoinositides in regulating actin polymerization. There is a report that PA has a much lower effect in binding with profilin to induce actin polymerization than PIP and PIP_2 (19), but other actin binding proteins have not been studied.

To determine if PA has a more general role in inducing actin polymerization, the present studies need to be extended to other cells, particularly those in which agonists elevate PA. More interestingly, the mechanism(s) by which PA alters actin assembly/disassembly needs to be elucidated. An attractive possibility, suggested by previous work, is that the phospholipid interacts with actin-binding proteins. However, a role for a PA-activated protein kinase also needs to be explored, as does the apparent negative effects of PKC and tyrosine kinase. Although the phenomenon of agonist-induced PC hydrolysis by PLD is widespread (10), the physiological role of the PA generated remains obscure. The present findings suggest an important function for this lipid.

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