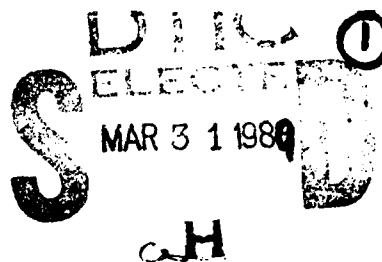


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Inflammation, Vol. 13, No. 1, 1989



MODULATION OF PHOSPHOLIPASE A_2 LYTIC ACTIVITY BY ACTIN AND MYOSIN¹

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Abstract—Prostacyclin (PGI_2) production is closely coupled with endothelial cell shape and F-actin distribution in vitro. These findings may implicate cytoskeletal constituents in a mechanism regulating eicosanoid metabolism. To determine the potential for such a regulatory mechanism, cytoskeletal protein effects on the rate-limiting eicosanoid cascade enzyme (phospholipase A_2 , PLA_2) were studied. Membrane phospholipid degradation was indirectly determined by spectrophotometric measurement of PLA_2 -induced rat red blood cell ghost (RBC-G) hemolysis. PLA_2 was incubated with actin (skeletal, smooth, or nonmuscle cell) at a nonmuscle cell concentration (100 μM) and then exposed to the RBC-G. Comparisons in the presence or absence of actin revealed that F-actin stimulated whereas G-actin suppressed PLA_2 lytic behavior significantly ($P < 0.05$). When a 10: or 100:1 F-actin to myosin ratio was used, the F-actin stimulatory effect was significantly ($P < 0.05$) reduced. These findings suggest that the in vitro correlation between PGI_2 production and endothelial cell shape may be the result of PLA_2 regulation by cytoskeletal elements that impart cellular form. *Red blood cell ghost (RBC-G)*

INTRODUCTION

Prostacyclin (PGI_2) affects the cardiovascular system in numerous ways (1), one of which is augmentation of actin stress fiber formation (2). These are cytoskeletal elements that in vitro appear to promote endothelial cell junctional integrity (3). Thus, this eicosanoid may influence permeability, and knowledge

¹The investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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of the mechanisms that control its production might provide insight to alterations in endothelial cell barrier function.

The rate-limiting enzyme of the PGI_2 cascade is phospholipase A_2 (PLA_2) (4). This enzyme is soluble or membrane associated (5) and may be influenced by the modulatory effects of calcium, cAMP, hormones, and membrane structure (6). Cell shape is perhaps another regulatory factor. For example, bradykinin stimulates PGI_2 production by substrate-attached endothelial cells (7). In contrast, endothelial cells (8) or transformed fibroblasts (7) in suspension are not stimulated by bradykinin. The cell shape differences of attached and suspended cells perhaps explain this phenomenon. Moreover, PGI_2 synthesis by the small endothelial cells in contact-inhibited cultures might exceed that of large cells in nonconfluent cultures because of cell shape differences (9). Cell shape is of greater significance than cell-to-cell contact, since in the absence of contact inhibition, small endothelial cells still generate more PGI_2 than large cells (10). Small endothelial cells in culture have a diffuse F-actin distribution and lack the F-actin stress fibers seen in large cells (Figure 1). Enhanced PGI_2 synthesis occurs with a diffuse F-actin distribution (Figure 1A) and production is reduced when complexes of F-actin, myosin, and other cytoskeletal proteins (stress fibers; Figure 1B) are present (10).

Changes in the distribution and polymerized state of actin alter cell shape. Moreover, actin binds many proteins (11) and regulates glycolysis by allosteric modulation of glycolytic enzyme catalytic site (12–15). Thus, cytoskeletal factors that influence cell shape are also associated with metabolic control mechanisms. Because cell shape (7–10) and F-actin distribution (10) correlate with PGI_2 production, PLA_2 regulation by a cytoskeletal-mediated mechanism may be operational in eicosanoid metabolism. To determine the potential for such a mechanism, the effect of actin and actin–myosin complexes on extracellular PLA_2 lytic behavior was determined. These cytoskeletal elements were associated with an alteration in PLA_2 lytic activity, which further suggests the control of intracellular PLA_2 by the constituents that impart cellular form.

MATERIALS AND METHODS

Calcium chloride, ATP, potassium chloride, guanidine hydrochloride, rabbit muscle actin, chicken muscle actin, chicken gizzard actin, bovine muscle myosin, chicken gizzard myosin, bovine albumin, phalloidin, and PLA_2 derived from bee venom ($\text{PLA}_2\text{-B}$) or porcine pancreas ($\text{PLA}_2\text{-P}$) were obtained from Sigma Chemical Co., St. Louis, Missouri. Human platelet actin was procured from Calbiochem Biochemicals, San Diego, California. Sodium chloride and sodium acetate were purchased from Mallinckrodt, Paris, Kentucky. Fisher Scientific (Fairlawn, New Jersey) was the source for Tris and EDTA.

The compositions of buffers and solutions were as follows: isotonic saline, 0.144 M sodium

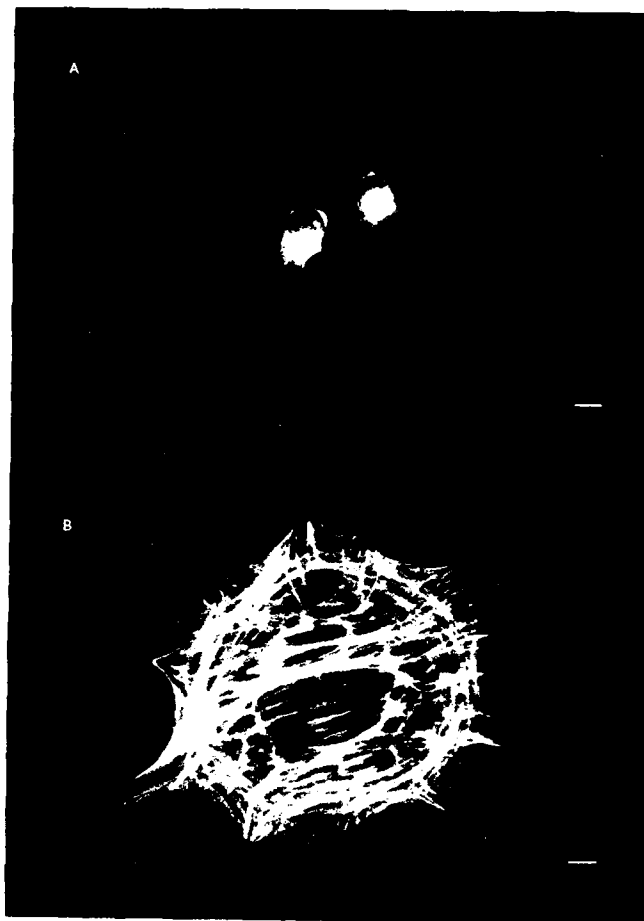


Fig. 1. The effects of substrate adhesive capacity on bovine aortic endothelial (BAE) cell shape, F-actin distribution, and actin stress fiber formation. BAE cells were seeded on bacteriological plastic or tissue culture plastic. After 24 h, cells were fixed (3.7% formalin, 30 min), treated with acetone (80%, 10 min), and exposed to rhodamine phalloidin (0.165 μ M, 10 min, Junction City, Oregon). A Zeiss microscope equipped with the appropriate filters for the excitation of rhodamine was used to observe the cell fluorescence. Photographs of the fluorescence were taken using Tri-X Pan film (Kodak, 400 ASA). BAE cells on a substrate of reduced adhesive capacity (bacteriological plastic, A) were small with a diffuse F-actin distribution and appeared to lack stress fibers. Such cells on an adhesive substrate (tissue culture plastic, B) were large with many discrete actin stress fibers. Bar equals 10 μ m.



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chloride, 2.0 mM calcium chloride, pH 7.5; hemolysis buffer, 0.05 M sodium chloride, 1.0 mM EDTA; resealing solution, 1.5 M sodium chloride, 1.0 mM EDTA; actin polymerizing solution, 50.0 mM potassium chloride, 2.0 mM calcium chloride, 1.0 mM ATP; actin depolymerizing solution, 1.5 M guanidine hydrochloride, 1.0 M sodium acetate, 1.0 mM ATP, 20.0 mM Tris, 6.0 mM calcium chloride, pH 7.5

Red blood cell ghosts (RBC-G) were prepared as previously described (16, 17). Blood was obtained from anesthetized (52 mg penobarbital sodium/kg; intravenous) rats. After centrifugation (2500g) and supernatant aspiration, the red blood cells were washed in saline three times. To each 5-ml pellet volume, 50 ml of hemolysis buffer was added and stirred at room temperature for 30 min. A sufficient volume of resealing solution was then added to restore isotonicity. This mixture was stirred slowly for 1 h. After resealing, ghosts were collected by centrifugation (15,000g). With gentle resuspension, ghosts were separated from the lysis-resistant red blood cells that comprised the dark red button in the pellet center. Ghosts were washed in saline until the absorbance value (418 nm) remained constant. RBC-G were then adjusted to a 20% hematocrit.

Polymerizing solution was employed to generate F-actin (18). Actin (5 mg/ml) was suspended in this solution for 2 h at 5°C. Polymerization increased solution viscosity. Actin (5 mg/ml) was suspended in depolymerizing solution to obtain G-actin (19). To account for any possible direct polymerizing or depolymerizing buffer effects, these solutions were used as diluents for PLA₂ incubated in the absence of cytoskeletal protein.

Measurement of PLA₂ activity via RBC-G lysis followed previously described procedures (16, 17). RBC-G (20% hematocrit) were diluted in isotonic saline (1:5.33) to a 9.0 ml volume (Figure 2). Four 250- μ l samples were then collected, centrifuged (1 min, 15,000g), the supernatant diluted (1:10) in distilled water, and measured in a spectrophotometer at 418 nm to determine background absorbance before PLA₂ addition. PLA₂-B (88 units/ml) was incubated (30 min, 5°C) alone or with G- or F-actin (100 μ M) from bovine or chicken skeletal muscle. Similar PLA₂-P (300 units/ml) studies employed actin derived from human platelets, chicken skeletal muscle, or chicken gizzard. Some PLA₂-P studies used incubations with phalloidin (100 μ M) or bovine albumin (100 μ M). Other studies involved incubations first with F-actin (chicken skeletal muscle) and then phal-

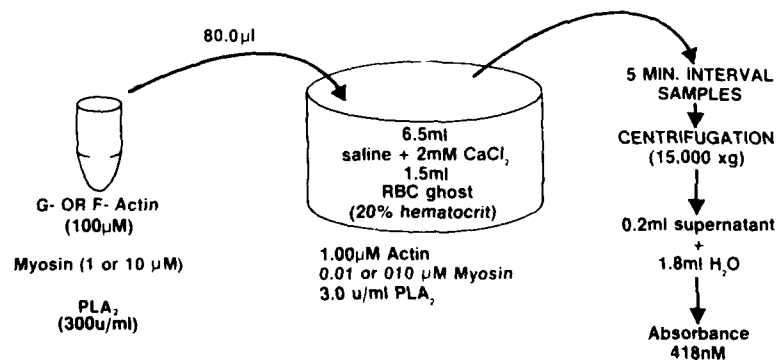


Fig. 2. Phospholipase A₂ (PLA₂) was incubated (30 min, 5°C) in the presence or absence of cytoskeletal protein prior to exposure to the red blood cell (RBC) ghost bath. Control experiments conducted in the absence of F- or G actin used enzyme incubated with polymerizing or depolymerizing solution, respectively. PLA₂ activity was followed by measurement of sample supernatant absorbance at 418 nm at 5-min intervals over a 30-min period.

loidin. PLA₂-P was also incubated with F-actin (chicken skeletal muscle or human platelet) and then myosin (30 min, 5°C, bovine muscle or chicken gizzard, 10 or 1 μ M). After these incubation conditions, an aliquot (80.0 μ l) was added to the RBC-G (8 ml) and gently stirred at 37°C. Over 30 min, at 5-min intervals, four 250- μ l samples were collected and supernatant absorbance determined.

Each PLA₂ lytic activity study was repeated in triplicate. From the 12 samplings (4 samples per 5-min time period $\times$ 3 replications), a mean absorbance value was calculated and a third-degree regression generated that described PLA₂ lytic activity with time. To determine significant ($P < 0.05$) differences, lytic activity curves were studied by analysis of variance and Tukey computation (20).

RESULTS

PLA₂ action on the RBC-G phospholipid membranes resulted in lysis. As measured by an increased bath solution supernatant absorbance, hemoglobin was released, which indirectly determined PLA₂ activity (16, 17). Control studies demonstrated that actin, myosin, or the small volume (80 μ l) of polymerizing or depolymerizing solution in the absence of PLA₂ did not induce ghost lysis (data not shown). Although enzyme activity was not significantly altered by incubation in polymerizing or depolymerizing solution, activity was significantly enhanced by depolymerizing solution when compared to incubation in saline (Figure 3A). Moreover, differences in the RBC-G from preparation to preparation altered the PLA₂ kinetics (Figure 3B). To account for these assay characteristics, PLA₂ was incubated with polymerizing or depolymerizing solution in the absence of F- or G-actin, and only activity curves generated from the same RBC-G preparation were compared. Under identical experimental conditions, PLA₂ lytic activity curves were replicated without significant differences (Figure 3C). Results were similar using PLA₂-B or PLA₂-P incubated in polymerizing or depolymerizing solution.

Significantly, prior incubation of PLA₂-B with polymerized chicken or rabbit muscle actin stimulated, while G-actin reduced, lytic activity when the same RBC-G preparation was used (Figure 4). Similar actin effects on PLA₂-P activity were noted, even with different RBC-G preparations (Figure 5). For skeletal muscle (chicken muscle, Figure 5A and B), smooth muscle (chicken gizzard, Figure 5C and D), or nonmuscle cell (human platelet, Figure 5E and F) actin, F-actin significantly stimulated, while G-actin reduced, RBC-G lysis. As illustrated (Figure 5C and E), prior PLA₂-P incubation with F-actin could occasionally be associated with an initial suppression in enzyme activity over the first 10 min in the RBC-G bath. Incubation with F-actin followed by myosin exposure significantly reduced the stimulation of PLA₂-P seen after incubation with F-actin alone (Figure 6). This was true for both the 100:1 and 10:1 F-

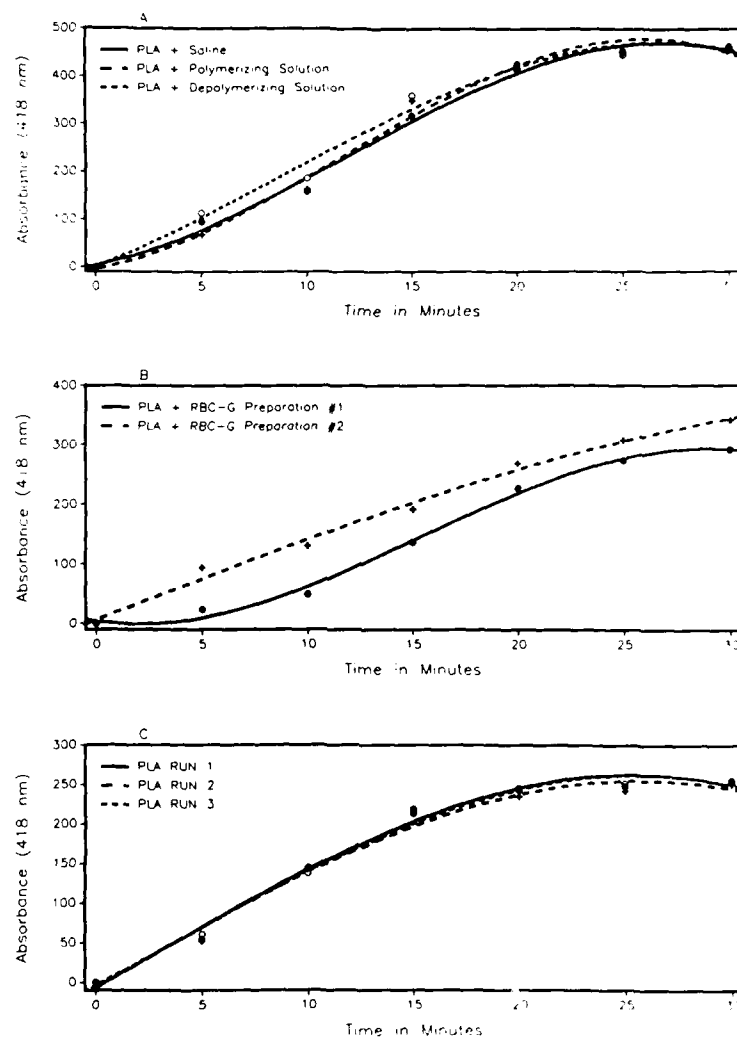


Fig. 3. Effect of different enzyme incubation conditions (saline, polymerizing, or depolymerizing solution in the absence of cytoskeletal protein) on phospholipase A₂ (PLA) lytic activity (A). Demonstration of the changes in PLA kinetics associated with different ghost preparations (B). Illustration of the close replication of PLA lytic activity curves when the same enzyme source (bee venom or porcine pancreas), enzyme incubation conditions (polymerizing or depolymerizing solution in the absence of cytoskeletal protein), and red blood cell ghost preparation were used (C). For A, B, and C, similar results were obtained using PLA derived from bee venom or porcine pancreas.

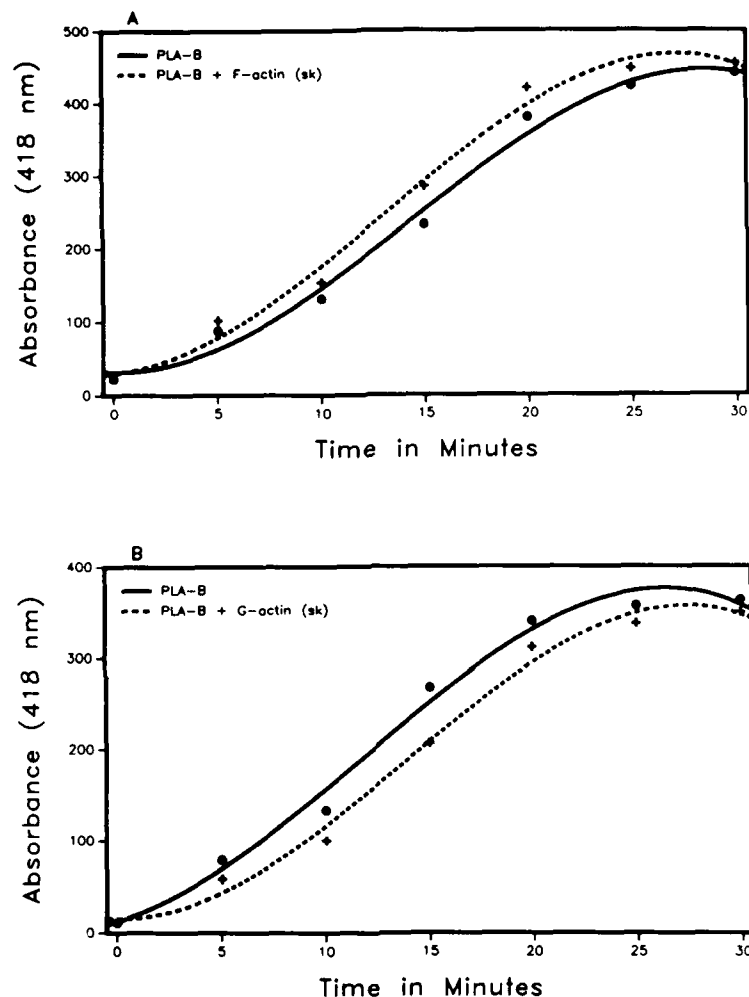


Fig. 4. Effects of prior incubation with F-actin (A, 100 μ M) or G-actin (B, 100 μ M) derived from chicken skeletal muscle (sk) on bee venom phospholipase A₂ (PLA-B) lytic activity. Results were similar using rabbit skeletal muscle actin. In the absence of cytoskeletal protein, PLA-B was incubated with polymerizing or depolymerizing solution. The same ghost preparation was used throughout.

actin-to-myosin ratios employed. Results were similar using skeletal muscle actin and myosin or nonmuscle cell actin with smooth muscle myosin. Myosin (10 μ M) had no independent inhibitory effects on lytic activity (data not shown).

Albumin resulted in significantly greater PLA₂ suppression than did G-

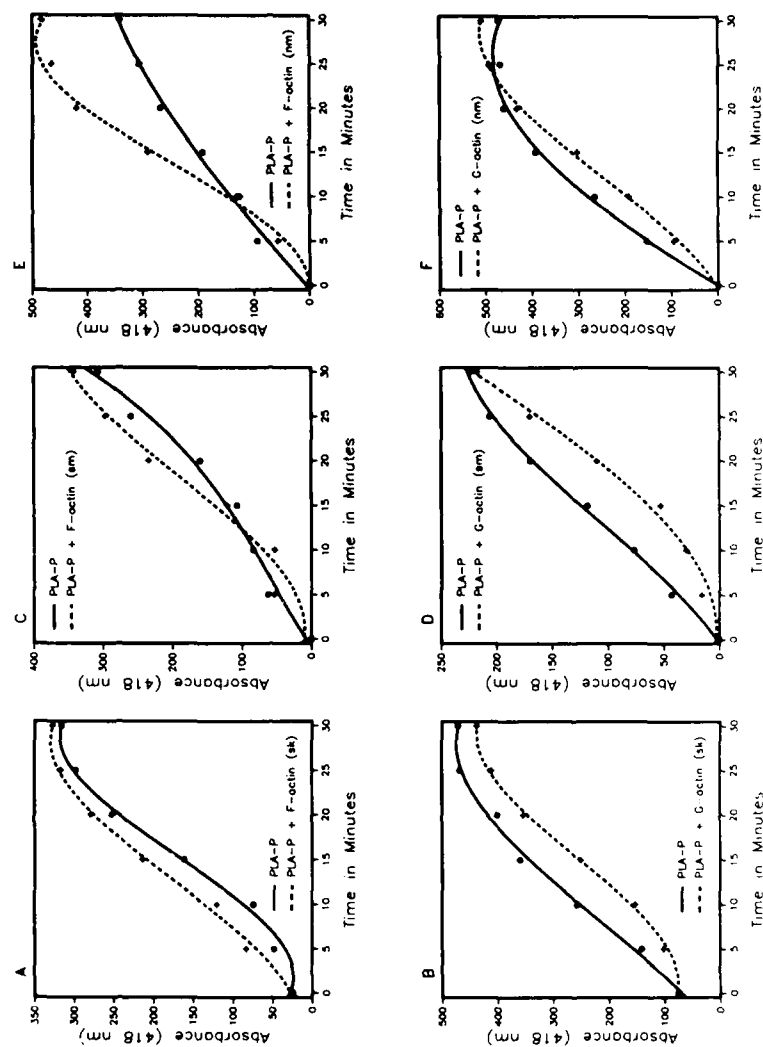


Fig. 5. Effects of prior incubation with F-actin (A, C, and E; 100 μ M) or G-actin (B, D, and F; 100 μ M) from chicken skeletal muscle (sk), chicken smooth muscle (sm), or human platelet nonmuscle (nm) cell sources on porcine pancreatic phospholipase A₂ (PLA-P) lytic activity. In the absence of cytoskeletal protein, PLA-P was incubated with polymerizing or depolymerizing solution. The same ghost preparation was used within each study, but different preparations were used between studies.

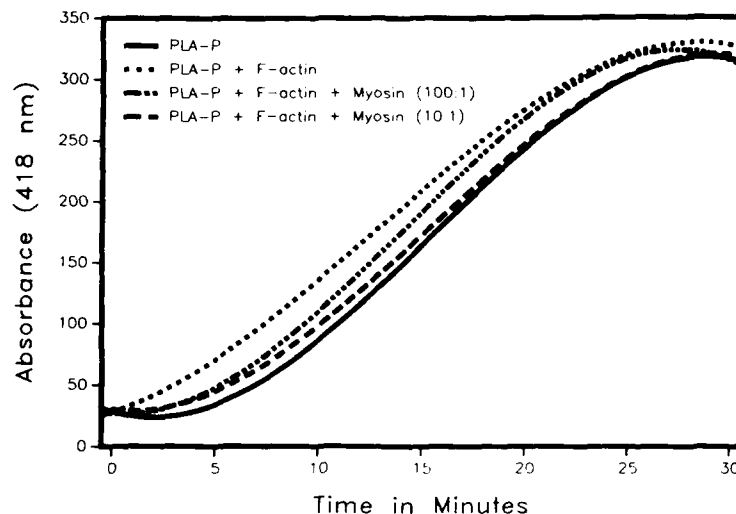


Fig. 6. Effects of prior incubation with F-actin (100 μ M) or F-actin (100 μ M) plus myosin (10 or 1 μ M) on porcine pancreatic phospholipase A₂ (PLA-P) lytic activity. Similar results were obtained using chicken skeletal muscle actin and such actin (skeletal muscle cell) in combination with bovine muscle myosin or human platelet actin and such actin (nonmuscle cell) in combination with chicken gizzard myosin. In the absence of cytoskeletal protein, PLA-P was incubated with polymerizing solution. The same ghost preparation was used throughout.

actin (Figure 7A), while phalloidin, another noncytoskeletal protein, had no effect (Figure 7B). When PLA₂-P was first incubated with F-actin (chicken skeletal muscle) and then phalloidin, the stimulatory effect of polymerized actin was significantly improved (Figure 7C).

DISCUSSION

Studies of endothelial cells in culture demonstrate a significant correlation among cell shape, F-actin distribution, and PGI₂ synthesis (7-10). The findings obtained in this current report support an explanation for this phenomenon based on the modulation of PLA₂ activity by cytoskeletal constituents that impart cellular form.

RBC-G lysis determined PLA₂ activity. Lysis is associated with phospholipid hydrolysis, since in the absence of hydrolysis erythrocytes are not lysed (16), while in ghosts the major phospholipid classes are degraded (21), and significant phospholipid breakdown correlates with ghost lysis (22). Therefore, RBC-G lysis is an indirect measure of phospholipid hydrolysis by PLA₂.

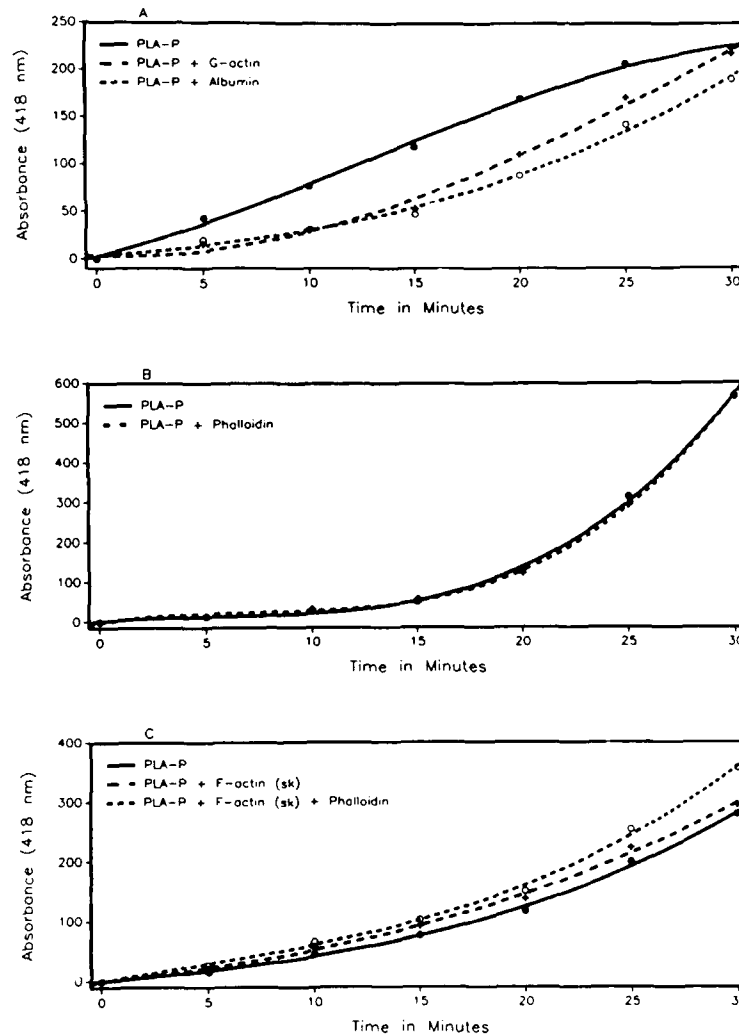


Fig. 7. Effects of prior incubation with albumin ($100\ \mu\text{M}$) or G-actin (chicken smooth muscle; $100\ \mu\text{M}$) on porcine pancreatic phospholipase A_2 (PLA-P) lytic activity (A). In the absence of albumin or G-actin, PLA-P was incubated with depolymerizing solution. The albumin incubation solution also contained depolymerizing solution. Effect of phalloidin exposure ($100\ \mu\text{M}$) on enzyme function (B). Incubations in the presence or absence of phalloidin used polymerizing solution. Demonstration of the enhancement effect by the presence of phalloidin ($100\ \mu\text{M}$) on chicken skeletal muscle F-actin (sk; $100\ \mu\text{M}$) stimulation of PLA-P lytic activity (C). In the presence or absence of F-actin or F-actin plus phalloidin, all enzyme incubations were conducted in the presence of polymerizing solution. Within each study the same RBC-G preparation was used, but different preparations were used between studies.

Although an indirect determination may have limitations, it was preferable to those assays that directly measure hydrolysis of an isolated phospholipid, which is not part of a membrane composed of the many elements that influence enzyme-membrane interactions. Because this model accounted for some of the adversities associated with the PLA₂ active site gaining access to its substrate in a complex membrane, it closely approximated the *in vivo* conditions under which intracellular PLA₂ must function. The use of extracellular PLA₂ to draw inference about the nature of intracellular PLA₂ is an approach used by others (6). Moreover, intracellular PLA₂ molecular weight is similar to PLA₂-P and, like the extracellular form, intracellular PLA₂ may exist as a zymogen (6). Thus, these forms of PLA₂ may not be unique, and factors that influence one form may be relevant to the other.

Under identical conditions (same PLA₂ source, incubation environment, and RBC-G preparation), the assay stability was demonstrated by activity curve replication without significant differences (Figure 3C). After actin incubation, PLA₂ from diverse sources was stimulated by F-actin, but inhibited by G-actin. This was true using the same RBC-G preparation throughout (Figure 4) or when different preparations were employed (Figure 5), even though such differences could alter the PLA₂ kinetics (Figure 3B). Any possible effects of the polymerizing or depolymerizing solution were eliminated by PLA₂ incubation with the solution in the absence of cytoskeletal protein. Finally, the dependence of lytic behavior on actin polymerization state excluded any participation by contaminants in the actin, since lysis influenced by contaminants should not change with actin state. Thus, only the effects of actin could account for these changes in PLA₂ activity.

When incubated with enzyme, actin (5 mg/ml) exceeded the critical concentration required for polymerization (11). The RBC-G bath conditions were not as favorable for F-actin (0.05 mg/ml). However, the F-actin state was maintained for a sufficient time, because lytic activity was enhanced by F-actin, but not G-actin (Figures 4 and 5). The further enhancement of lytic activity by phalloidin (Figure 7C) was additional evidence that the F-actin state was essential to lysis stimulation. Phalloidin binds to F-actin in a 1:1 ratio (23), lowers the critical concentration for polymerization (24, 25), and stabilizes the F-actin state by reducing monomer dissociation (24).

Occasionally, F-actin initially suppressed lytic activity (Figure 5C and E). This may be related to an actin fiber length that impeded enzyme-membrane interactions, which was overcome by depolymerization to a more optimal fiber size. The augmentative effect of phalloidin on stimulated PLA₂ activity with F-actin (Figure 7C) was evidence for depolymerization in the RBC-G bath in the absence of phalloidin. Thus, fiber length may affect F-actin stimulation of PLA₂. Since this suppression was not consistently noted, initial actin fiber length in the RBC-G bath was perhaps an uncontrolled variable.

Actin and myosin are major stress fiber proteins. Myosin had no independent inhibitory effects (data not shown), but it reduced the F-actin stimulation of lysis (Figure 6). Tropomyosin and α -actinin stress fiber proteins (10 μ M) did not alter stimulation (data not shown). Thus, if stress fibers have an impact, it is perhaps due to myosin.

Phalloidin, in the absence of F-actin, had no effect (Figure 7B). However, albumin significantly reduced PLA₂ lytic behavior (Figure 7A). Albumin's effect is indirect, since it alters the form of the substrate presented to PLA₂ (26). The greater inhibition by albumin relative to G-actin (Figure 7A) suggests that these proteins may influence enzyme activity by different mechanisms. As demonstrated by the phalloidin effect, not all proteins altered lytic activity. This finding (Figure 7B) and others (Figure 3C) illustrated how closely lytic curves were replicated when activity was not changed. Although the actin-associated modulations in PLA₂ activity may appear small, because the activity curves within each condition were so closely replicated, these changes were significant.

Although the mechanism of cytoskeletal protein action on PLA₂ is not known, it may be relevant to various cell types, since skeletal, smooth, and nonmuscle cell actin isoforms influenced lytic behavior (Figure 5). The relevance to endothelial cells was further enhanced by the use of actin (100 μ M) and myosin (1 μ M) concentrations reported for nonmuscle cells (27). Furthermore, because diverse extracellular PLA₂ sources (PLA₂-B and PLA₂-P) were similarly affected by actin, this may be a common PLA₂ attribute.

These findings support a potential for cytoskeletal-mediated regulation of eicosanoid metabolism. A diffuse F-actin distribution in small, substrate-attached endothelial cells (Figure 1A) permits greater PGI₂ production (10), because PLA₂ may be stimulated by F-actin free of other cytoskeletal proteins (Figures 4A and 5A,C,E). Reduced PGI₂ production by large, substrate-attached cells (10) (Figure 1B) might be the result of enzyme suppression by the actin-myosin complexes of stress fibers (Figure 6). Since reduced cell adhesion and F-actin stress fiber level correlate directly (10, 28) (Figure 1), in the absence of adhesion, G-actin may dominate to perhaps decrease PLA₂ activity (Figures 4B and 5B,D,F) and PGI₂ synthesis in suspended cells (7, 8). Endothelial cell stress fiber promotion by PGI₂ (2) also supports this mechanism. Factors that induce an actin-myosin dissociation (stress fiber disruption) might enhance PLA₂ activity. The resulting PGI₂ would mediate the return of actin-myosin complexes (stress fibers) to perhaps suppress PLA₂ and further PGI₂ synthesis. Therefore, PGI₂ and cytoskeletal proteins may function in a reciprocal signaling loop to modulate cell shape and eicosanoid metabolism (Figure 8). Since the cytoskeleton influences junctional integrity (3), if this mechanism were functional in vivo, it might play a role in the alteration of permeability within the endothelium.

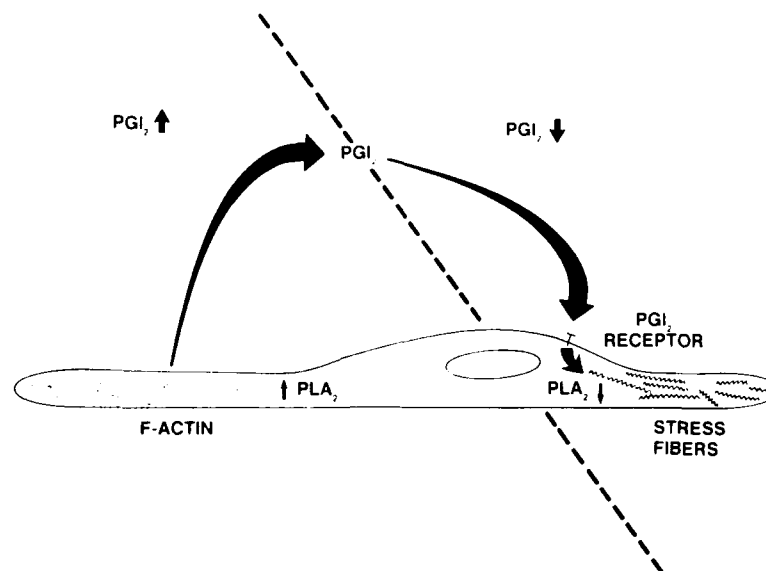


Fig. 8. Illustration of a potential reciprocal signaling loop that perhaps functions as a mechanism in the regulation of eicosanoid metabolism and cytoskeletal structure. Factors that cause a dissociation of F-actin and myosin (stress fiber disruption) may stimulate the rate-limiting enzyme of the eicosanoid cascade, phospholipase A₂ (PLA₂). This stimulation would induce enhanced arachidonic acid release and elevate prostacyclin (PGI₂) synthesis. The binding of PGI₂ to its putative membrane receptor would initiate the formation of complexes of F-actin, myosin, and other cytoskeletal proteins (stress fibers) to alter the cytoskeleton and cell shape. Stress fibers might suppress PLA₂ activity and reduce PGI₂ metabolism. The dotted diagonal line distinguishes the on (left, PGI₂ synthesis increased)/off (right, PGI₂ synthesis decreased) signal within this loop.

Acknowledgments—The authors acknowledge the excellent technical support of Mr. Michael Lukason and Robert Carpenter.

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