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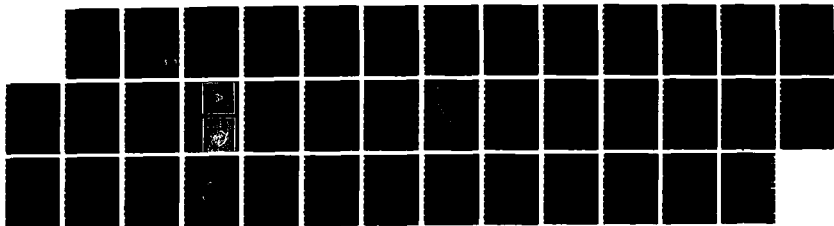
MODULATION OF PHOSPHOLIPASE A2 ACTIVITY BY ACTIN AND
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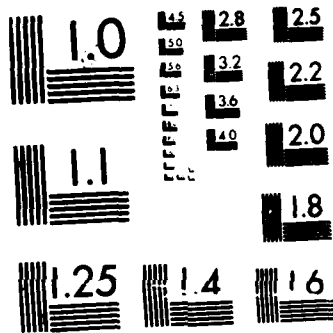
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Modulation of Phospholipase A₂ Activity by Actin and Myosin

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Running title: Actin and myosin PLA₂ regulation

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ABSTRACT

Endothelial cell prostacyclin (PGI_2) production is closely coupled with cell shape. Since shape change implies cytoskeletal modulations, this finding may be an important clue to implicate cytoskeletal constituents in a mechanism regulating eicosanoid metabolism. Endothelial cells with a diffuse F-actin distribution generate more PGI_2 than cells with many discrete F-actin stress fibers. Because actin can modulate enzyme activity, the myofibril protein effects on phospholipase A_2 (PLA_2), the rate limiting eicosanoid cascade enzyme, were studied. PLA_2 action on rat red blood cell ghost (RBC-G) membrane phospholipids resulted in ghost hemolysis. Enzyme activity was followed by measurement of the RBC-G bath supernatant absorbance at 418 nm. PLA_2 was exposed to actin (skeletal, smooth, or nonmuscle cell) at physiologically relevant concentrations ($100 \mu\text{M}$) for nonmuscle cells and then allowed to hemolyze the RBC-G. Comparisons of PLA_2 activity in the presence or absence of actin revealed that significantly ($p < 0.05$) F-actin stimulated whereas G-actin suppressed enzyme activity. When myosin was introduced at a 10 or 100:1 F-actin to myosin ratio, the F-actin stimulatory effect was significantly ($p < 0.05$) reduced. These findings suggest that the correlation of endothelial cell PGI_2 metabolic modulation to cell shape and actin distribution was perhaps due to changes in PLA_2 activity as a direct result of alterations in the polymerized state of actin and the degree of actin-myosin stress fiber formation.



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INTRODUCTION

Phospholipase A₂ (PLA₂)¹ is the rate limiting eicosanoid cascade enzyme which initiates endothelial cell prostacyclin (PGI₂) metabolism (14). Other than a potential calcium regulatory role (25), little is known about the cellular mechanisms which control synthesis of this important arachidonic acid metabolite. However, cell shape is one factor which may impact eicosanoid metabolism. Bradykinin stimulates PGI₂ production by substrate-attached endothelial cells (10). In contrast, trypsin-detached endothelial cells in suspension are not stimulated by bradykinin (13). Moreover, transformed fibroblasts, whether detached by trypsin or with a rubberpoliceman, also lose their capacity to respond to bradykinin (10). Thus, the responses of substrate-attached and suspended cells are perhaps the result of cell shape differences and not due to the action of trypsin on membrane receptors.

Differences in cell shape might also explain the phenomenon in which PGI₂ synthesis by the small, rounded endothelial cells in contact-inhibited cultures is greater than that of large, flat cells in nonconfluent cultures (5). Cell shape is perhaps of more significance to this phenomenon than cell to cell contact, for in the absence of contact inhibition, small, rounded endothelial cells still generate significantly greater quantities of PGI₂ than large, flat cells (4). Cell shape change implies modulation of the cytoskeleton. As illustrated in Fig. 1, small, rounded endothelial cells have a diffuse F-actin distribution and appear to lack F-actin stress fibers, whereas large, flat cells have many stress fibers. A significant enhancement in PGI₂ synthesis is associated with a diffuse F-actin distribution (Fig. 1a) and such synthesis is reduced when complexes of F-actin, myosin, and other myofibril proteins (stress fibers; Fig. 1b) are present (4).

Actin is partly responsible for cellular shape and form. Changes in its distribution and polymerized state affect cell morphology. Actin binds a number of proteins (12) and modulates glycolytic enzyme activity (21-23). The control of glycolysis by the actin allosteric alteration of enzyme catalytic site is proposed (16). This suggests that the actin cytoskeleton is associated with mechanisms for the control of metabolism. Because cell shape (4,5,10,13) and F-actin distribution (4) correlate with PGI₂ production, a PLA₂ regulatory mechanism associated with actin may be operational in endothelial cells.

To determine the potential for a cytoskeletal-mediated regulatory mechanism, the effect of actin and complexes of F-actin with myosin on PLA₂ activity was determined. Evidence is presented which demonstrates that actin state of polymerization and actin-myosin complexes altered PLA₂ activity in vitro. This indicates that the correlation of endothelial cell PGI₂ synthesis to cell shape and actin distribution may be the result of metabolic control of the eicosanoid pathway by a cytoskeletal-directed mechanism.

MATERIALS and METHODS

Materials

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 PLA₂ derived from bee venom (PLA₂-B) or porcine pancreas (PLA₂-P) were obtained from Sigma Chemical Co., St. Louis, MO. Human platelet actin was procured from Calbiochem Biochemicals, San Diego, CA. Sodium chloride and sodium acetate were purchased from Mallinckrodt, Paris, KY. Fisher Scientific (Fairlawn, NJ) was the source for Tris and EDTA. Penobarbital sodium used to anesthetize rats came from Abbott Laboratories, North Chicago, IL.

The compositions of buffers and solutions were as follows: Isotonic saline: 0.144 M sodium chloride, 2.0 M 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.

Preparation of Red Blood Cell Ghosts (RBC-G)

RBC-G were prepared as previously described (3,9,19,29). Blood was obtained from anesthetized (52 mg penobarbital sodium/kg: iv) laboratory rats. After centrifugation (2,500g), the supernatant and buffy coat were aspirated. The red blood cells were washed in isotonic saline. 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. Following resealing, the ghosts were collected by centrifugation (15000g). Ghosts were gently resuspended in isotonic saline, in such a manner, as not to disturb the dark red button in the center of the pellet. The suspended ghosts were separated from the lysis-resistant red blood cells comprising this red button. Ghosts were washed in isotonic saline until the wash solution absorbance value (418 nm) remained constant. The RBC-G were then adjusted to a 20% hematocrit.

Preparation of F- and G-actin

Polymerizing solution was employed to generate F-actin (17). Actin (5 mg/ml: chicken skeletal muscle, chicken smooth muscle, or human platelet nonmuscle cell) 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 (8). To

account for possible direct polymerizing or depolymerizing buffer effects, these solutions were used as diluents for PLA₂ incubated in the absence of myofibril protein.

Lysis of RBC-G by Phospholipase A₂

Lysis of the RBC-G followed the procedures as illustrated in Fig. 2. RBC-G (20% hematocrit) were diluted in isotonic saline (1:5.33) to a final volume of 9.0 ml. The mixture was slowly stirred at 37°C. When well suspended, four 250 μl samples were collected; centrifuged (15,000g) for 1 min; 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 in the presence of actin (100 μM; G- or F-actin from bovine or chicken skeletal muscle). PLA₂-P (300 units/ml) studies employed G- or F-actin derived from chicken skeletal muscle, chicken smooth muscle, or human platelet nonmuscle cell sources (100 μM). Some PLA₂-P studies used incubations with phalloidin (100 μM) or bovine albumin (100 μM). Other PLA₂-P studies involved incubations first with F-actin (chicken skeletal muscle; 100 μM) and then phalloidin (100 μM). PLA₂-P was also incubated with F-actin (chicken skeletal muscle or human platelet; 100 μM) and then myosin (bovine muscle or chicken gizzard; 10 or 1 μM). Following enzyme incubation, an aliquot (80.0 μl) was added to the RBC-G suspension (8 ml) and gently stirred at 37°C. At 5 min intervals over a 30 min test period, four 250 μl samples were collected and supernatant absorbance determined as described above. Each study of PLA₂ activity was repeated in triplicate.

From the 12 samplings (4 samples per 5 min time period X 3 replications), a mean absorbance value was calculated and a third degree regression curve generated which described PLA₂ activity with time. PLA₂ activity curves were studied by analysis of

variance and Tukey computation (30) to determine significant ($p < 0.05$) differences in enzyme activity following exposure to myofibrillar or nonmyofibrillar proteins.

RESULTS

Lysis of RBC-G by Phospholipase

Fig. 3 demonstrates the reproducibility of PLA_2 activity curves under identical experimental conditions. PLA_2 action on the RBC-G membranes resulted in phospholipid hydrolysis and ghost lysis. Hemoglobin was released to the RBC-G bath and increased bath solution supernatant absorbance. By measurement of absorbance, the degree of ghost lysis and PLA_2 activity could be determined. No significant ($p < 0.05$) differences were noted in PLA_2 activity when experimental conditions were the same. Results were similar using PLA_2 -B or PLA_2 -P.

Effects of Actin on the Activity of PLA_2 Derived from Bee Venom

As illustrated in Fig. 4, prior incubation of PLA_2 -B with actin ($100 \mu M$) altered the activity of this enzyme. Polymerized chicken muscle actin significantly ($p < 0.05$) stimulated PLA_2 -B activity (Fig. 4a). Incubation in the presence of monomeric actin had the opposite effect, for PLA_2 -B was significantly ($p < 0.05$) inhibited (Fig. 4b). Similar results were recorded when rabbit muscle actin was employed (data not shown).

Effects of Actin and Myosin on the Activity of PLA_2 Derived from Porcine Pancreas

The effects of prior incubation with actin ($100 \mu M$) on PLA_2 -P were similar to those noted for PLA_2 -B (Fig. 5). For skeletal muscle (chicken muscle: Fig. 5a,b).

smooth muscle (chicken gizzard; Fig. 5c,d). or nonmuscle cell (human platelet; Fig. 5e,f) actin. F-actin (100 μ M) significantly ($p < 0.05$) stimulated, while G-actin (100 μ M) significantly ($p < 0.05$) inhibited PLA₂-P activity. As illustrated in Figures 5c and 5e, prior PLA₂-P incubation with smooth muscle or nonmuscle cell F-actin could be associated with an initial suppression in enzyme activity over the first 10 min in the RBC-G bath. Significant stimulation occurred only after this time period had elapsed.

While prior incubation with albumin (100 μ M) significantly ($p < 0.05$) inhibited PLA₂-P activity (Fig. 6a), phalloidin (100 μ M) had no effect (Fig. 6b). However, when PLA₂-P was first incubated in F-actin (chicken skeletal muscle) and then phalloidin, the stimulatory effect of polymerized actin was significantly ($p < 0.05$) improved (Fig. 6c). Furthermore, incubation with F-actin (100 μ M) followed by myosin (10 or 1 μ M) exposure significantly ($p < 0.05$) reduced the stimulation of PLA₂-P seen after incubation with F-actin alone (Fig. 7). Results were similar using chicken skeletal muscle actin with bovine muscle myosin or human platelet actin with chicken gizzard myosin. Incubation of PLA₂-P with myosin (10 μ M) in the absence of F-actin had no independent inhibitory PLA₂-P effects (data not shown).

DISCUSSION

PLA₂ activity was followed by the determination of hemoglobin release, as a result of RBC-G lysis. Under identical conditions, activity curves were replicated with no significant differences (Fig. 3). Therefore, changes in enzyme activity within experiments were not the result of variability inherent to this approach. Moreover, this model was appropriate, for the use of an intact phospholipid membrane as the substrate approximated the in vivo conditions under which this enzyme functions.

While incubation with phalloidin, in the absence of other protein, had no effect (Fig. 6b), albumin inhibited PLA₂ (Fig. 6a). The lack of a phalloidin effect demonstrated that not all proteins at a concentration of 100 μM altered PLA₂ activity. Albumin binds many proteins and serves as a transport vehicle for materials within the vascular fluids (7). Albumin's PLA₂ inhibitory effect indicated that it perhaps functions to suppress deleterious enzymes that gain access to the blood.

PLA₂ activity was also altered by the state of actin polymerization. Fibrous actin stimulated, while the monomeric form reduced PLA₂ activity (Figs. 4 and 5). Moreover, the F-actin stimulatory effect was reduced by myosin (Fig. 7). Though any direct connection to actual cellular events must be made with utmost caution, these *in vitro* findings suggest a potential for PLA₂ regulation by a cytoskeletal-directed mechanism.

Traditional methods were used to promote actin polymerization (17). When incubated with enzyme, actin (5 mg/ml) exceeded the critical concentration required for *in vitro* polymerization (12). Because of actin dilution (0.05 mg/ml), the RBC-G bath conditions were not as favorable for fibrous actin. However, the PLA₂/F-actin interaction was maintained for a sufficient time period, because activity was enhanced by prior incubation under conditions which supported fibrous actin, but not G-actin (Figs. 4 and 5). Differences in PLA₂ activity were not due to the polymerizing and depolymerizing buffers, because these solutions were used as diluents when PLA₂ was incubated in the absence of actin. The further enhancement of enzyme activity by phalloidin (Fig. 6c) was additional evidence that the F-actin state was essential to PLA₂ stimulation. Phalloidin binds to F-actin in a 1:1 ratio (26) and lowers the critical concentration for polymerization (2,6). Furthermore, it stabilizes F-actin by preventing monomer dissociation (2). Therefore, fibrous actin stabilization perhaps explains the further enhancement of PLA₂ activity by F-actin with phalloidin. This augmentative effect of phalloidin (Fig. 6c) also indicated that depolymerization occurred in the RBC-G bath.

As illustrated in Figs. 5c and 5e, F-actin suppressed PLA₂ activity during the first 10 min in the RBC-G bath. This might be explained by an initial inhibition by a fiber length that impeded enzyme-substrate interactions which was overcome by actin depolymerization to a more optimal fiber size. The phalloidin effect (Fig. 6c) was evidence for depolymerization in the RBC-G bath. Thus, though F-actin stimulated PLA₂, fiber length may be another modulating factor. Because such an effect was not consistently noted, initial RBC-G bath fiber length was perhaps an uncontrolled variable.

Actin and myosin are major stress fiber constituents. Myosin had no independent inhibitory effects (data not shown), however, it reduced the F-actin stimulation of PLA₂ (Fig. 7) which suggests that stress fiber formation may also affect enzyme activity. Tropomyosin and α -actinin (10 μ M) stress fiber proteins did not alter the PLA₂ stimulatory effect of F-actin (data not shown). Thus, if myofibril protein complexes (stress fibers) alter PLA₂ activity, it is perhaps due to the presence of myosin.

The mechanism by which cytoskeletal proteins altered PLA₂ activity was not known. However, the model for glycolytic enzyme modulation by cytoskeletal constituents may be germane (16). In this model, enzyme binding to cytoskeletal structures alters the catalytic site to change metabolic rate. If such a model is applicable to our findings, then the PLA₂/G-actin interaction may have modified the catalytic site to reduce enzyme activity. However, F-actin must have further changed enzyme configuration to permit more efficient membrane phospholipid hydrolysis. If PLA₂ inhibition by G-actin were merely due to the effect of a single actin molecule obstructing enzyme access to its substrate, then further inhibition would be expected when the enzyme was associated with actin polymers. Since this was not the case (Figs. 4 and 5), the actin effect on PLA₂ was perhaps the result of enzyme catalytic site modulation.

A reduction in the F-actin stimulation of PLA₂ by myosin may be the result of any of the aforementioned mechanisms. Myosin binding to actin could further modulate enzyme configuration, directly obstruct enzyme active site, or affect fiber length such that substrate and enzyme could not easily associate. In addition, myosin binding might result in a dissociation of PLA₂ from F-actin.

Such mechanisms may be relevant to various cell types, for all actin isoforms altered PLA₂ activity (Fig. 5). Its potential relevance to endothelial cells was further enhanced by the use of actin (100 μM) and myosin (1 μM) concentrations reported for nonmuscle cells (18). Furthermore, a nonmuscle cell ratio of actin (skeletal muscle or nonmuscle cell) to myosin (skeletal or smooth muscle cell) of a 100:1 effectively suppressed the PLA₂ stimulation by F-actin (Fig. 7). Because several endothelial cell PLA₂ types might function to initiate the eicosanoid cascade (14), the finding that diverse PLA₂ sources (bee venom and porcine pancreas) were similarly affected by actin was important, for it suggested that this may be a common PLA₂ attribute. Also, eicosanoid enzymes are regarded to be membrane bound, however, PLA₂ may be bound or soluble in the cytosol (1). Moreover, an enzyme's existence in a particulate or soluble phase is a variable dependent on the cell metabolic status (11,15,22,28). Finally, enzyme-membrane associations do not necessarily exclude these potential actin-myosin modulatory effects. Therefore, these PLA₂ findings may be pertinent to endothelial cell PLA₂ activity and explain the correlation of PGI₂ synthesis to cell shape and actin distribution.

A diffuse F-actin distribution in small, rounded, substrate-attached endothelial cells (Fig. 1a) supports greater PGI₂ production (4), because PLA₂ may be maximally stimulated by F-actin free of other myofibril proteins (Fig. 5a,c,e). In large, flat, substrate-attached cells (Fig. 1b), actin and myosin complexed in stress fibers might reduce enzyme activity (Fig. 7) and PGI₂ production (4). Because of the correlation

between stress fiber formation and cell adhesion (4,27; Fig. 1). in the total absence of adhesion, G-actin may become dominant within suspended cells to suppress PLA₂ activity (Figs. 4b and 5b,d,f) and PGI₂ synthesis (10,13). Furthermore, suspended endothelial cells are stimulated to undergo significant calcium influx (20). However, suspended cells do not generate PGI₂ (10,13) and endothelial cell PLA₂ may be calcium independent (14). Calcium does not appear to offer an adequate explanation for PLA₂ regulation and calcium PLA₂ stimulatory actions (25) could be the result of calcium effects on endothelial cell cytoskeletal structure. Therefore, the correlation of PGI₂ production with endothelial cell shape and actin distribution, coupled with this PLA₂ study supports a cytoskeletal-mediated mechanism for eicosanoid metabolic regulation.

Since PGI₂ markedly promotes endothelial cell stress fiber formation (24), a cytoskeletal-mediated mechanism was further supported. As illustrated (Fig. 8), factors which cause an actin-myosin dissociation might support PLA₂ activation. The resulting PGI₂ would mediate the return of actin-myosin complexes. Such complexes might reduce PLA₂ activity to suppress further PGI₂ synthesis. In conclusion, because PGI₂ augments stress fiber formation and the cytoskeletal proteins actin and myosin could potentially modulate PLA₂ activity, PGI₂ in concert with the cytoskeleton may act in a signalling loop in the control of endothelial cell eicosanoid metabolism.

FIGURE LEGENDS

Figure 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, OR). 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 (Kodac; 400 ASA). BAE cells on a substrate of reduced adhesive capacity (bacteriological plastic; Fig 1a) were small and rounded with a diffuse F-actin distribution which appeared to lack stress fibers. Such cells on an adhesive substrate (tissue culture plastic; Fig. 1b) were large and flat with many discrete actin stress fibers.

Figure 2. Phospholipase A₂ (PLA₂) was incubated (30 min; 5° C) in the presence or absence of myofibril protein prior to exposure to the red blood cell (RBC) ghost bath. Enzyme activity was followed by measurement of sample supernatant absorbance at 418 nm.

Figure 3. Illustration of phospholipase A₂ (PLA₂) activity curves replicated under identical experimental conditions. Similar PLA₂ activity curves were obtained using PLA₂ derived from bee venom or porcine pancreas

Figure 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 (PLA₂-B) activity.

Figure 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 (PLA₂-P) activity.

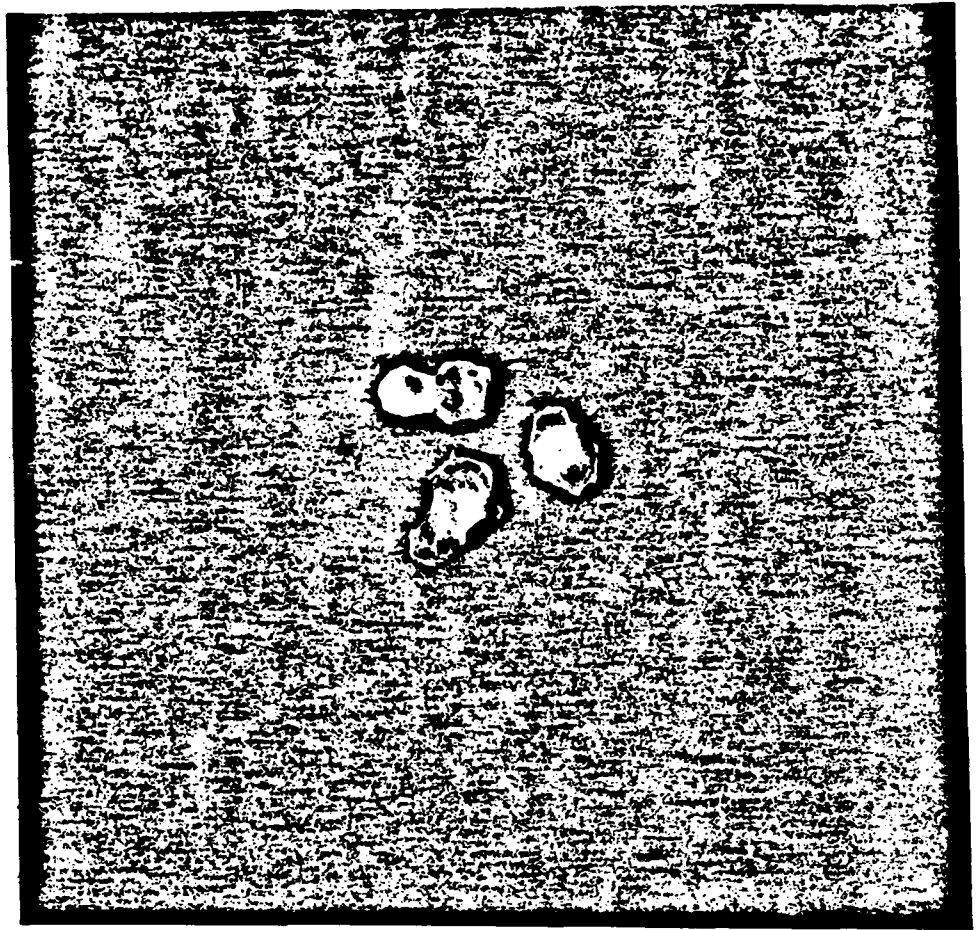
Figure 6. Effects of prior incubation with albumin (A: 100 μ M), phalloidin (B: 100 μ M), or chicken skeletal muscle (sk) F-actin plus phalloidin (C: 100 μ M) on porcine pancreatic phospholipase A₂ (PLA₂-P) activity.

Figure 7. 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 (PLA₂-P) activity. Similar PLA₂ activity curves 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.

Figure 8. Illustration of a potential cytoskeletal-prostaglandin (PGI₂) signalling loop which perhaps functions as an eicosanoid metabolic regulatory mechanism. Factors which cause a dissociation of F-actin from other myofibril proteins (i.e., myosin) may maximally stimulate the rate limiting enzyme of the eicosanoid cascade, phospholipase A₂ (PLA₂). Such stimulation would induce enhanced arachidonic acid release from cell membranes and elevate PGI₂ synthesis. The binding of prostacyclin to its putative membrane receptor would initiate the formation of complexes of F-actin, myosin, and other myofibril proteins (stress fibers). The association of PLA₂ with such complexes might suppress PLA₂ activity and reduce PGI₂ metabolism.

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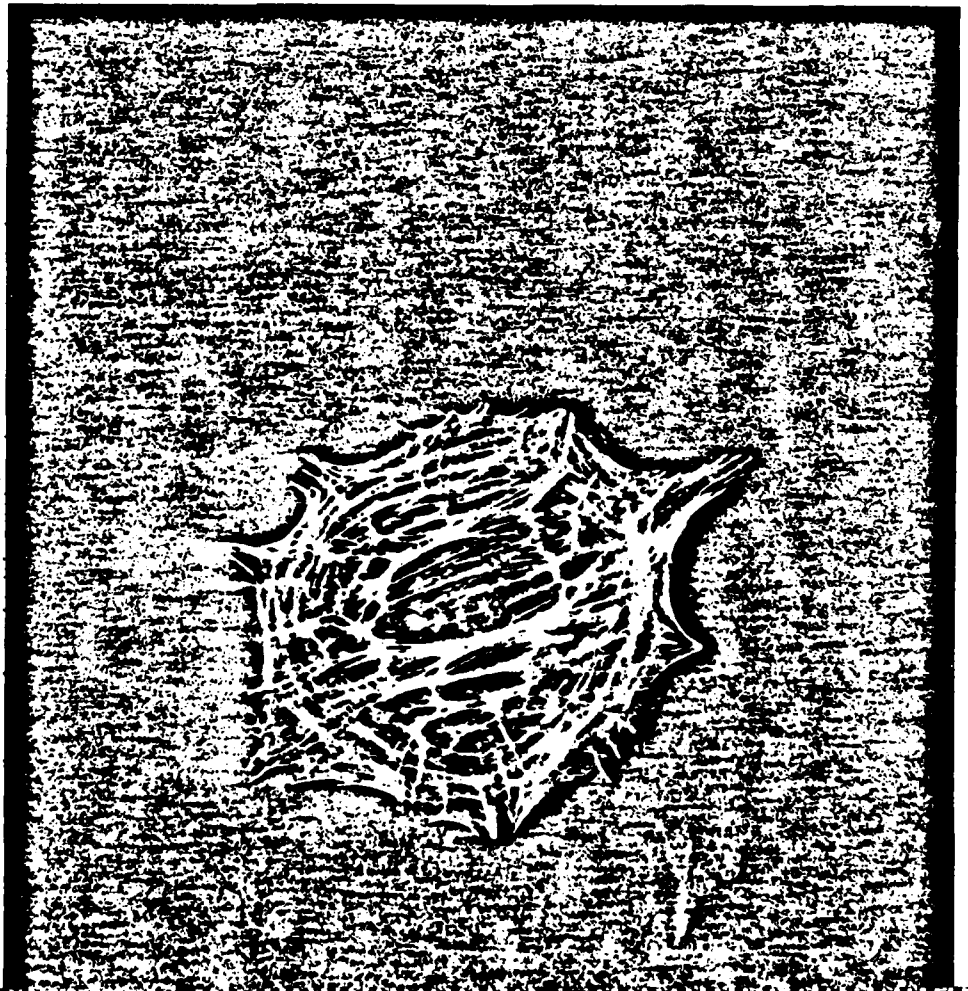


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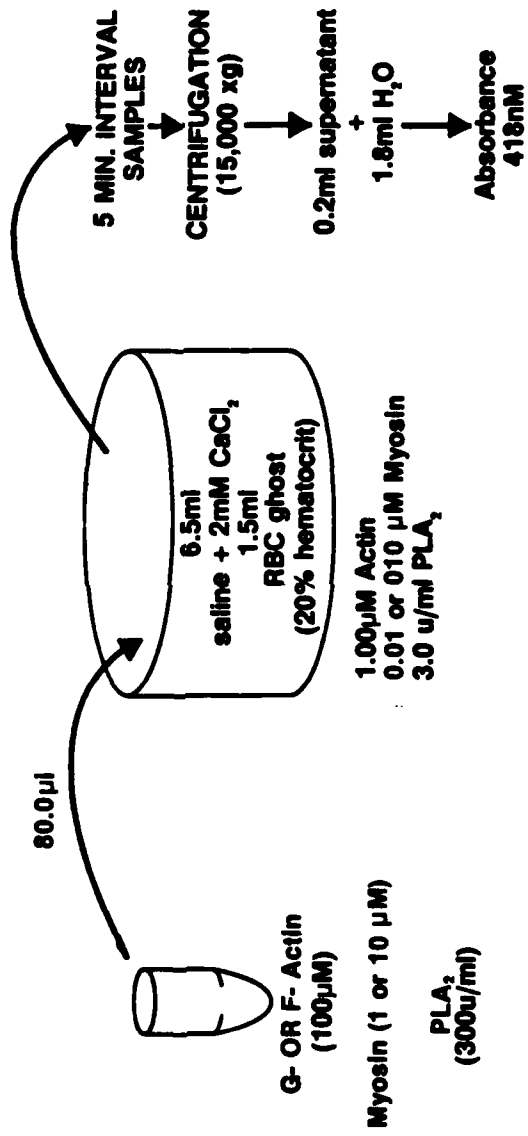


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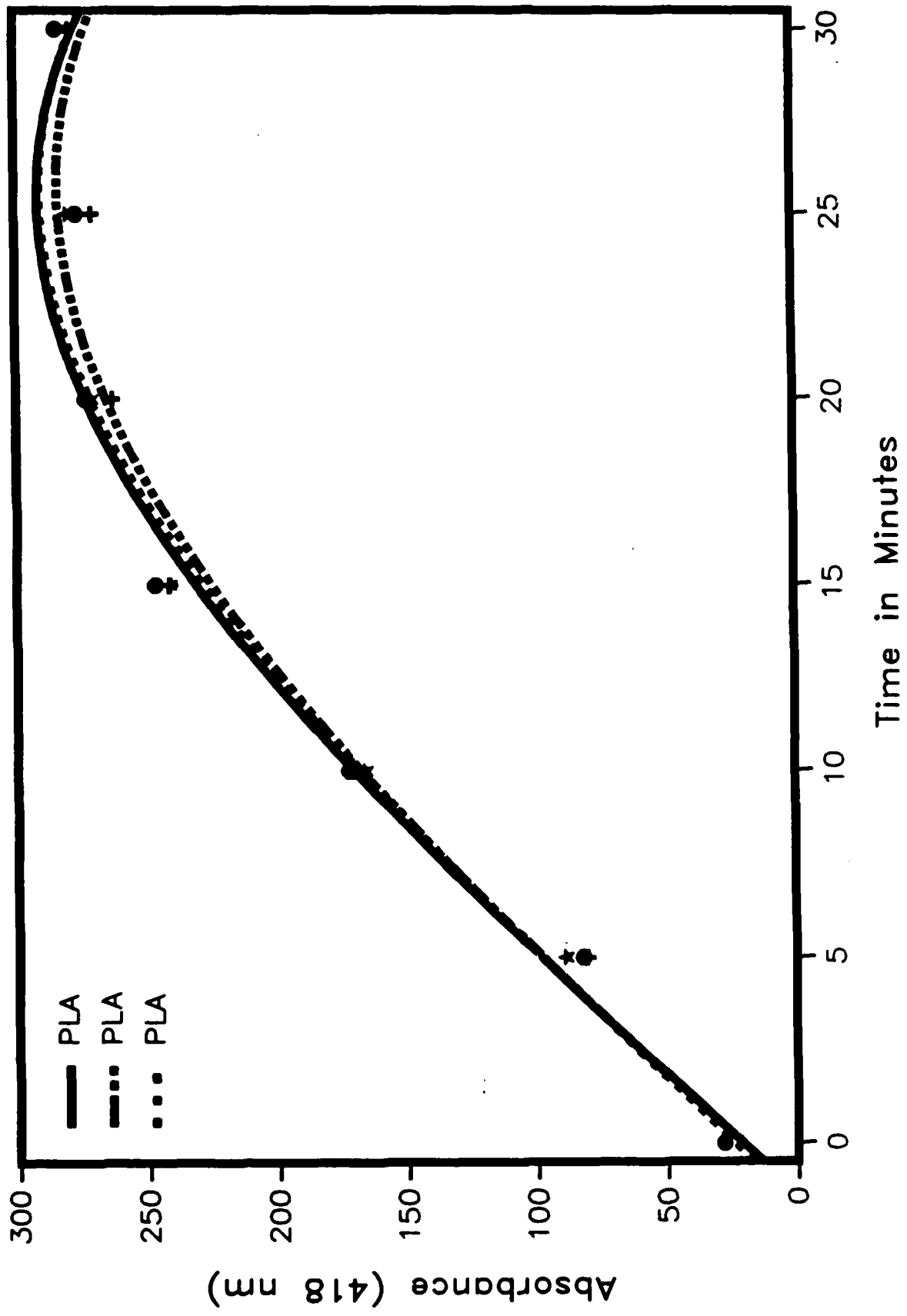


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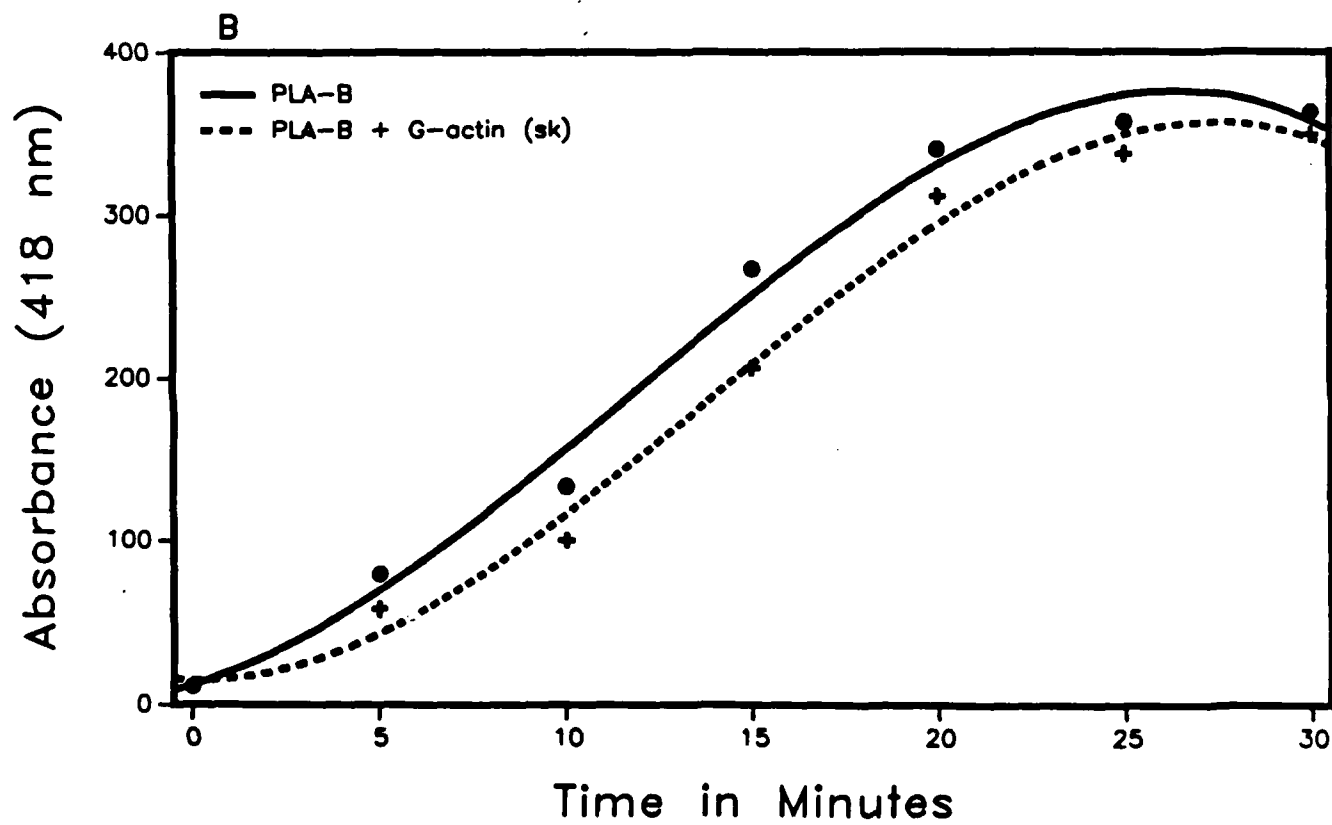
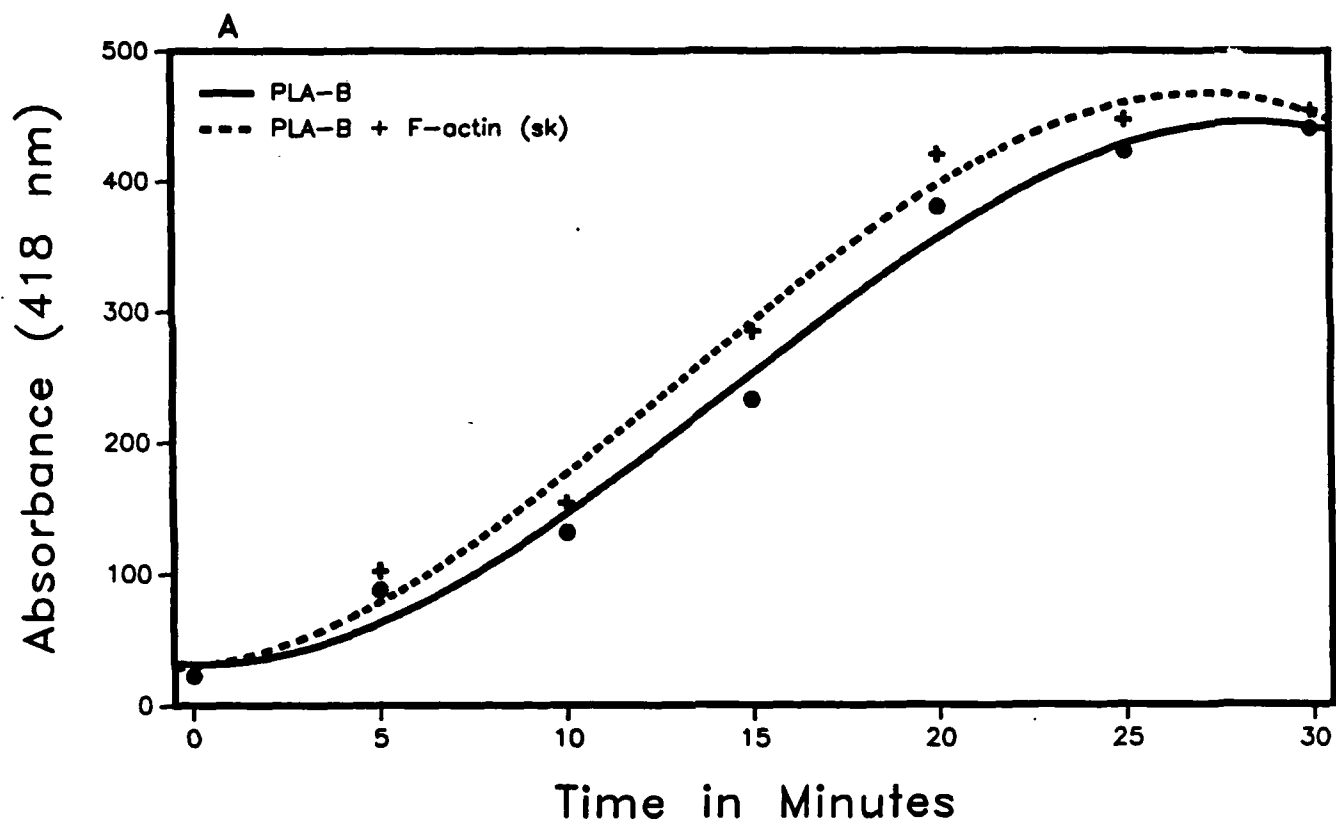


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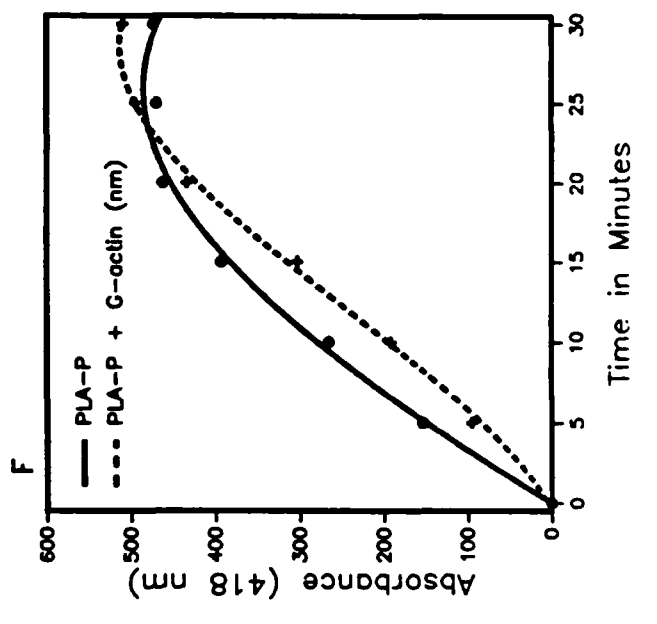
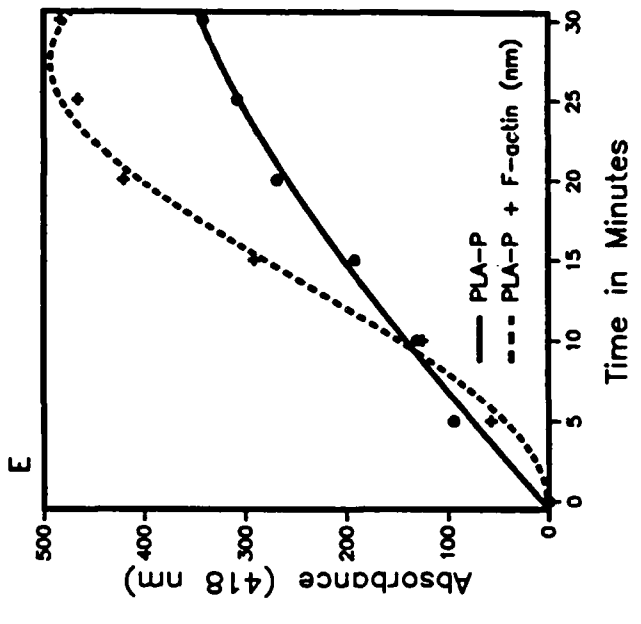
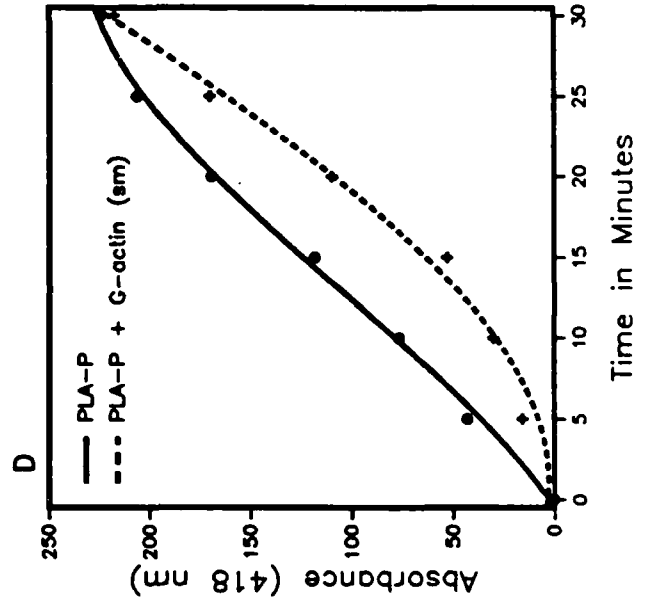
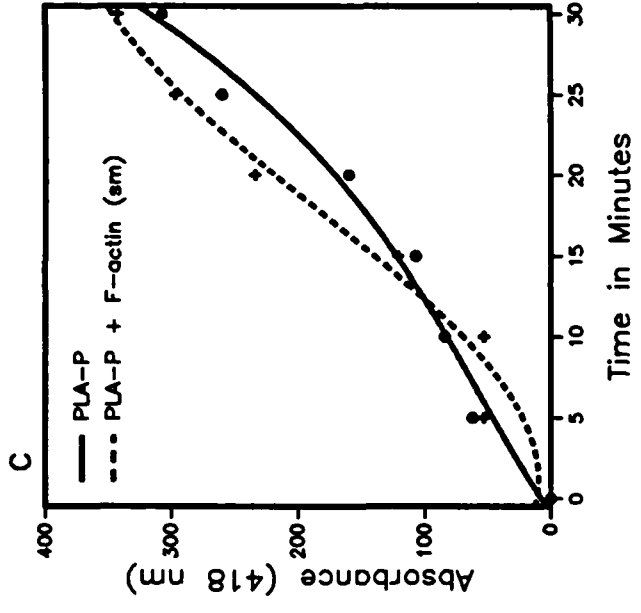
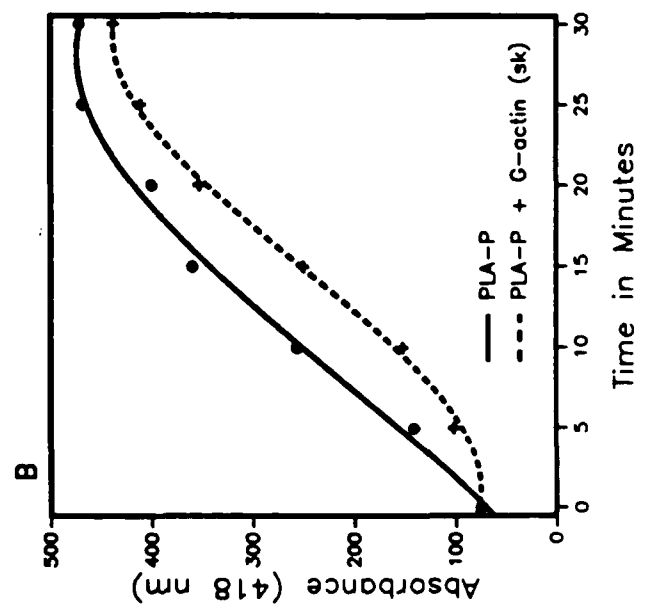
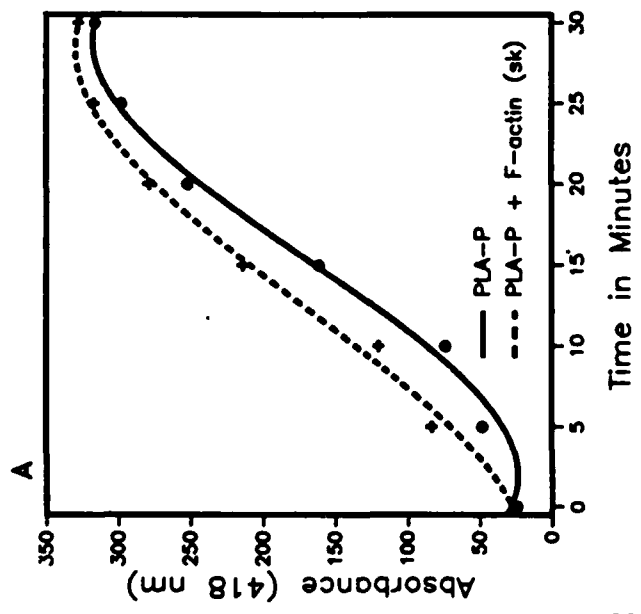


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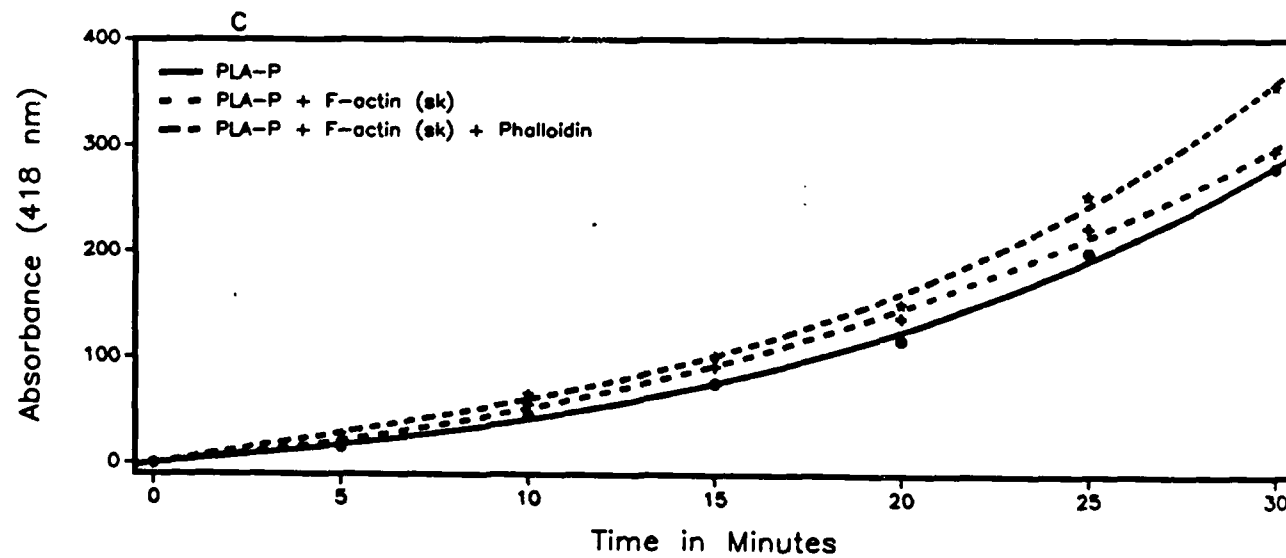
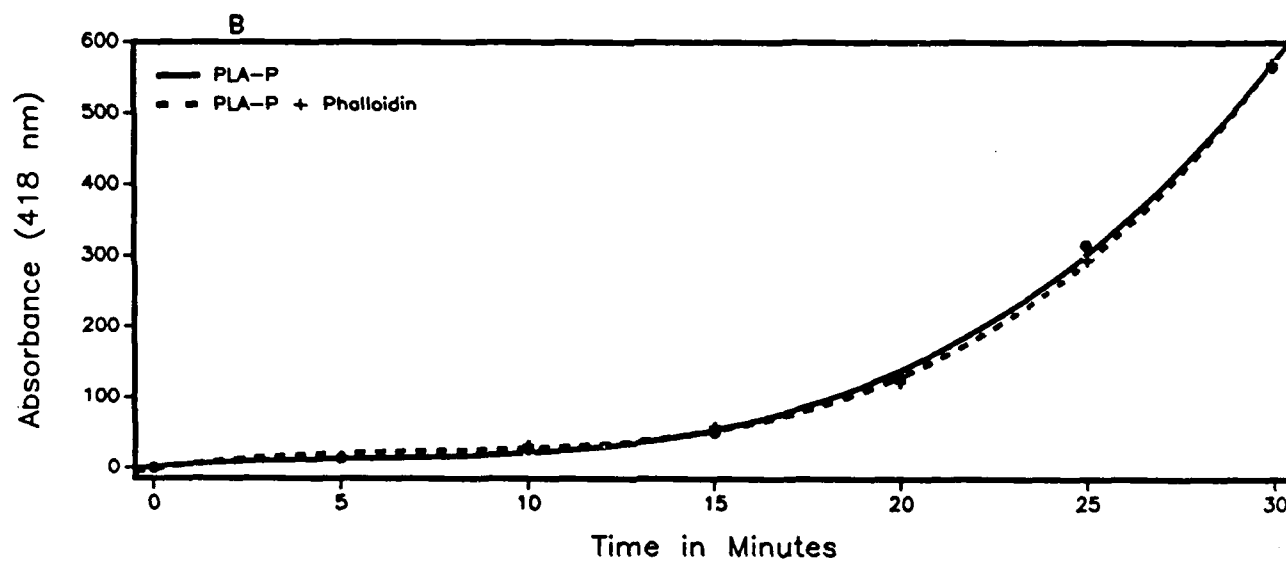
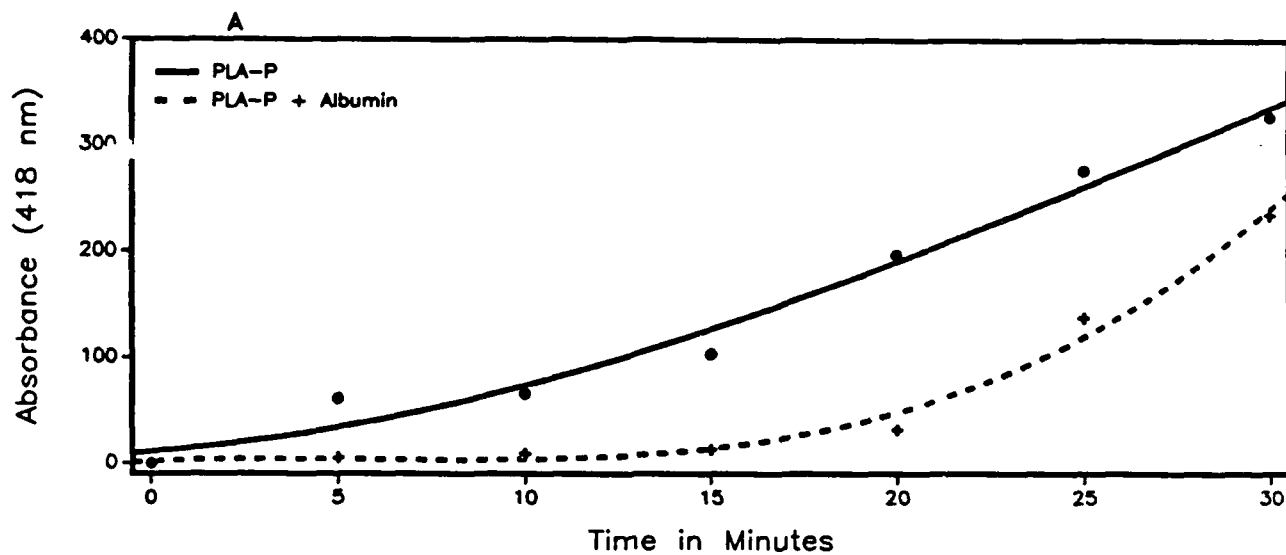


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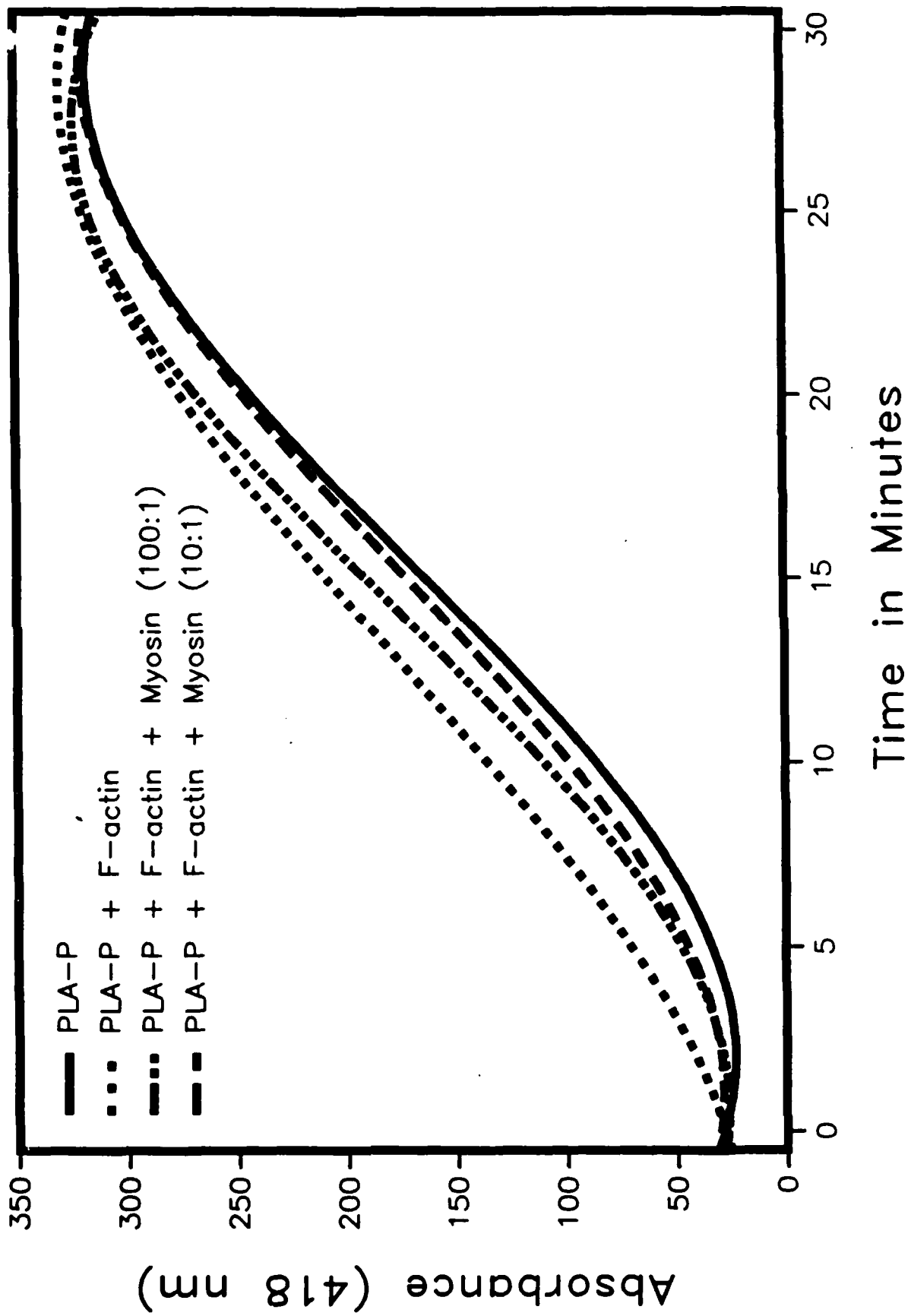
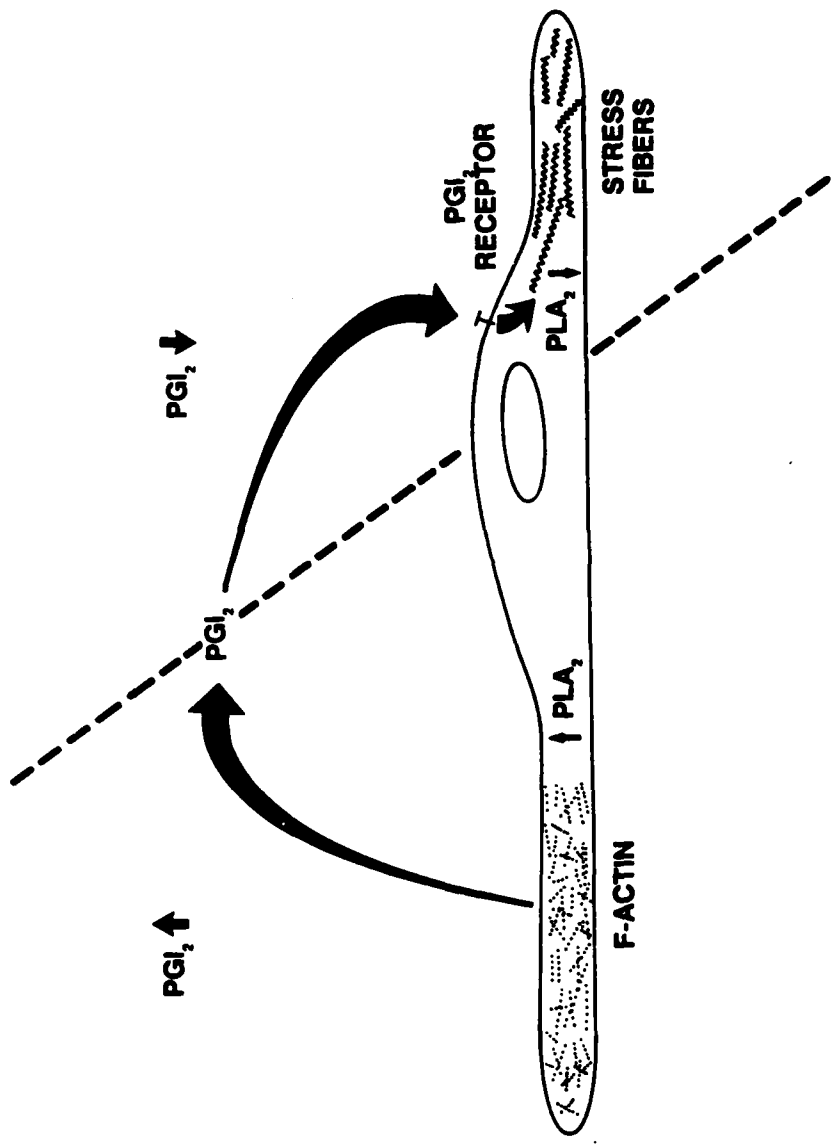


Figure 8. Illustration of a potential cytoskeletal-prostacyclin (PGI_2) signalling loop which perhaps functions as an eicosanoid metabolic regulatory mechanism. Factors which cause a dissociation of F-actin from other myofibril proteins (i.e., myosin) may maximally stimulate the rate limiting enzyme of the eicosanoid cascade, phospholipase A_2 (PLA_2). Such stimulation would induce enhanced arachidonic acid release from cell membranes and elevate PGI_2 synthesis. The binding of prostacyclin to its putative membrane receptor would initiate the formation of complexes of F-actin, myosin, and other myofibril proteins (stress fibers). The association of PLA_2 with such complexes might suppress PLA_2 activity and reduce PGI_2 metabolism.



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1. Abbreviations used: PLA_2 , phospholipase A_2 ; PLA_2 -B, phospholipase A_2 derived from bee venom; PLA_2 -P, phospholipase A_2 derived from porcine pancreas; PGI_2 , prostacyclin, RBC-G, red blood cell ghosts.

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

DISCLAIMER

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REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED		1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY		3. DISTRIBUTION / AVAILABILITY OF REPORT UNLIMITED	
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE			
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
6a. NAME OF PERFORMING ORGANIZATION U.S. Army Research Institute of Environmental Medicine	6b. OFFICE SYMBOL (if applicable)	7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) Natick, MA 01760		7b. ADDRESS (City, State, and ZIP Code)	
8a. NAME OF FUNDING / SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (if applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code)		10. SOURCE OF FUNDING NUMBERS	
		PROGRAM ELEMENT NO. 3M161102BS	PROJECT NO. 10 Bs10C
		TASK NO. 611102S1	WORK UNIT ACCESSION NO. CA WU-01
11. TITLE (Include Security Classification) Modulation of Phospholipase A ₂ Activity by Actin and Myosin			
12. PERSONAL AUTHOR(S) DuBose, D.A., Shepro, D. (Boston University), and Hechtman, H.B. (Harvard Uni.			
13a. TYPE OF REPORT Final	13b. TIME COVERED FROM 4/1/86 TO 4/1/87	14. DATE OF REPORT (Year, Month, Day) 87-04-15	15. PAGE COUNT 35
16. SUPPLEMENTARY NOTATION (PHOSPHOLIPASE A ₂ ...)			
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) Prostacyclin, endothelial cell, cytoskeleton, metabolism regulation, enzyme allosteric modulation
FIELD	GROUP	SUB-GROUP	
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Endothelial cell prostacyclin (PGI ₂) production is closely coupled with cell shape. Since shape change implies cytoskeletal modulations, this finding may be an important clue to implicate cytoskeletal constituents in a mechanism regulating eicosanoid metabolism. Endothelial cells with a diffuse F-actin distribution generate more PGI than cells with many discrete F-actin stress fibers. Because actin can modulate enzyme activity, the myofibril protein effects on phospholipase A (PLA), the rate limiting eicosanoid cascade enzyme, were studied. F-actin stimulated whereas G-actin suppressed enzyme activity. Myosin in the presence of F-actin, the F-actin stimulatory effect was significantly reduced. These findings suggest that the correlation of endothelial cell PGI metabolic modulation to cell shape and actin distribution was perhaps due to changes in PLA activity as a direct result of alterations in the polymerized state of actin and the degree of actin-myosin stress fiber formation.			
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED	
22a. NAME OF RESPONSIBLE INDIVIDUAL David A. DuBose		22b. TELEPHONE (Include Area Code) 617-651-4264	22c. OFFICE SYMBOL SGRD-UE-CR

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6-87

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