Biochemical and Biophysical Research Communications **280,** 259–264 (2001)
doi:10.1006/bbrc.2000.4083, available online at http://www.idealibrary.com on IDEAL®

Selective Secretion of Protein Kinase C Isozymes by Thrombin-Stimulated Human Platelets

Travis Jon Hillen, Annayya R. Aroor, and Shivendra D. Shukla[1]
Department of Pharmacology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

Received November 16, 2000

The protein kinase C (PKC) was secreted from thrombin-stimulated human platelets in a time- and dose-dependent manner. The PKC specific inhibitors Ro31-8220 (0.05 μM) and GF 109203X (0.5 μM) totally inhibited the secreted kinase activity. Western blot analysis of the secretory components showed reactivity to PKCα, PKCβII, and PKCδ antibodies, but not to PKCβI, and p42/44 MAPK, although they were present in lysed platelets. The fractionation of platelets secreted components showed that PKC activity increased in both soluble and microparticle fractions after thrombin treatments. This is the first report demonstrating that activated human platelets selectively secrete protein kinase C isozymes. Protein kinase C secreted by platelets in this unique manner may have an extracellular role in the plasma, and may regulate cellular functions, including remodeling of vascular endothelial cells.

Key Words: **protein kinase C; platelets; platelet microparticles; secretion; protein kinase C isoenzymes; thrombin.**

Upon vascular wall injury, platelets become activated resulting in shape change, aggregation, and secretion (1–3). In addition to their role in thrombosis, platelets are also involved in atherosclerosis (4). The interactions among platelets, leukocytes and endothelium play pivotal roles in vascular homeostasis and competent immune-inflammatory response *in vivo* and *in vitro* (5–7). The stimulation of platelet aggregation results in the generation of membrane derived microparticles and secretion of a multitude of inflammatory and vasoactive substances including mediators (5-HT), nucleotides (ADP, ATP), ions (Ca^{2+}, Mg^{2+}), proteins (von Willebrand's factor), and growth factors (e.g., PDGF). These platelet-derived molecules or microparticles have been shown to contribute to complementary signaling in leukocytes and endothelial cells to regulate leukocyte and endothelial cell gene expression during vascular inflammatory responses (8–12).

Recent studies have shown secretion of protein kinase A (PKA) and casein kinase from activated platelets. PKA secreted by human platelets can act as an exo-protein kinase and phosphorylates vitronectin, a protein secreted by activated platelets (13). Casein kinase has been shown to phosphorylate the plasma protein C3. The protein kinase C isozymes are one class of intracellular signaling mediators involved in multiple functions in cells including platelets (14–16). In platelets, several PKC isozymes have been identified including PKCs α, β, δ, ζ, η and θ (17), but their secretion from activated platelets is not known. We have investigated the secretion of protein kinase C from thrombin activated human platelets.

MATERIALS AND METHODS

Materials. Human platelet concentrate was obtained from the regional American Red Cross facility in Columbia, Missouri. Protein kinase A inhibitor was purchased from Sigma Chemical Co. Myelin basic protein (MBP) was purchased from GIBCO-BRL. Protein kinase C antibodies were purchased from Santa Cruz. FITC-conjugated Annexin-V was purchased from PharMingen. Ro-31-8220 and GF 109203X were purchased from BioMol. [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) was bought from DuPont-NEN. Nitrocellulose and goat-anti [rabbit IgG] secondary antibody were purchased from BioRad.

Isolation of washed platelets. Human platelet concentrate was centrifuged at 250*g* for 8 min at 25°C to remove red blood cell contamination. The platelet rich plasma (PRP) was collected and the red blood cells discarded. The PRP was centrifuged at 750*g* and platelets were washed with Hepes platelet buffer (pH 7.1) containing the following: (5 mM Hepes, 148 mM NaCl, 2.5 mM KCl, 1 mM KH_2PO_4, 5 mM glucose, and 0.1 mM EGTA). This step was repeated and the washed platelets were then counted using a Coulter counter and brought to a final concentration of 2.0×10^9/ml for treatment with thrombin. These platelets were viable as routinely monitored by their aggregation and [^{3}H] serotonin secretion responses to thrombin.

Abbreviations used: MAPK, mitogen-activated protein kinase; PKC, protein kinase C; LDH, lactate dehydrogenase; PDGF, platelet-derived growth factor; PKA, protein kinase A; MBP, myelin basic protein; PRP, platelet rich plasma; HPB, Hepes platelet buffer; FITC, fluorescein isothiocyanate conjugated; DTT, dithiothreitol; TBST, Tris-buffered saline-Tween 20.

[1] To whom correspondence should be addressed. Fax: (573) 884-4558. E-mail: shuklasd@missouri.edu.

0006-291X/01 $35.00

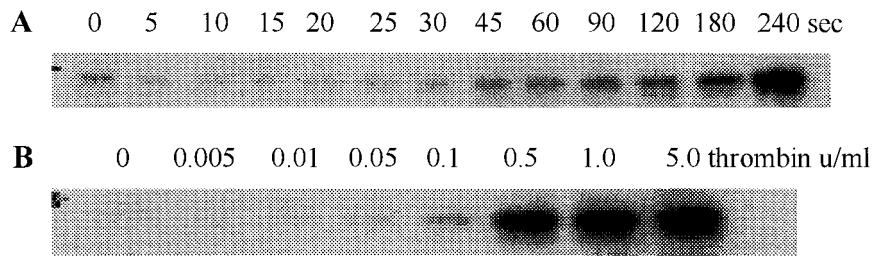

FIG. 1. Protein kinases present in the secretory component. Washed platelets (2×10^9 cells/ml) were stimulated for differing time periods with 1 unit/ml thrombin (A) or with differing concentrations of thrombin (0.005–5 units/ml) for 4 min (B). After stimulation, platelets were centrifuged and 10 μl of resulting supernatant was used for an in-gel protein kinase assay using MBP and [γ-^{32}P] ATP (see Materials and Methods). Data are from one of three similar experiments.

Platelet activation and collection of secretory products. Washed platelets were treated with thrombin (0.01–5 units/ml) for varying lengths of time (5 s–30 min). After treatments, platelets were centrifuged at 750*g* for 15 min. The resulting secretory component was collected and microparticles were isolated as described previously (18). Briefly, supernatant was centrifuged at 13,000*g* for 45 min at 4°C and the resulting platelet secretory and microparticle components were collected and placed immediately on ice. Both fractions were used for flow cytometry (Becton-Dickinson FACS Vantage TurboSort) to characterize distribution of the microparticles by using 2 μm beads. The flow cytometer was set to gate 5,000 events in the 2 μm range as described previously (19). The ratio of events in the microparticle region versus the bead region were used to calculate the concentration of microparticles.

Lactate dehydrogenase (LDH) assay. In a disposable cuvette, 1500 μl of phosphate buffer (50 mM phosphate, pH 7.5, and 0.63 mM pyruvate) and 50 μl of secretory component were added. To start the reaction, 25 μl of 11.3 mM β-NADH buffer was added. Spectrophotometer readings at wavelength 340 nm were taken every 30 s for 3 min. LDH activity was expressed as units per liter (U/L).

Assay for protein kinases. The filter-binding assay was performed as described previously (20). Ro31-8220 (0.05 μM) and GF 109203X (0.5 μM) were used as inhibitors of protein kinase C in corresponding reaction mixtures and their concentrations were selected based on reported values. The in-gel protein kinase assay was performed by SDS electrophoresis in a 10% polyacrylamide resolving gel containing 5 mg/ml MBP as described earlier (21).

SDS–PAGE and immunoblotting. The proteins contained in 10 μl of secretory component in Laemmli buffer were separated by SDS electrophoresis in a 10% polyacrylamide resolving gel. Proteins were electrophoretically transferred to nitrocelluluse membrane and probed with specific PKC antibodies or p42/p44 MAPK antibody. Autoradiograms were obtained with enhanced chemiluminescense (ECL) using Pierce Super Signal Reagent.

RESULTS

Protein Kinase C Activity in Secretions Derived from Thrombin Activated Platelets

Thrombin (0.01–5 units/ml) treatment for 5 s to 5 min was used to induce platelet aggregation and secretion. At 1 unit/ml thrombin, maximal [^{3} H] serotonin secretion was observed within 30 s, whereas aggregation was complete by 60 s (results not shown). The secreted component was utilized for protein kinase C assay. First, to decipher what kinases are being secreted by platelets, an in-gel MBP kinase assay of the secretory component was performed. Upon autoradiography, the in-gel MBP kinase assay showed sharp bands at around 80 kDa intensifying in both a time- (Fig. 1A) and thrombin dose-dependent manner (Fig. 1B). PKC isozymes have similar molecular weight. The phosphorylation of MBP did not appreciably increase until 30 s after stimulation, but increased steadily thereafter (Fig. 1A). The phosporylation of MBP was detected at as low as 0.05 and 0.1 units/ml of thrombin but increased appreciably at thrombin concentrations of 0.5 units/ml or higher (Fig. 1B). An aliquot of the secretory component was used for lactate dehydrogenese (LDH) assay. It was evident that the above treatments did not result in platelet lysis as shown by the lack of LDH release (Fig. 2).

Treatment of the secretory component with the kinase reaction mixture containing MBP and [^{32} P] ATP resulted in a time-dependent phosphorylation of MBP (Fig. 3). We also employed a PKC specific substrate peptide (acetylated MBP 4–14 aa) derived from the myelin basic protein sequence (GIBCO-BRL, Grand Island, NY) in these assays and similar results were obtained. Treatment of the secretory component with

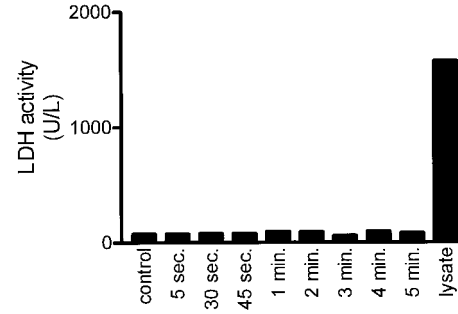

FIG. 2. Determination of lactate dehydrogenase activity in platelet secretory fraction. Washed platelets (2×10^9cells/ml) were stimulated with thrombin (1 unit/ml) for different times (0–5 min), centrifuged, and 50 μl of secretory product was used for lactate dehydrogenase (LDH) assay (see Materials and Methods). A similar aliquot of platelet lysate was also assayed for total LDH activity. The figure is representative of five separate experiments.

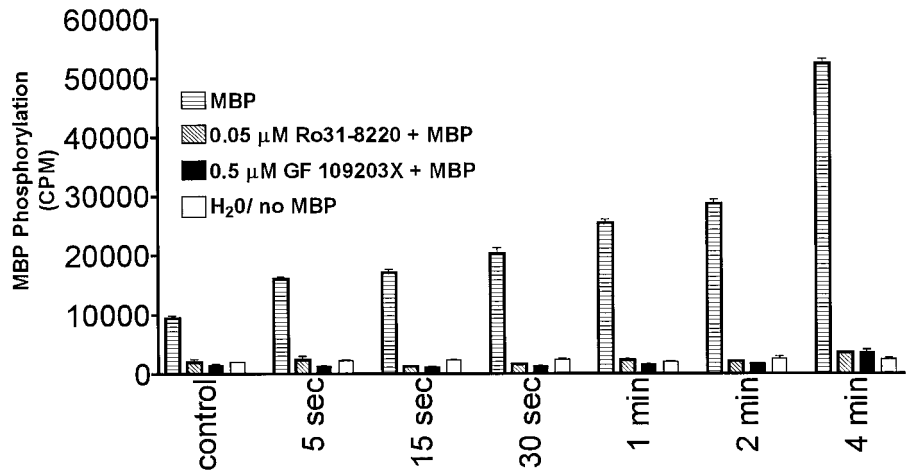

FIG. 3. Detection of protein kinase activity in secretory components. Washed platelets (2×10^9 cells/ml) were stimulated with thrombin (1 unit/ml) for varying lengths of time, centrifuged, and 12.5 μl of secretory component was used for kinase activity using a MBP filter-binding assay. The inhibitors Ro31-8220 (0.05 μM) and GF 109203X (0.5 μM) were added to the reaction mixture as indicated under Materials and Methods. The results are mean ± SE of three experiments.

the PKC specific inhibitors Ro31-8220 (0.05 μM) or GF 109203X (0.5 μM) resulted in near complete inhibition of the kinase (Fig. 3). Taken together, these results suggest that PKC is being secreted by human platelets upon thrombin stimulation.

Characterization of PKC Isozymes Secreted by Thrombin-Stimulated Human Platelets

Equal amounts of secretory component from thrombin-stimulated platelet samples were electropho-

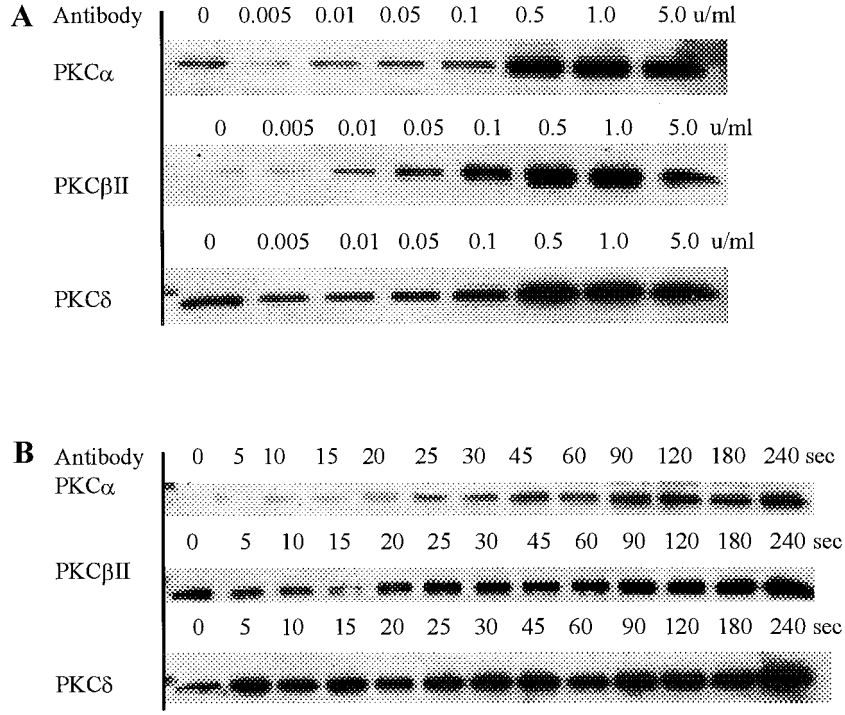

FIG. 4. Western blot analysis of the dose-dependent (A) and time-dependent (B) secretion of PKC isoenzymes. Washed platelets (2×10^9 cells/ml) were stimulated with thrombin (0.005–5 units/ml) for 4 min (A) or were stimulated with 1 units/ml thrombin for varying times (0–4 min) (B). Following stimulation, secretory components were obtained (see Materials and Methods) and their equal volumes were subjected to 10% SDS–PAGE and analyzed using antibodies raised against PKCα, PKCβII, and PKCδ. The figure is representative of three separate experiments.

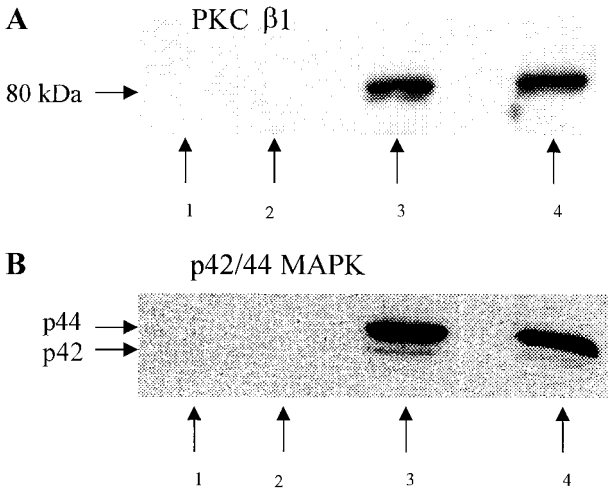

FIG. 5. Western blot analysis of thrombin (1 unit/ml)-induced secretion of PKCβ1 (A) and p42/44 MAPK (B). Washed platelets (2×10^9 cells/ml) were stimulated with thrombin (1 unit/ml) for 5 min. Equal volumes of the secretory components were subjected to 10% SDS–PAGE and analyzed using antibodies raised against PKCβI and p42/44 MAPK; secretory component following 5 min stimulation (lanes 1 and 2) and lysed platelets used for positive control (lanes 3 and 4). The figure is representative of three separate experiments.

resed and probed with PKC specific primary antibodies. Three PKC isozymes, PKC α, PKC βII, and PKC δ were detected. These isozymes were secreted in both a thrombin dose- (Fig. 4A) and time-dependent (Fig. 4B) manner. The secretion of PKC did not change until thrombin concentrations of 0.5 unit/ml and higher were used (Fig. 4A). Further, PKC secretion did not appreciably increase until 30 s but increased steadily thereafter (Fig. 4B). PKCβI, another isozyme, was present in lysed platelets but not in the secretory component (Fig. 5A). MAPK (p42/p44) was present in the platelet lysate, but was not found in the secretory component isolated from the activated platelets (Fig. 5B).

Protein Kinase C Activity in Microparticles

The platelet secreted fraction was separated into soluble and microparticle fractions. The flow cytometry data showed that the concentration of microparticles in the supernatant fraction was negligible (data not shown). The distribution of PKC activity within these components was determined. Significant activity of protein kinase C was found in the stimulated platelet soluble fraction, which was 4.5-fold higher than the control supernatant (Fig. 6A). However, protein kinase C activity was also present in the microparticle fraction. Microparticles derived from activated platelets showed threefold higher PKC activity compared to the control microparticles (Fig. 6A). The microparticles were lysed in 1% Triton X-100, sonicated, and PKC activity determined in the Triton treated portion versus the native microparticle fraction (Fig. 6B). The Triton X-100 treated microparticles had a sixfold increase in PKC activity as compared to the native microparticle fraction. This concentration of Triton X-100 does not directly affect PKC enzyme activity. The re-

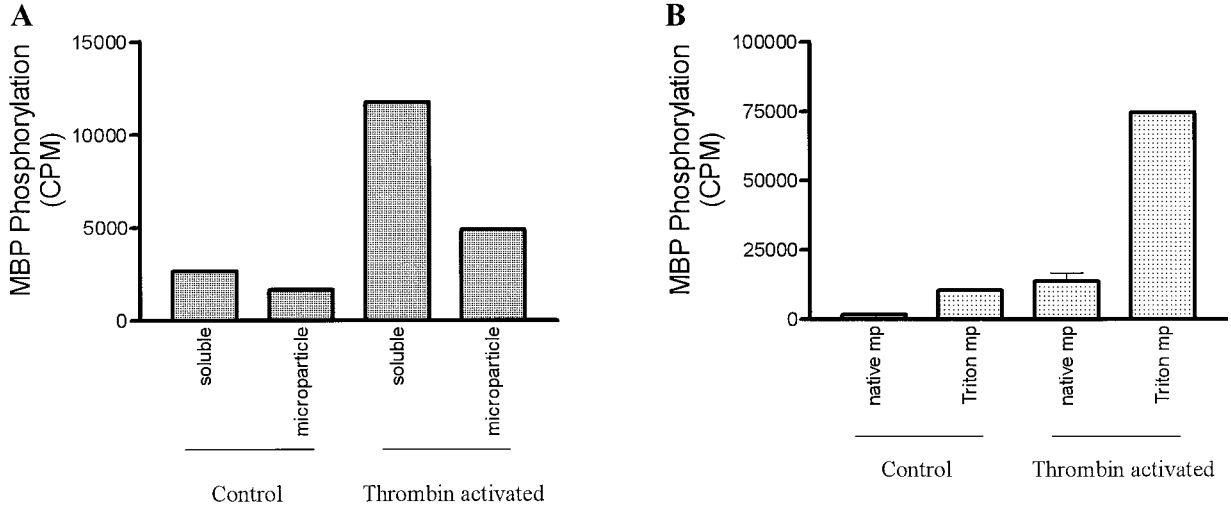

FIG. 6. The distribution of protein kinase C activity within the soluble supernatant and microparticle fractions. (A) Thrombin (1 unit/ml) activated human platelets were centrifuged at 750*g* for 15 min at 4°C. Supernatant was collected and subsequently centrifuged at 13,000*g* for 45 min at 4°C. Soluble and microparticle fractions were isolated and their protein kinase activity determined using filter-binding assay (see Materials and Methods). Microparticle fraction was normalized to the number of microparticles present in both the control and thrombin-activated fractions. The figure is representative of three separate experiments. (B) The microparticle fractions of both control and thrombin-activated platelets were subjected to treatment with 1% Triton X-100. Protein kinase C activity was determined in native microparticle fractions and Triton solublized fractions by the filter-binding assay (see Materials and Methods). The figure is representative of one of four separate experiments.

sults suggest that PKC is present both inside and at the surface of microparticles.

DISCUSSION

This is the first report of protein kinase C secretion by thrombin-activated human platelets in a time- and dose-dependent manner. The kinase activity of the enzymes secreted by the platelets was inhibited by the PKC specific inhibitors Ro31-8220 (0.05 μM) and GF 109203X (0.5 μM) suggesting the secreted kinase is protein kinase C. It was further established that PKC isozymes α, βII, and δ were secreted in both time- and dose-dependent manner. PKC βI was not secreted even though it was present in the platelet lysate. These results demonstrated selectivity of protein kinase C secretion. Although MAPK (p42/44, kDa) is abundantly present in platelets, it was also not secreted. The lack of secretion of PKCβ_1 or MAPK or of LDH release also ruled out lysis in these experiments. The time dependent secretion of PKC has an interesting characteristic in that it was detectable 20 to 30 s after thrombin activation of platelets and increased thereafter. In contrast, serotonin secretion was maximal by 30 s. This delayed secretion of PKC may have important bearing upon its physiological role at the site of platelet plug and the injured endothelium. It is also noteworthy that human platelets are activated by PKC antibody and a role for surface phosphorylation by PKC in hemostasis has been proposed (22). The secretion of protein kinase C isozymes is an exciting development considering the recent discovery of exo- and ecto-protein kinase activities and their role in cellular regulation (13, 23, 24). Potential functions of secreted protein kinase C in phosphorylations of plasma components, e.g., fibrinogen and vitronectin (25–27) or cell surface proteins of blood cells and endothelial cells can be envisaged. Platelet secreted PKC may have a pivotal role in phosphorylating proteins critical to immune, inflammatory and remodelling responses.

ACKNOWLEDGMENTS

The authors are thankful to Mr. Yu-I Weng for experimental help and to Ms. Pam Burgess for word processing this manuscript. Travis Hillen was supported by a University of Missouri Columbia Molecular Biology Program Fellowship.

REFERENCES

1. George, J. N. (2000) Platelets. *Lancet* **355,** 1531–1539.
2. Lèvy-Toledano, S. (1999) Platelet signal transduction pathways: could we organize them into a "hierarchy"? *Haemostasis* **29,** 4–15.
3. Ross, R. (1999) Atherosclerosis-an inflammatory disease. *New Eng. J. Med.* **340,** 115–126.
4. Theilmeier, G., Lenaerts, T., Remacle, C., Collen, D., Vermylen, J., and Hoylaerts, M. F. (1999) Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. *Blood* **94,** 2725–2734.
5. Ostrovsky, L., King, A. J., Bond, S., Mitchell, D., Lorant, D. E., Zimmerman, G. A., Larsen, R., Niu, X. F., and Kubes P. (1998) A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process. *Blood* **91,** 3028–3036.
6. Simon, D. I., Xu, H., Ortlepp, S., Rogers, C., and Rao, N. K. (1997) 7E3 monoclonal antibody directed against the platelet glycoprotein IIb/IIIa cross-reacts with leukocyte integrin Mac-1 and blocks adhesion to fibrinogen and ICAM-1. *Arterioscler. Thromb. Vasc. Biol.* **17,** 528–535.
7. Massberg, S., Enders, G., Leiderer, R., Eisenmenger, S., Vestweber, D., Krombach, F., and Messmer K. (1998) Platelet-endothelial cell interactions during ischemia/reperfusion: The role of P-selectin. *Blood* **92,** 507–515.
8. Smith, R. D., and Owen, W. G. (1999) Platelet responses to compound interactions with thrombin. *Biochemistry* **38,** 8936–8947.
9. McNicol, A., and Israels, S. J. (1999) Platelet dense granules: Structure, function and implications for haemostasis. *Thromb. Res.* **95,** 1–18.
10. Barry, O. P., Praticò, D., Savani, R. C., and FitzGerald, G. A. (1998) Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J. Clin. Invest.* **102,** 136–144.
11. Nomura, S., Suzuki, M., Katsura, K., Xie, G. L., Miyazaki, Y., Miyake, T., Kido, H., Kagawa, H., and Fukuhara, S. (1995) Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus. *Atherosclerosis* **116,** 235–240.
12. Heijnen, H. F. G., Schiel, A. E., Fijnheer, R., Geuze, H. J., and Sixma, J. J. (1999) Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and α-granules. *Blood* **94,** 3791–3799.
13. Shaltiel, S., Schvartz, I., Korc-Grodzicki, B., and Kreizman, T. (1993) Evidence for an extra-cellular function for protein kinase A. *Mol. Cell. Biochem.* **127/128,** 283–291.
14. Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C., and Fisher, G. J. (1992) Translocation of protein kinase C isozymes in thrombin-stimulated human platelets. *J. Biol. Chem.* **267,** 15585–15590.
15. Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9,** 484–496.
16. Johannes, F.-J., Prestle, J., Eis, S., Overhagemann, P., and Pfizenmaier, K. (1994) PKCu is a novel, atypical member of the PKC family. *J. Biol. Chem.* **269,** 6140–6148.
17. Wang, F., Naik, U. P., Ehrlich, Y. H., Freyberg, Z., Osada, S., Ohno, S., Kuroki, T., Suzuki, K., and Kornecki, E. (1993) A new protein kinase C, nPKCη^1 and nPKCθ are expressed in human platelets: Involvement of nPKCη^1 and nPKCθ in signal transduction stimulated by PAF. *Biochem. Biophys. Res. Commun.* **191,** 240–246.
18. Fourcade, O., Simon, M.-F., Viodé, C., Rugani, N., Leballe, F., Ragab, A., Fournié, B., Sarda, L., and Chap, H. (1995) Secretory phospholipase A_2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. *Cell* **80,** 919–927.
19. Miller, D. T., and Bode, A. P. (1987) Quantitation of microparticles in platelet suspensions by flow cytometry. *Thromb. Haemost.* **58,** 484.
20. Kansra, S. V., and Shukla, S. D. (2000) Reverse relationship between mitogen activated protein kinase and human platelet aggregation. *Clin. Exper. Hypertension* **22,** 145–154.
21. Reddy, M. A., and Shukla, S. D. (1996) Potentiation of mitogen-

activated protein kinase by ethanol in embryonic liver cells. *Biochem. Pharm.* **51,** 661–668.

22. Babinska, A., Ehrlich, Y. H., and Kornecki, E. (1996) Activation of human platelets by protein kinase C antibody: Role for surface phosphorylation in homeostasis. *Amer. J. Physiol.* **271,** H2134–H2144.
23. Chen, W., Wieraszko, A., Hogan, M. V., Yang, H. A., Kornecki, E., and Ehrlich, Y. H. (1996) Surface protein phosphorylation by ecto-protein kinase is required for the maintenance of hippocampal long-term potentiation. *Proc. Nat. Acad. Sci. USA* **93,** 8688–8693.
24. Ekdahl, K. N., and Nilsson, B. (1999) Alterations in C3 activation and binding caused by phosphorylation by a casein kinase released from activated human platelets. *J. Immunol.* **162,** 7426–7433.
25. Blomback, B., Blomback, M., Edman, P., and Hessel, B. (1962) Amino-acid sequence and the occurrence of phosphorus in human fibrinopeptides. *Nature* **193,** 883–886.
26. Rand, M. D., Kalafatis, M., and Mann, K. G. (1994) Platelet coagulation factor Va: The major secretory platelet phosphoprotein. *Blood* **83,** 2180–2190.
27. Mehringer, J. H., Weigel, C. J., and Tollefsen, D. M. (1991) Cyclic AMP-dependent protein kinase phosphorylates serine 378 in vitronectin. *Biochem. Biophys. Res. Commun.* **179,** 655–660.