

Regulation of Protease-Activated Receptor (PAR) 1 and PAR4 Signaling in Human Platelets by Compartmentalized Cyclic Nucleotide Actions^S

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ABSTRACT

Thrombin potently regulates human platelets by the G protein-coupled receptors protease-activated receptor (PAR) 1 and PAR4. Platelet activation by thrombin and other agonists is broadly inhibited by prostacyclin and nitric oxide acting through adenylyl and guanylyl cyclases to elevate cAMP and cGMP levels, respectively. Using forskolin and YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole] to selectively activate the adenylyl and guanylyl cyclases, respectively, and the membrane-permeable analogs *N*⁶,2'-*O*-dibutyryl-adenosine-3'-5'-cAMP (dibutyryl-cAMP) and 8-(4-parachlorophenylthio)-cGMP (8-pCPT-cGMP), we sought to identify key antiplatelet steps for cyclic nucleotide actions in blocking platelet activation by PAR1 versus PAR4. Platelet aggregation by PAR1 or PAR4 was inhibited with similar EC₅₀ of 1.2 to 2.1 μM forskolin, 31 to 33 μM YC-1, 57 to 150 μM dibutyryl-cAMP, and 220 to 410 μM 8-pCPT-cGMP. There was a marked left shift in the inhibitory

potencies of forskolin and YC-1 for α-granule release and glycoprotein IIb/IIIa/integrin αIIbβ3 activation (i.e., EC₅₀ of 1–60 and 40–1300 nM, respectively) that was not observed for dibutyryl-cAMP and 8-pCPT-cGMP (i.e., EC₅₀ of 200–600 and 40–140 μM, respectively). This inhibition was essentially instantaneous, and measurements of cyclic nucleotide levels and kinase activities support a model of compartmentation involving the cyclic nucleotide effectors and regulators and the key molecular targets for this platelet inhibition. The different sensitivities of PAR1 and PAR4 to inhibition of calcium mobilization and dense granule release identify key antiplatelet steps for cyclic nucleotide actions and are consistent with the signaling models for these receptors. Specifically, PAR4 inhibition depends on the regulation of both calcium mobilization and dense granule release, and PAR1 inhibition depends predominantly on the regulation of dense granule release.

Thrombin is the major protease in the coagulation cascade whose pleiotropic actions can ultimately lead to thrombosis and tissue injury. Thrombin also has multiple direct effects on vascular cells and is the most potent activator of platelets identified to date, functionally connecting tissue damage to inflammatory and hemostatic responses (Viles-Gonzalez et al., 2005). Many of the cellular effects of thrombin are initi-

ated by protease-activated receptors (PARs), which are G protein-coupled receptors. PAR are unique among G protein-coupled receptors in that activation occurs through proteolytic generation of a tethered ligand. Thrombin acts through PAR1 and PAR4 on human platelets to signal activation responses including calcium mobilization, release of procoagulant molecules from α-granules (e.g., P-selectin), release of small molecules from dense granules (e.g., ADP), activation of glycoprotein IIb/IIIa/integrin αIIbβ3 (GPIIb/IIIa), and aggregation (Brass, 2003; Coughlin, 2005).

Platelet activation by thrombin and other agonists can be prevented by physiologic platelet antagonists that increase intraplatelet levels of nucleotide 3'-5'-cyclic monophosphates (Schwarz et al., 2001). Prostacyclin inhibits platelets by stimulating the Gs-coupled prostacyclin IP receptor leading to the activation of adenylyl cyclase and production of cAMP. Nitric

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ABBREVIATIONS: PAR, protease-activated receptor; GPIIb/IIIa, glycoprotein IIb/IIIa/integrin αIIbβ3; NO, nitric oxide; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PDE, phosphodiesterase; PAR-AP, protease-activated receptor agonist peptide; YC-1, 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole; dibutyryl-cAMP, *N*⁶,2'-*O*-dibutyryl-adenosine-3'-5'-cAMP; 8-pCPT-cGMP, 8-(4-parachlorophenylthio)-cGMP; IBMX, 3-isobutyl-1-methylxanthine; VASP, vasodilator-stimulated phosphoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AM, acetoxymethyl ester; PLD, phospholipase D.

oxide (NO) is highly membrane-permeable and inhibits platelets by directly activating the soluble guanylyl cyclase, resulting in the production of cGMP. It has been recently recognized that NO exerts cGMP-independent effects on platelets by mechanisms that involve *S*-nitrosylation of proteins (for recent example and discussion, see Morrell et al., 2005). Cyclic nucleotides are known to function in platelets by binding the regulatory subunit of their respective cyclic nucleotide-dependent serine/threonine kinases, PKA and PKG. Whether cyclic nucleotides function in platelets by kinase-independent mechanisms such as gated ion channels or other cyclic nucleotide binding proteins is an open question. Genetic studies in the mouse revealed that platelet PKG is essential to prevent intravascular adhesion and aggregation of platelets after ischemia (Massberg et al., 1999). Furthermore, the PKG defect was not compensated by cAMP/PKA, supporting the existence of distinct regulatory mechanisms for cAMP and cGMP. Cyclic nucleotide levels in platelets are directly regulated by at least three different phosphodiesterases (PDEs): the cGMP-stimulated, nonselective PDE2; the cGMP-inhibited, cAMP-selective PDE3; and the cGMP-stimulated, cGMP-specific PDE5 (Schwarz et al., 2001).

Although cyclic nucleotide signaling pathways have been studied in platelets since the late 1960s, cyclic nucleotide and platelet-activating signals share complex relationships that remain poorly delineated. Moreover, PAR4 is a recently discovered platelet receptor for thrombin (Kahn et al., 1998; Xu et al., 1998), and studies of cyclic nucleotide-mediated platelet inhibition to date have largely used thrombin and PAR1 selective agonist peptides. Our investigations have shown that PAR1 and PAR4 activate platelets by distinct signaling pathways (Holinstat et al., 2006, 2007). We sought to identify key antiplatelet events for cyclic nucleotide actions and hypothesized that cAMP and cGMP engage distinct mechanisms to inhibit PAR1 and PAR4 activation of human platelets. We defined threshold concentrations for maximal platelet aggregation by the PAR-selective agonist peptides PAR1-AP and PAR4-AP. Using these concentrations of PAR1-AP and PAR4-AP and detailed kinetic analyses of platelet activation, we compared the antiplatelet effects of adenylyl and guanylyl cyclase-activating compounds and membrane-permeable cAMP and cGMP analogs. These kinetic analyses and measurements of cyclic nucleotide levels and kinase activities support a model of cyclic nucleotide actions in which the cyclic nucleotide effector (e.g., PKA or PKG) and regulators (e.g., PDE) and the key molecular targets for platelet inhibition share specific spatiotemporal relationships. Although such compartmentation is widespread and particularly well recognized in the heart (Steinberg and Brunton, 2001; Fischmeister et al., 2006; Smith and Scott, 2006; Zaccolo, 2006), this model has not been previously established in platelets. The kinetic analyses also show that the inhibition of platelet aggregation by cyclic nucleotide signaling pathways best correlates with the inhibition of calcium mobilization and dense granule release. In this regard, PAR1 and PAR4 have distinct sensitivities to cyclic nucleotide actions that are consistent with the current signaling models for these receptors. Namely, PAR4 signals platelet aggregation by calcium mobilization and purinergic signaling pathways, and PAR1 is predominantly dependent on a purinergic signaling component at threshold PAR1-AP

concentrations (Holinstat et al., 2006; Murugappa and Kunapuli, 2006).

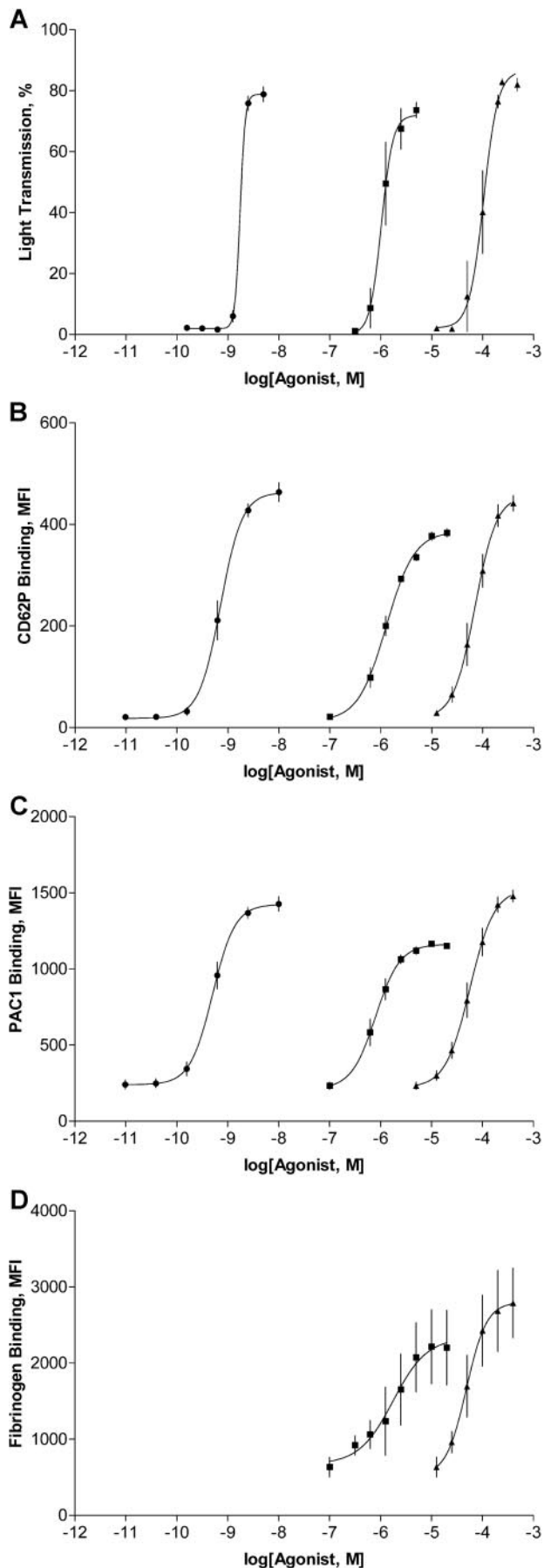
Materials and Methods

Materials. Recombinant human α -thrombin (2700 NIH units/mg) was purchased from Enzyme Research Laboratories (South Bend, IN). Activating peptides for PAR1 (PAR1-AP, SFLLRN) and PAR4 (PAR4-AP, AYPGKF) were purchased from GL Biochem (Shanghai, China). Forskolin and YC-1 were purchased from A.G. Scientific, Inc. (San Diego, CA). *N*⁶,2'-*O*-Dibutyryladenine-3'-5'-cAMP (dibutyryl-cAMP) and 8-(4-parachlorophenylthio)-cGMP (8-pCPT-cGMP) were purchased from Axxora LLC (San Diego, CA). 3-Isobutyl-1-methyl-xanthine (IBMX) was purchased from Sigma-Aldrich (Saint Louis, MO). PAC1 (antibody to GPIIb/IIIa) conjugated to fluorescein isothiocyanate and CD62P (antibody to P-selectin) conjugated to *R*-phycoerythrin were purchased from BD Biosciences (San Jose, CA). Phospho-vasodilator-stimulated phosphoprotein (VASP) (Ser157) rabbit antibody was purchased from Cell Signaling Technology (Danvers, MA). Anti-phospho-VASP (Ser239) mouse antibody was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse antibody was purchased from Abcam, Inc. (Cambridge, MA). Goat anti-rabbit IgG antibody conjugated to IRDye 800 was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA). Goat anti-mouse IgG antibody conjugated to AlexaFluor 680 and Fura-2-AM were purchased from Invitrogen (Carlsbad, CA). Glass cuvettes and disposable siliconized stir bars for aggregometry were obtained from Chrono-Log Corporation (Havertown, PA).

Human Platelets. Human platelets were isolated from whole blood obtained after informed consent from healthy volunteers at Vanderbilt University Medical Center. These studies were approved by the Vanderbilt University institutional review board. All steps in platelet isolation were performed at room temperature. Blood was collected using BD Vacutainer tubes containing buffered sodium citrate. After centrifugation at 200g for 15 min, the platelet-rich plasma phase was transferred to a conical tube. A 1:10 (v/v) acid citrate dextrose solution [2.5% (w/v) sodium citrate tribasic, 1.5% citric acid, and 2.0% D-glucose] was added, and washed platelets were prepared by centrifugation at 800g for 10 min. Platelets were suspended in Tyrode's buffer (140 mM sodium chloride, 5.0 mM potassium chloride, 0.4 mM sodium phosphate, 1.0 mM magnesium chloride, 5.0 mM D-glucose, and 10 mM HEPES, pH 7.4) and counted as 3- to 30-fl particles using a Z1 series Coulter Counter (Beckman Coulter, Fullerton, CA) equipped with a 50 μ m aperture.

Aggregation. Washed platelet concentration was adjusted to 2.0×10^8 platelets/ml, and 0.5 ml of suspension was transferred to a cuvette containing a stir bar. Platelets were prewarmed to 37°C for at least 5 min. Unless otherwise indicated, the cyclic nucleotide agonists were added 5 min before platelet stimulation. Thrombin, PAR1-AP, and PAR4-AP were added after 2 min of 1000 rpm stirring in the presence of 1.25 mM calcium chloride. Platelet aggregation was measured by light transmission using a Chrono-Log Aggregometer according to the manufacturer's protocol.

α -Granule Release and GPIIb/IIIa Activation. Washed platelet concentration was adjusted to 1.7×10^7 platelets/ml, and 30 μ l of suspension (5.0×10^5 platelets) was transferred to a 5-ml round bottom tube. PAC1 and CD62P antibodies (10 μ l each) or an equal volume of Tyrode's buffer for controls were added (i.e., final volume 50 μ l). Unless otherwise indicated, the cyclic nucleotide agonists were added 5 min before platelet stimulation. After 10 min of stimulation with thrombin, PAR1-AP, or PAR4-AP, platelets were fixed for 10 min by addition of 50 μ l of 2% formaldehyde. The suspension was diluted by addition of 400 μ l of Tyrode's buffer and then submitted for flow cytometric analysis. Platelets were distinguished on the basis of side- and forward-light scatter, and 10,000 platelets were analyzed per condition.



Cyclic Nucleotide Levels. Washed platelet concentration was adjusted to 1.0×10^9 platelets/ml, and $80 \mu\text{l}$ of suspension was used for each reaction. Platelets were treated with forskolin or YC-1 for 3 min. cAMP and cGMP levels were measured in duplicate using the CatchPoint Fluorescent Assay Kit as described by the manufacturer (Molecular Devices Corp., Sunnyvale, CA). In some experiments, platelets were treated for 10 min with 1 mM IBMX, a nonselective PDE inhibitor, before stimulation with forskolin or YC-1.

VASP Phosphorylation. Washed platelets were adjusted to 2.0×10^8 platelets/ml and treated with cyclic nucleotide agonists at 37°C for 10 min unless otherwise indicated. Reactions were stopped by addition of $5\times$ sample lysis buffer (10% sodium dodecyl sulfate, 50% glycerol, and 0.5 M Tris, pH 6.8) and boiling for 5 min. Platelet lysate ($15 \mu\text{l}$) was separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. Phosphorylated VASP was detected using 1:2000 dilutions of phospho-VASP (Ser157) rabbit antibody and anti-phospho-VASP (Ser239) mouse antibody. Anti-GAPDH mouse antibody (1:10,000 dilution) was used as an internal control for protein loading. Primary antibodies were detected using 1:5000 dilutions of IRDye 800-conjugated anti-rabbit IgG antibody and AlexaFluor 680-conjugated anti-mouse IgG antibody and measured using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Dense Granule Release. Washed platelet concentration was adjusted to 2.2×10^8 platelets/ml, and $270 \mu\text{l}$ of suspension was transferred to a cuvette containing a stir bar. Platelets were pre-warmed to 37°C for at least 5 min in the presence of $30 \mu\text{l}$ of Chrono-Lume reagent, which detects ATP release. Cyclic nucleotide agonists were added 5 min before platelet stimulation. Thrombin, PAR1-AP, and PAR4-AP were added after 2 min of 1000 rpm stirring in the presence of 1.25 mM calcium chloride. Dense granule secretion was measured by luminescence using a Chrono-Log Lumi-Aggregometer according to the manufacturer's protocol.

Calcium Mobilization. Washed platelets were suspended in a volume of Tyrode's buffer equal to the platelet-rich plasma volume, and 2.5 ml of suspension was transferred to a 15-ml conical tube pre-filled with 7.5 ml of platelet buffer containing $25 \mu\text{g}$ of Fura-2-AM and 0.4 units of apyrase. After incubation at 37°C for 30 min, 1:10 (v/v) acid citrate dextrose solution was added, and platelets were centrifuged at $800g$ for 10 min. Fura-2-AM loaded platelets were suspended to a final concentration of 0.4×10^8 platelets/ml, and calcium chloride was added to a final concentration of 1.25 mM. Platelet suspension (1 ml) was transferred to a cuvette and pre-warmed to 37°C for at least 5 min. Cyclic nucleotide agonists were added 5 min before platelet stimulation. Calcium mobilization in response to PAR1-AP and PAR4-AP was measured by A340 and A380 using a Cary Eclipse spectrofluorometer (Varian, Inc., Walnut Creek, CA).

Calculations and Statistical Analysis. All data are expressed as mean \pm S.E. Determinations of best fit curves and EC_{50} and Hill coefficient values were made using Prism software (GraphPad Software Inc., San Diego, CA). Comparison between experimental conditions was performed by the paired Student's *t* test. Differences in mean values were considered significant for $p < 0.05$.

Results

Platelet Activation by Thrombin Receptors. To identify the threshold concentrations for maximal platelet aggreg-

Fig. 1. Activation of platelet thrombin receptors. Dose-dependent responses of platelets to thrombin (●), PAR1-AP (■), or PAR4-AP (▲) were measured using aggregometry (A) and flow cytometry (B–D). α -Granule release (B) and GPIIb/IIIa activation (C) were simultaneously measured using directly conjugated PE-CD42P (P-selectin) and fluorescein isothiocyanate-PAC1 antibodies, respectively. In separate experiments, fibrinogen binding (D) was measured using directly conjugated fluorescein isothiocyanate-fibrinogen. Points are mean \pm S.E. ($n = 6$ for aggregation, and $n = 4$ for flow cytometry studies). Curves represent a best fit analysis as described.

TABLE 1

Maximal effect, molar EC₅₀, and Hill coefficient for PAR agonist activation of platelets^a

	Thrombin			PAR1-AP			PAR4-AP		
	Maximal Effect ^b	EC ₅₀	Hill Coefficient	Maximal Effect ^b	EC ₅₀	Hill Coefficient	Maximal Effect ^b	EC ₅₀	Hill Coefficient
Aggregation	79 ± 2	1.7 ± 0.2 × 10 ⁻⁹	8.8 ± 1.3	74 ± 3	1.4 ± 0.3 × 10 ⁻⁶	3.8 ± 2.0	80 ± 1	1.2 ± 0.2 × 10 ⁻⁴	3.1 ± 1.3
P-selectin	460 ± 20	7.2 ± 0.7 × 10 ⁻¹⁰	2.0 ± 0.5	380 ± 10	1.3 ± 0.1 × 10 ⁻⁶	1.6 ± 0.2	440 ± 10	7.1 ± 0.9 × 10 ⁻⁵	2.1 ± 0.6
GPIIb/IIIa	1430 ± 40	5.0 ± 0.5 × 10 ⁻¹⁰	2.0 ± 0.4	1150 ± 10	8.2 ± 0.9 × 10 ⁻⁷	1.8 ± 0.4	1480 ± 30	5.7 ± 0.6 × 10 ⁻⁵	1.8 ± 0.4
Fibrinogen				2200 ± 400	2.2 ± 0.9 × 10 ⁻⁶	1.3 ± 1.3	2790 ± 400	4.8 ± 1.6 × 10 ⁻⁵	2.2 ± 1.9

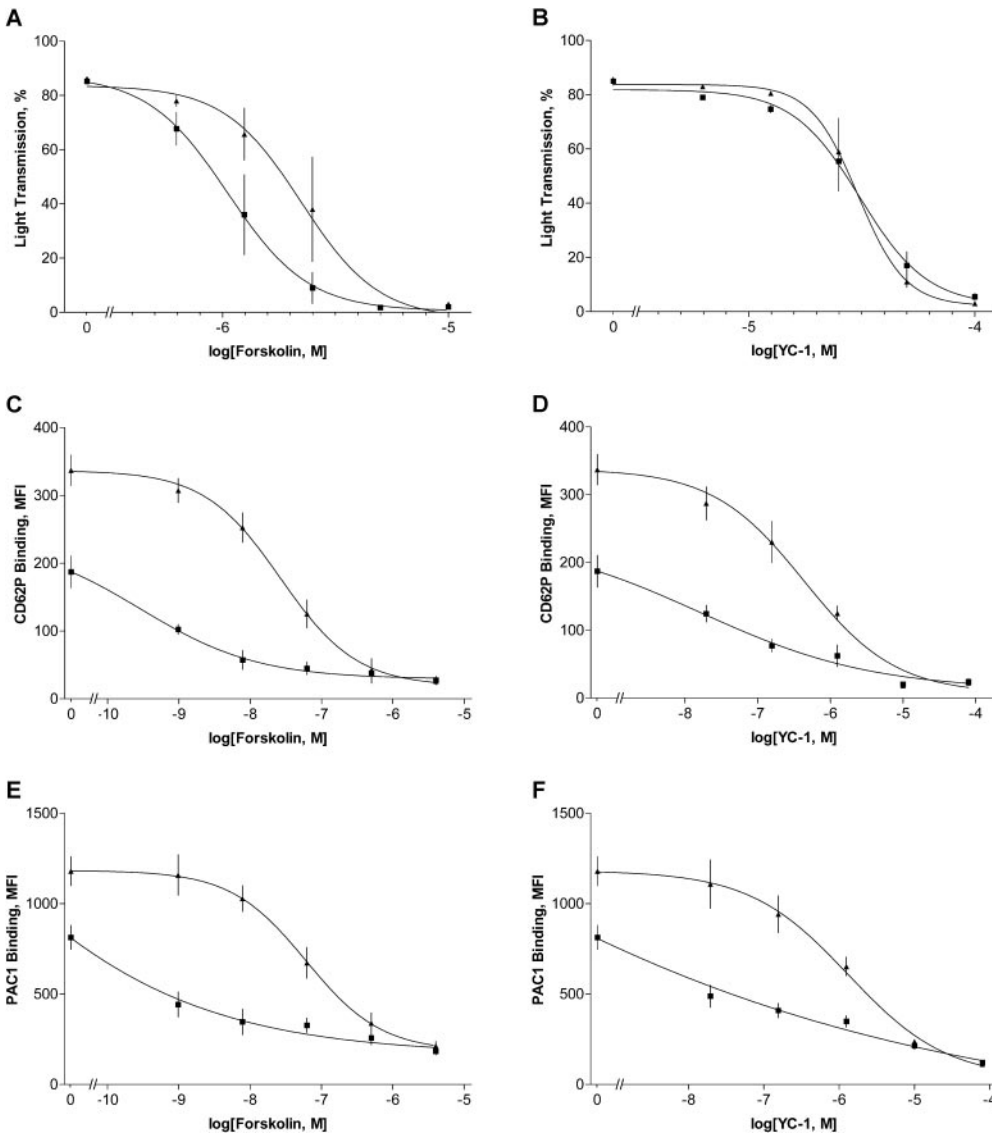
^a Values are mean ± S.E. (*n* = 3–6).^b Units for maximal effect are percent light transmission for aggregation and MFI for P-selectin, PAC1 (GPIIb/IIIa), and fibrinogen binding. Differences between PAR1-AP and thrombin are significant for P-selectin expression (*p* < 0.007) and GPIIb/IIIa activation (*p* < 0.02). Differences between PAR1-AP and PAR4-AP are significant for P-selectin expression (*p* < 0.007), GPIIb/IIIa activation (*p* < 0.004), and fibrinogen binding (*p* < 0.02). Differences in aggregation between thrombin, PAR1-AP, and PAR4-AP and in P-selectin expression and GPIIb/IIIa activation between thrombin and PAR4-AP are not significant (*p* > 0.05).

Fig. 2. Dose-dependent inhibition of platelets by cyclic nucleotide agonists. Platelets were treated in a dose-dependent manner with forskolin (A, C, and E) to increase cAMP production or YC-1 (B, D, and F) to increase cGMP production for 5 min before stimulation with 2.8 μ M PAR1-AP (■) or 240 μ M PAR4-AP (▲). Platelet aggregation (A and B), α -granule release (C and D), and GPIIb/IIIa activation (E and F) were measured as described. Points are mean \pm S.E. (*n* = 4 for aggregation and *n* \geq 3 for flow cytometry studies). Curves represent a best fit analysis as described.

gation, we measured the dose-dependent response of platelets to PAR1-AP and PAR4-AP (Fig. 1A). Platelet response to thrombin was used to define maximal aggregation. Table 1 shows the maximal effect, EC₅₀, and Hill coefficient for each agonist. The maximal response for the agonist peptides were similar to thrombin (*p* > 0.05), and the EC₅₀ values are in agreement with other published values (Kahn et al., 1998; Xu et al., 1998; Chung et al., 2002). We also evaluated

the dose-dependent activation of platelets as measured by α -granule release (i.e., P-selectin expression by CD62P binding, Fig. 1B), GPIIb/IIIa activation (i.e., PAC1 binding, Fig. 1C), and fibrinogen-binding (Fig. 1D). The maximal response by PAR1-AP for these events was consistently less than that achieved by thrombin or PAR4-AP (Table 1). The EC₅₀ values of thrombin and each agonist peptide for these markers of platelet activation were similar to the

TABLE 2
Molar EC_{50} for inhibition of platelets by cyclic nucleotide agonists^a

Mechanism		Aggregation		GPIIb/IIIa		P-selectin	
		PAR1	PAR4	PAR1 ^b	PAR4	PAR1 ^b	PAR4
Forskolin	AC activator	$1.2 \pm 0.3 \times 10^{-6}$	$2.1 \pm 0.4 \times 10^{-6}$	1×10^{-9}	$6.0 \pm 2.5 \times 10^{-8}$	1×10^{-9}	$2.6 \pm 0.9 \times 10^{-8}$
Dibutyryl-cAMP	cAMP analog	$1.5 \pm 0.2 \times 10^{-4}$	$5.7 \pm 0.9 \times 10^{-5}$	2×10^{-4}	$2.0 \pm 0.4 \times 10^{-4}$	6×10^{-4}	$2.0 \pm 0.3 \times 10^{-4}$
YC-1	GC activator	$3.1 \pm 0.5 \times 10^{-5}$	$3.3 \pm 0.5 \times 10^{-5}$	2×10^{-7}	$1.3 \pm 0.6 \times 10^{-6}$	2×10^{-7}	$4.1 \pm 1.6 \times 10^{-7}$
8-pCPT-cGMP	cGMP analog	$4.1 \pm 0.5 \times 10^{-4}$	$2.2 \pm 0.2 \times 10^{-4}$	5×10^{-5}	$1.4 \pm 0.5 \times 10^{-4}$	4×10^{-5}	$1.2 \pm 0.7 \times 10^{-4}$

^a Platelets were stimulated with 2.8 μ M PAR1-AP or 240 μ M PAR4-AP. Values are mean \pm S.E. ($n = 3-6$).

^b Approximate concentrations.

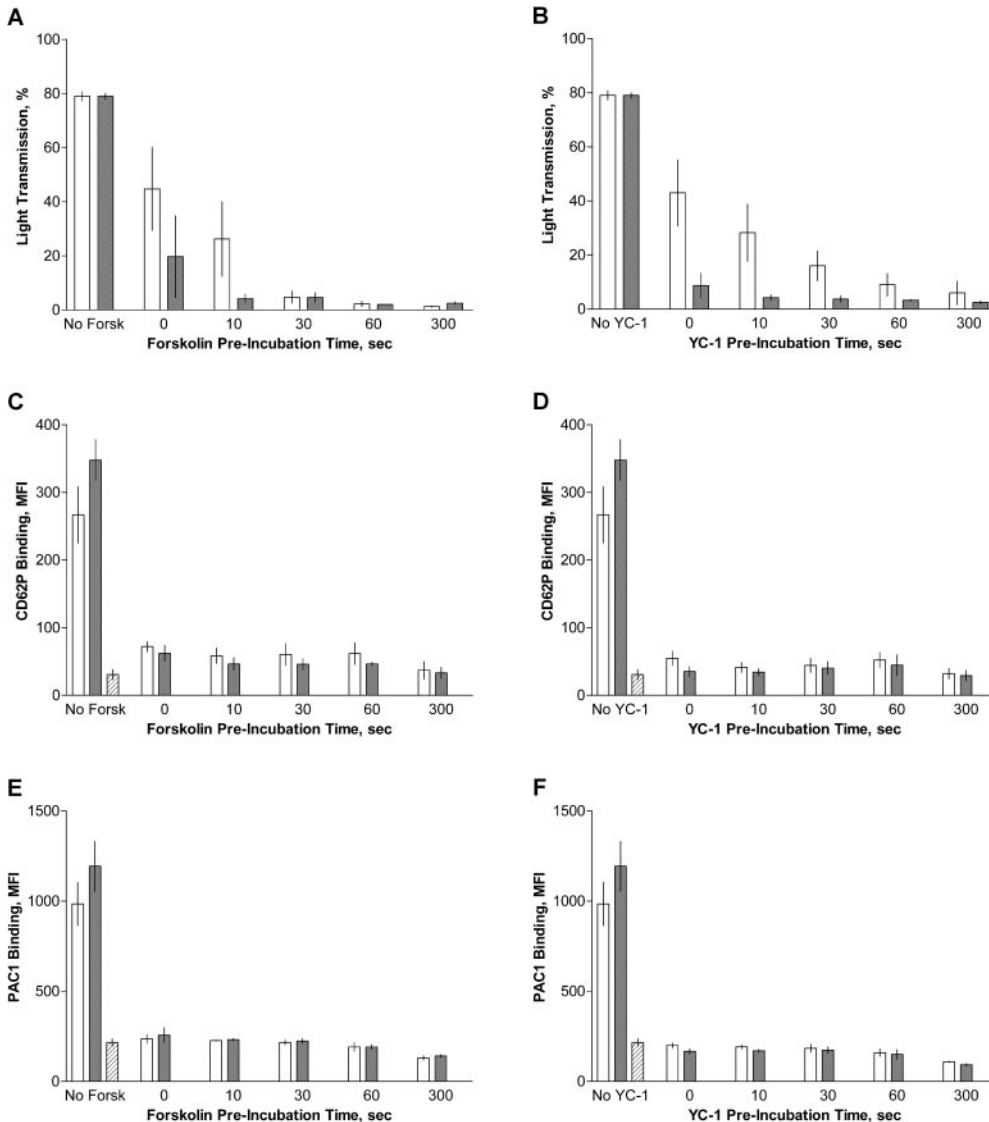


Fig. 3. Time-dependent inhibition of platelets by cyclic nucleotide agonists. Platelets were treated with 4.0 μ M forskolin (A, C, and E) or 80 μ M YC-1 (B, D, and F) simultaneous or prior to stimulation with 2.8 μ M PAR1-AP (open bars) or 240 μ M PAR4-AP (shaded bars). Platelet aggregation (A and B), α -granule release (C and D), and GPIIb/IIIa activation (E and F) were measured as described. Hashed bars (C-F) represent basal levels in untreated platelets. Bars, mean \pm S.E. ($n = 4$ for aggregation and $n = 3$ for flow cytometry studies).

respective EC_{50} for aggregation. The Hill coefficients were generally similar among the agonists with the exceptions of aggregation by thrombin in comparison with the agonist peptides and fibrinogen binding by PAR1-AP in comparison with PAR4-AP. Based on these data, we defined the threshold concentrations for maximal platelet aggregation by each agonist to be 2.0 nM thrombin, 2.8 μ M PAR1-AP, and 240 μ M PAR4-AP.

Kinetic Analysis of Cyclic Nucleotide Agonists. Using the threshold concentrations of PAR1-AP and PAR4-AP defined above, we measured the dose- and time-dependent inhibition of platelets by cAMP and cGMP signaling pathways.

To increase intraplatelet cAMP, we used forskolin, an activator of adenylyl cyclases (Siegl et al., 1982), or dibutyryl-cAMP, a cell-permeable cAMP analog that selectively activates PKA (Schwede et al., 2000). To increase intraplatelet cGMP levels, we used YC-1, an activator of soluble guanylyl cyclase (Wu et al., 1995), or 8-pCPT-cGMP, a cell-permeable cGMP analog that selectively activates PKG and does not significantly affect cGMP-regulated phosphodiesterases (Schwede et al., 2000).

Agonist peptide stimulated aggregation was inhibited in a dose-dependent manner by forskolin and YC-1 (Fig. 2, A and B), as were α -granule release (Fig. 2, C and D) and GPIIb/IIIa

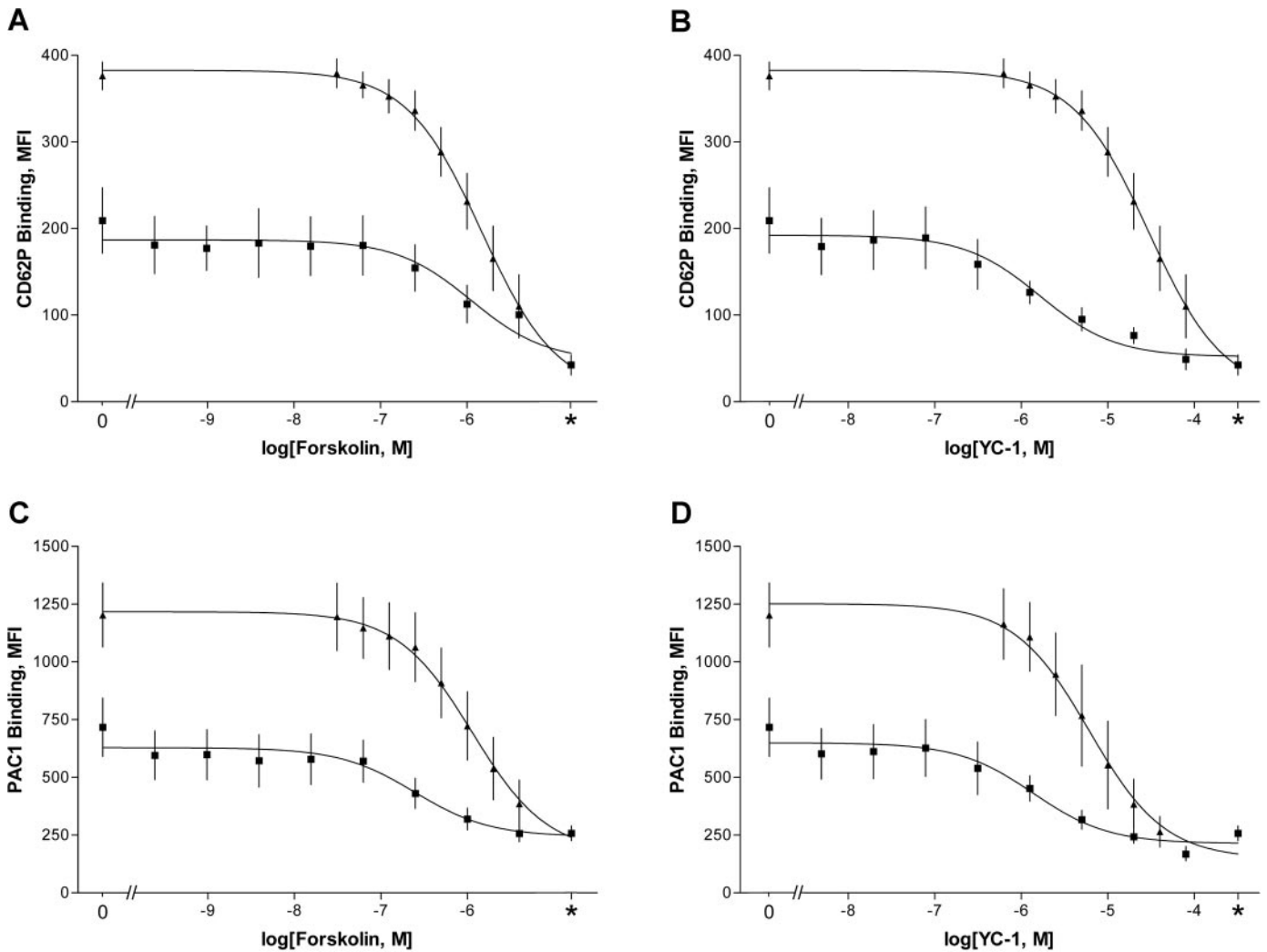


Fig. 4. Dose-dependent instantaneous inhibition of platelets by cyclic nucleotides agonists. Platelets were treated in a dose-dependent manner with forskolin (A and C) or YC-1 (B and D), applied simultaneously with 2.8 μ M PAR1-AP (■) or 240 μ M PAR4-AP (▲). α -Granule release (A and B) and GPIIb/IIIa activation (C and D) were measured as described. Points at asterisks (*) represent basal levels in untreated platelets. Points are mean \pm S.E. ($n = 4$). Curves represent a best fit analysis as described.

activation (Fig. 2, E and F). Each compound had nearly equal potency for inhibiting PAR1 versus PAR4 (Table 2), and the EC_{50} values of forskolin and YC-1 for inhibiting platelet aggregation agree with previously reported values (Siegl et al., 1982; Wu et al., 1995). Based on EC_{50} , forskolin was at least 15 times more potent than YC-1. Forskolin and YC-1 were 25 to 1200 times more potent for inhibiting α -granule release and GPIIb/IIIa activation in comparison with inhibiting aggregation. Although α -granule release and GPIIb/IIIa activation were fully inhibited by 0.5 μ M forskolin and 10 μ M YC-1, the threshold concentrations for inhibiting platelet aggregation were 4.0 μ M forskolin and 80 μ M YC-1 (Supplemental Figs. 1 and 2).

Likewise, we measured the inhibitory effects of dibutyryl-cAMP and 8-pCPT-cGMP (Table 2; data not shown). Dibutyryl-cAMP was slightly more potent than 8-pCPT-cGMP for inhibiting aggregation and PAR1-stimulated α -granule release and GPIIb/IIIa activation. The cyclic nucleotide analogs had essentially equal potency for inhibiting PAR4-stimulated α -granule release and GPIIb/IIIa activation. Forskolin was at least 25 times more potent than dibutyryl-cAMP for inhibiting platelet functions, and YC-1 was at least six times more

potent than 8-pCPT-cGMP (Table 2). Interestingly, the potency of dibutyryl-cAMP and 8-pCPT-cGMP for inhibiting aggregation versus α -granule release or GPIIb/IIIa activation changed very little (i.e., 0–10 times more potent for inhibiting the latter), especially compared with the potency differences observed for forskolin and YC-1 (Table 2). Threshold concentrations for dibutyryl-cAMP and 8-pCPT-cGMP to fully inhibit platelet functions were 0.5 to 1.0 mM.

Cyclic nucleotide actions that lead to platelet inhibition are rapidly activated upon exposure to forskolin and YC-1 (Fig. 3). Aggregation by PAR1 was fully inhibited within 30 s of forskolin exposure and 60 s of YC-1 exposure (Fig. 3, A and B). Aggregation by PAR4 was fully inhibited within 10 s of exposure to either forskolin or YC-1 (Fig. 3, A and B). Consistent with this rapid onset of action, α -granule release and GPIIb/IIIa activation were inhibited essentially instantaneously by simultaneous application of either forskolin or YC-1 with agonist peptide (Fig. 3, C–F). There was a dose-dependent relationship for this instantaneous inhibition with a general decrease in the potency of forskolin and YC-1 as compared with longer exposure times (Fig. 4). Specifically, the instantaneous inhibition of α -granule release and GPI-

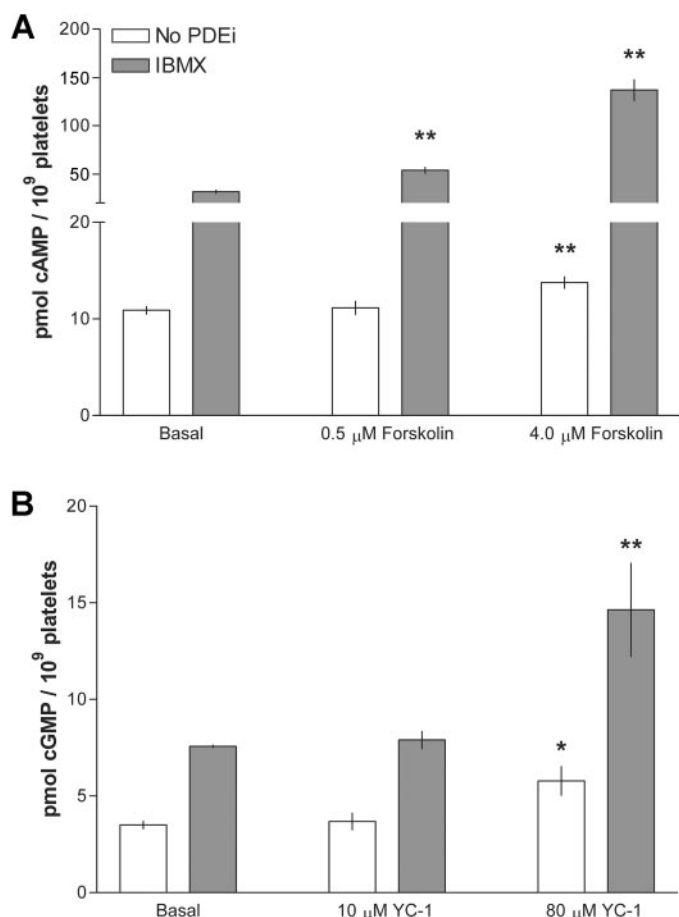


Fig. 5. Elevation of cAMP and cGMP in platelets by cyclic nucleotide agonists. Platelets were treated in the absence (open bars, “No PDEi”) or presence of 1 mM IBMX, a nonselective PDE inhibitor (shaded bars), with forskolin (A) or YC-1 (B). Bars are mean \pm S.E. ($n = 5$ in the absence IBMX and $n = 3$ in the presence of IBMX). $p < 0.05$ (*) and $p < 0.01$ (**) relative to the corresponding “basal” control. Differences in cAMP and cGMP levels between the basal controls in the absence and presence of IBMX are significant ($p < 0.000004$).

IbIIIa activation by forskolin occurred with EC_{50} of 0.25 to 1.4 μ M for both agonist peptides (Fig. 4, A and C). There were more obvious potency differences for YC-1 in the instantaneous inhibition of PAR1 versus PAR4 with EC_{50} of 1.4 to 1.6 and 6.1 to 30 μ M, respectively (Fig. 4, B and D).

We also observed that 80 μ M YC-1 decreased GPIIbIIIa activation to $47.7 \pm 2.2\%$ of basal (95% confidence interval, 43.1–52.3%; $n = 12$, based on pooled data from Fig. 2F and data not shown from untreated platelets and platelets treated with YC-1 only; see also Supplemental Fig. 2, E and F). A less robust decrease in GPIIbIIIa activation was observed with 4.0 μ M forskolin to $80.3 \pm 4.1\%$ of basal (95% confidence interval, 72.1–88.5%; $n = 12$, based on pooled data from Fig. 2E and data not shown from untreated platelets and platelets treated with forskolin only). This degree of inhibition required more than 60 s of exposure to forskolin or YC-1 to become fully established and was not overcome by agonist peptide stimulation (Fig. 3, E and F). Dibutyryl-cAMP and 8-pCPT-cGMP were unable to produce this type of inhibition even when applied at 1 mM for 10 min or more (data not shown). Importantly, neither forskolin nor YC-1 decreased P-selectin levels below basal (Fig. 3, C and D; see also Supplemental Figs. 1, C and D, and 2, C and D), reflect-

ing the very low presence of P-selectin on the surface of unstimulated platelets. To our knowledge, this observation has not been previously reported.

Compartmentation is a well recognized model in other systems of cardiovascular biology that describes the existence of a specific spatiotemporal relationship between cyclic nucleotide signaling pathways and their molecular targets (Steinberg and Brunton, 2001; Fischmeister et al., 2006; Smith and Scott, 2006; Zaccolo, 2006). To explore the possibility that compartmentation in platelets could explain the relative potency differences of forskolin and YC-1 for inhibiting platelet aggregation versus α -granule release and GPIIbIIIa activation, we measured the ability of forskolin and YC-1 to elevate total platelet cAMP and cGMP levels (Fig. 5). Based on a mean platelet volume of 5.2 fl (Egenthaler et al., 1992), we determined cAMP and cGMP concentrations in unstimulated platelets to be 2.1 ± 0.1 and 0.67 ± 0.03 μ M, respectively. These values are in close agreement with concentrations reported by Egenthaler et al. (1992; i.e., 4.4 μ M cAMP and 0.4 μ M cGMP). Elevations in total platelet cAMP and cGMP levels were detectable only at 4.0 μ M forskolin (Fig. 5A) and 80 μ M YC-1 (Fig. 5B), respectively, concentrations of the compounds that fully inhibit platelet functions (Fig. 2). There were no detectable changes in the noncanonical cyclic nucleotide by forskolin or YC-1 (data not shown), although 80 μ M YC-1 appeared to stimulate a small, consistent elevation of cAMP that nearly reached statistical significance ($p = 0.076$). To define the contributions of PDE as regulators in the platelet compartmentation of cyclic nucleotide actions, we measured cAMP and cGMP levels in platelets treated with IBMX, a nonselective PDE inhibitor. PDE inhibition alone increased cAMP and cGMP concentrations in unstimulated platelets to 6.1 ± 0.3 and 1.5 ± 0.0 μ M, respectively (Fig. 5; based on mean platelet volume of 5.2 fl, Egenthaler et al., 1992). Although changes in cGMP levels by 10 μ M YC-1, a concentration that more selectively inhibits α -granule release and GPIIbIIIa activation, were not detectable even in the presence of IBMX (Fig. 5B), simultaneous PDE inhibition had a synergistic effect on ability of forskolin to elevate platelet cAMP levels and revealed the ability of 0.5 μ M forskolin to significantly elevate cAMP levels (Fig. 5A). Specifically, cAMP levels in the presence of IBMX were 2.9-, 4.8-, and 9.9-fold greater than cAMP levels in the absence of IBMX for unstimulated, 0.5 μ M forskolin, and 4.0 μ M forskolin conditions, respectively (Fig. 5A). In contrast, cGMP levels in the presence of YC-1 and IBMX changed proportionately to the increase observed for PDE inhibition alone (i.e., unstimulated, 10 μ M YC-1, and 80 μ M YC-1 conditions resulted in 2.2-, 2.1-, and 2.5-fold greater cGMP levels, respectively, in the presence of IBMX relative to cGMP levels in the absence of IBMX; Fig. 5B). These experiments demonstrate the key role of PDE in regulating basal and stimulated platelet cyclic nucleotide levels.

To further define the kinetics of cyclic nucleotide actions, we measured the dose- and time-dependent phosphorylation of VASP (Fig. 6), a well described target for PKA and PKG. In platelets, PKA and PKG phosphorylate VASP on Ser157, Ser239, and Thr278 (Butt et al., 1994). VASP migrates in SDS-polyacrylamide gel electrophoresis at two distinct molecular masses depending on Ser157 phosphorylation. The 46-kDa species represents nonphosphorylated and Ser239 phosphorylated VASP. The 50-kDa species represents

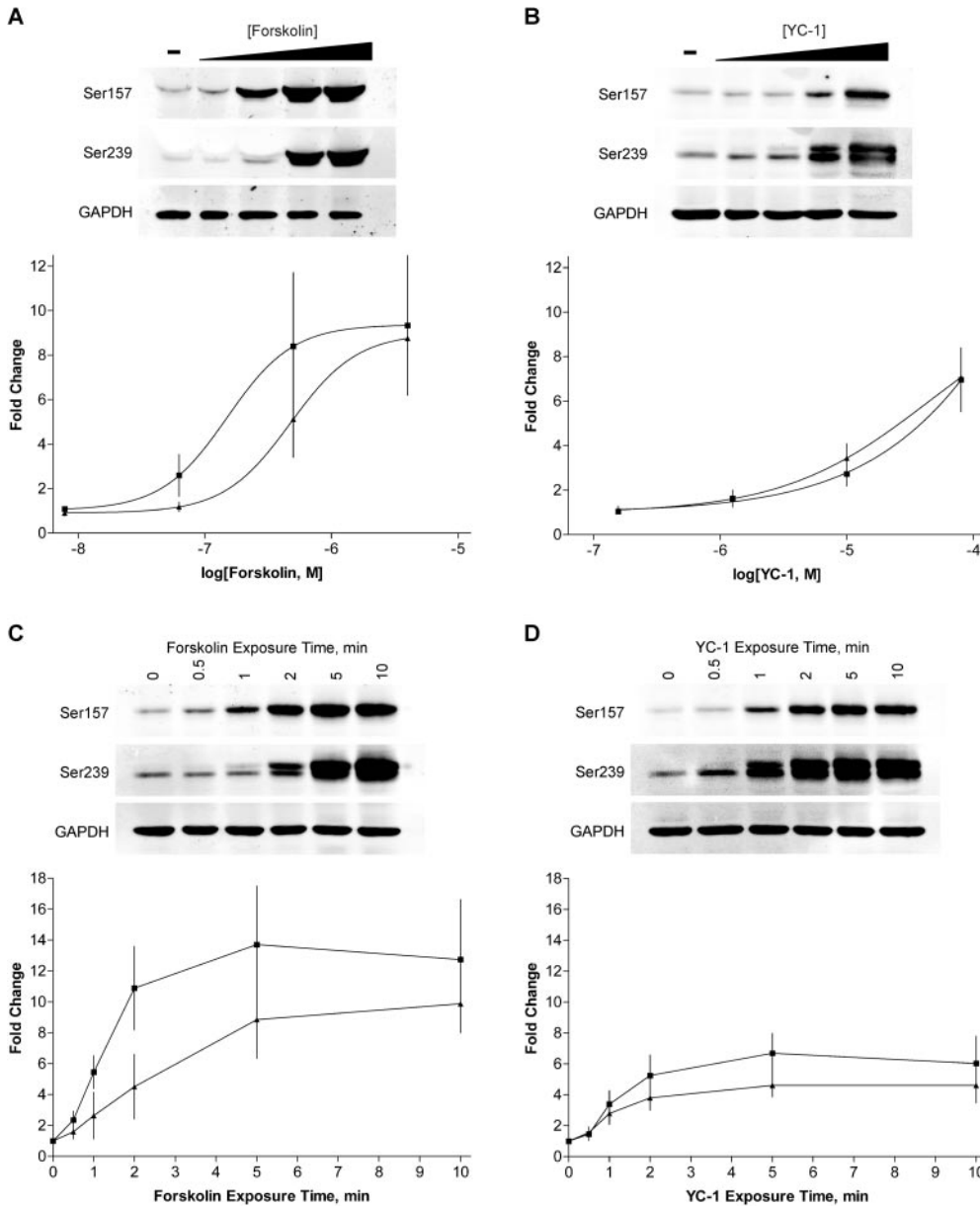


Fig. 6. Dose- and time-dependent phosphorylation of VASP by cyclic nucleotide agonists. Platelets were treated in a dose-dependent manner with forskolin (A) or YC-1 (B) for 10 min. In separate experiments, platelets were treated in a time-dependent manner with 4.0 μ M forskolin (C) or 80 μ M YC-1 (D). Reactions were stopped by addition of 5 \times lysis buffer and immediately boiling the sample. VASP phosphorylation was measured using Western assay of Ser157 (■) and Ser239 (▲) phosphospecific VASP antibodies. Data are normalized to GAPDH and expressed as the -fold change \pm S.E. relative to untreated platelets ($n = 4$ for dose-dependent studies and $n = 3$ for time-dependent studies). VASP phosphorylation stimulated by 0.5 and 4.0 μ M forskolin (A) and by 10 and 80 μ M YC-1 (B) relative to the unstimulated control are significant ($p < 0.05$).

Ser157 and possibly Ser239 phosphorylated VASP. Both species may be phosphorylated at Thr278. Ser157 and Ser239 are the preferred sites for PKA and PKG, respectively. Phosphorylation of VASP has been shown to closely correlate with fibrinogen receptor inhibition (Horstrup et al., 1994) and can be measured by Western assay using phosphospecific VASP antibodies. Forskolin and YC-1 stimulated robust kinase activation at concentrations that fully inhibit platelet functions (i.e., 4.0 and 80 μ M, respectively) and at concentrations that more selectively inhibit α -granule release and GPIIb/IIIa activation (i.e., 0.5 and 10 μ M, respectively; Fig. 6, A and B). Concentrations ≥ 250 μ M dibutyryl-cAMP or 8-pCPT-cGMP were required to stimulate significant VASP phosphorylation (data not shown). Full inhibition of platelet functions by forskolin correlated with an increase in VASP phosphorylation of 8 ± 3 -fold at Ser157 and 5 ± 1 -fold at Ser239 that was achieved only after 30 to 60 s of exposure (Fig. 6, A and C). Likewise, full inhibition of platelet functions by YC-1 correlated with an increase in VASP phosphorylation of 3 ± 1 -fold

at Ser157 and Ser239 that was achieved only after 30 s of exposure (Fig. 6, B and D).

We have previously shown that PAR4, but not PAR1, signals by both calcium mobilization and purinergic signaling (i.e., via the ADP receptor P2Y₁₂) to stimulate platelet aggregation (Holinstat et al., 2006). We hypothesized that these pathways were targeted by cyclic nucleotide signaling pathways and are the general mechanism for platelet inhibition. Interestingly, PAR1 and PAR4 had different sensitivities to the cyclic nucleotide actions that are consistent with the signaling models for these receptors in platelets (Holinstat et al., 2006; Murugappa and Kunapuli, 2006). Inhibition of PAR4 stimulated aggregation correlated closely with the ability of forskolin and YC-1 to inhibit both calcium mobilization (Fig. 7, C and D) and dense granule release (Table 3; Supplemental Fig. 2, G and H). Neither 4.0 μ M forskolin nor 80 μ M YC-1 fully inhibited calcium mobilization by PAR1 (Fig. 7, A and B), and the ability of forskolin and YC-1 to inhibit PAR1-stimulated aggregation best correlated with

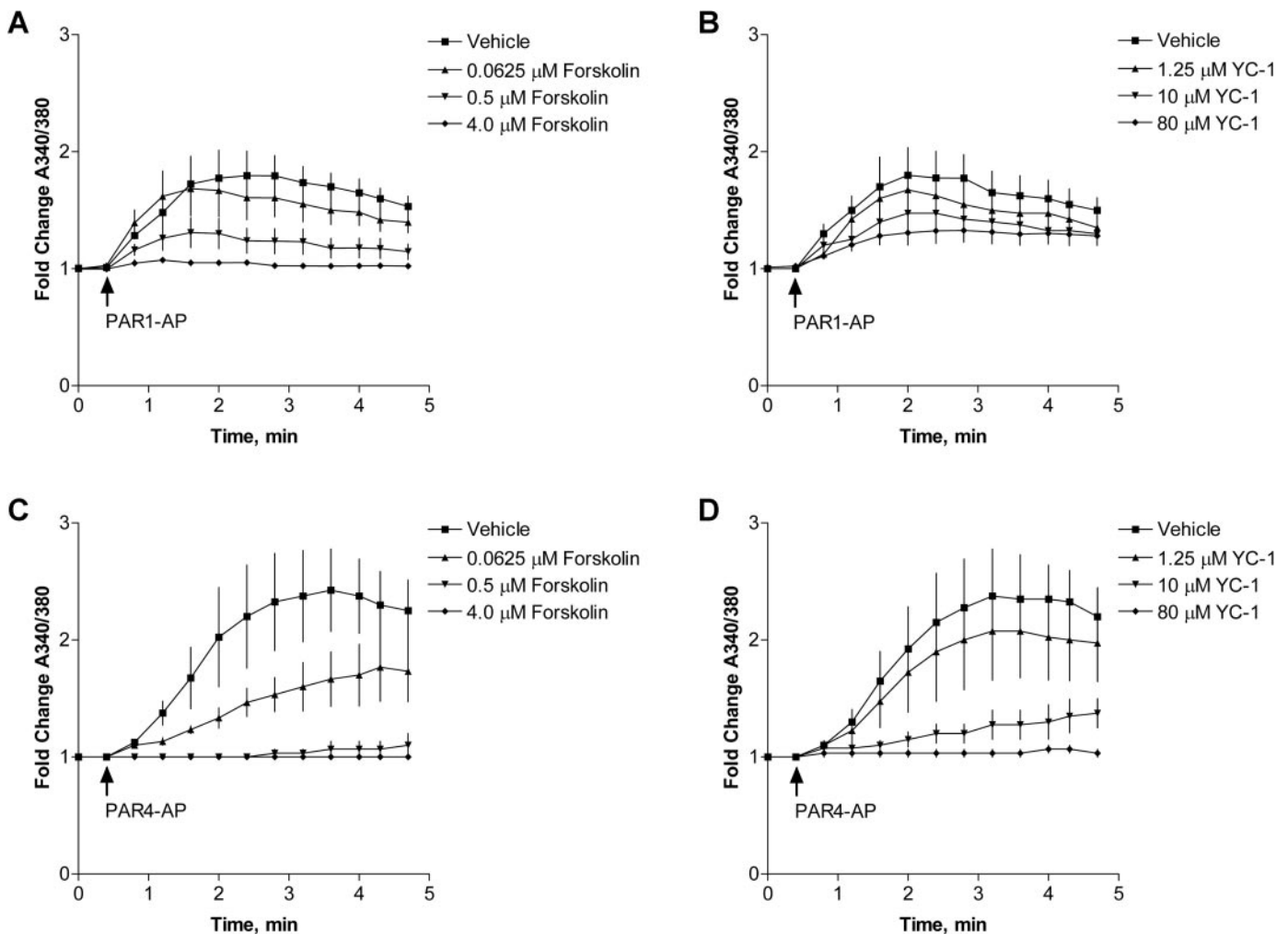


Fig. 7. Dose-dependent inhibition of platelet calcium mobilization by cyclic nucleotide agonists. Platelets were treated in a dose-dependent manner with forskolin (A and C) or YC-1 (B and D) for 5 min before stimulation with 2.8 μM PAR1-AP (A and B) or 240 μM PAR4-AP (C and D). Calcium mobilization was measured by absorbance fluorometry using Fura-2-AM. Points are mean ± S.E. of the fold change in $A_{340/380}$ from the baseline before stimulation with the agonist peptide ($n = 4$).

TABLE 3
Inhibition of platelet dense granule secretion by cyclic nucleotide agonists

	Vehicle	Forskolin			YC-1		
		0.5 μM	2.0 μM	4.0 μM	10 μM	40 μM	80 μM
PAR1	5.2 ± 0.3	0.2 ± 0.1	0.0	0.0	1.4 ± 0.5	0.2 ± 0.1	0.0
PAR4	5.5 ± 0.6	3.3 ± 0.8	0.0	0.0	4.0 ± 0.7	0.0	0.0

Platelets were stimulated with 2.8 μM PAR1-AP or 240 μM PAR4-AP. Values are mean micromolar ATP ± S.E. ($n = 3-5$).

the inhibition of dense granule release (Table 3; Supplemental Fig. 1, G and H).

Discussion

Our objectives were to determine the key antiplatelet events for cyclic nucleotide action on PAR signaling and to differentiate regulatory mechanisms for PAR1 and PAR4 and for cAMP and cGMP signaling pathways. We defined threshold concentrations for PAR-selective agonist peptides that result in maximal platelet aggregation (Fig. 1; Table 1). Using these agonist peptide concentrations, we performed kinetic analyses of platelet inhibition by selective activators of the adenylyl and guanylyl cyclases (forskolin and YC-1, respectively) and by the membrane-permeable analogs dibu-

tyryl-cAMP and 8-pCPT-cGMP. These experiments revealed surprising potency differences for the cyclase activators for inhibiting α -granule release and GPIIb/IIIa activation versus inhibiting aggregation (Fig. 2; Table 2). That is, 0.5 μM forskolin or 10 μM YC-1 were sufficient to inhibit α -granule release and GPIIb/IIIa activation, but aggregation required 4.0 μM forskolin or 80 μM YC-1 to be fully inhibited. Flow cytometry assays require greater dilution of platelets in comparison with other assays, and the apparent left shift in potency could be related to a concomitant dilution of released small molecule potentiators of platelet activation such as ADP. However, dibutyryl-cAMP and 8-pCPT-cGMP did not have similar left shifts in potency (Table 2), and the EC₅₀ of thrombin, PAR1-AP, and PAR4-AP for α -granule release

and GPIIb/IIIa activation concur with the respective EC₅₀ for aggregation (Table 1), supporting the notion that secondary platelet activators signal adequately in the flow cytometry assays. Another explanation for these left shifts in potency that also accounts for the rapid inhibitory kinetics of forskolin and YC-1 (Fig. 3) is that cyclic nucleotide signals share specific spatiotemporal relationships with their molecular targets. This compartmentation of cyclic nucleotide actions has not been described previously in platelets.

As further evidence for the compartmentation model in human platelets, we show that each cyclase activator is a considerably more potent inhibitor than its respective cyclic nucleotide analog (Table 2). This potency difference suggests that forskolin and YC-1, in directly activating their respective cyclase, may be advantaged by generating cyclic nucleotide bursts within a microdomain that includes the cyclic nucleotide effector (e.g., PKA or PKG) and regulators (e.g., PDE) and the key molecular target for inhibition of a platelet function. In contrast, dibutyryl-cAMP and 8-pCPT-cGMP, which importantly have in vitro kinase activation potencies similar to (in some cases, better than) their respective cyclic nucleotide (Schwede et al., 2000), randomly diffuse throughout the platelet, and actions comparable with a physiologically localized cyclic nucleotide burst may only be achieved at a relatively high concentration of the cyclic nucleotide analog. Measurements of cAMP and cGMP levels show that 4.0 μ M forskolin and 80 μ M YC-1 increase the total platelet concentration of cAMP to $2.6 \pm 0.1 \mu$ M and cGMP to $1.1 \pm 0.1 \mu$ M (Fig. 5; assuming mean platelet volume 5.2 fl, Eigenthaler et al., 1992). In comparison, 0.5 to 1.0 mM of the cyclic nucleotide analogs was required to achieve the degree of platelet inhibition mediated by 4.0 μ M forskolin and 80 μ M YC-1. Furthermore, measurements of cAMP and cGMP levels in the presence of IBMX, a nonselective PDE inhibitor, demonstrate the importance of PDE in regulating basal and stimulated platelet cyclic nucleotide levels. In particular, the ability of 0.5 μ M forskolin to elevate cAMP is revealed only in the presence of IBMX (Fig. 5A), supporting the essential function of PDE in limiting the dispersion of cAMP from its signaling microdomain (Fischmeister et al., 2006; Zaccolo, 2006). We were unable to detect a similar relationship for cGMP with 10 μ M YC-1 (Fig. 5B). However, measurements of VASP phosphorylation clearly show that 10 μ M YC-1 as well as 0.5 μ M forskolin activate the cyclic nucleotide-dependent kinases (Fig. 6, A and B).

Earlier studies identified calcium mobilization and purinergic signaling as major contributors to platelet aggregation by PAR4 and demonstrated the dependence of PAR1 on a purinergic signaling component at threshold maximum levels of activation (Holinstat et al., 2006; Murugappa and Knapuli, 2006). Our kinetic analyses are consistent with these models of signaling by PAR1 and PAR4. Inhibition of PAR4 stimulated aggregation closely correlates with the inhibition of both calcium mobilization (Fig. 7, C and D) and dense granule release (Table 3; Supplemental Fig. 2, G and H). Calcium mobilization by PAR1 is not completely inhibited by either forskolin or YC-1 (Fig. 7, A and B). Rather, inhibition of PAR1-stimulated aggregation best correlates with the inhibition of dense granule release (Table 3; Supplemental Fig. 1, G and H). Cyclic nucleotide signaling pathways probably inhibit calcium mobilization by several mechanisms such as inhibition of phospholipase C β by reduced substrate (i.e.,

phosphatidylinositol 4,5-bisphosphate) resynthesis (Rynning et al., 1998) and phosphorylation-dependent association of inositol-1,4,5-triphosphate-receptor-associated PKG substrate protein with the inositol-1,4,5-triphosphate receptor (Antl et al., 2007). Calcium mobilization and protein kinase C activation are essential for dense granule release (Rendu and Brohard-Bohn, 2001), and cyclic nucleotide signals also appear to inhibit protein kinase C (Doni et al., 1988, 1991; Kroll et al., 1988). With inhibition of dense granule release, there is a loss signaling by small molecule potentiators of platelet activation, in particular, ADP. Inhibition of this purinergic signal likewise involves several mechanisms including inhibition of its canonical signaling pathways (Marquis et al., 1969; Mellion et al., 1981; Siegl et al., 1982; Teng et al., 1997).

At doses of PAR1-AP above the threshold maximum concentrations, PAR1 loses purinergic dependence and distinctly signals through a phosphatidic acid pathway that seems to involve phospholipase D (PLD; Holinstat et al., 2006, 2007), a ubiquitous enzyme implicated in a wide array of cellular actions related to the production of phosphatidic acid including vesicle trafficking in Golgi, exocytosis and endocytosis, mitogenesis, superoxide production, actin cytoskeletal rearrangements, and lysophosphatidic acid formation (Exton, 2002). Inhibition of platelet aggregation by forskolin and YC-1 persists at higher PAR1-AP concentrations (M.L. Bilodeau, unpublished data). In neutrophils, cAMP signaling pathways have been shown to inhibit PLD activity (for a recent example and discussion, see Jang et al., 2004). How cyclic nucleotide signals regulate PLD in platelets remains to be explored.

We are unable to conclude whether cAMP and cGMP engage distinct antiplatelet mechanisms. Such observations must, in any regard, be interpreted cautiously because the discrimination of cyclic nucleotide binding proteins for cAMP and cGMP is quite varied, and the degree of functional selectivity is confounded by compartmentation of cyclases and cyclic nucleotide effectors and by experimental conditions that generate high cAMP or cGMP levels (Francis et al., 2005). Moreover, cGMP has been shown to activate PKA in platelets, presumably by regulating PDE3 (Li et al., 2003a), suggesting that some cGMP inhibitory mechanisms involve convergence on cAMP signaling pathways. Consistent with this model, 80 μ M YC-1 stimulated a small, consistent elevation of cAMP that nearly reached statistical significance ($p < 0.076$; data not shown). The Du group (Li et al., 2003b; Li et al., 2004; Marjanovic et al., 2005) has recently reported that platelets have a time- and concentration-dependent biphasic response to cGMP. Although high concentrations of NO and cGMP inhibit platelet activation, low concentrations synthesized following platelet activation stimulate platelet secretion and second wave aggregation. These conclusions have been questioned by two independent groups using similar methods (Gambaryan et al., 2004; Marshall et al., 2004). We did not detect a cGMP contribution to platelet activation, although the significance of this observation is weakened by differences in methodology (e.g., use of YC-1 in time-dependent assays).

Numerous drugs are used in current medical practice to prevent platelet activation. New molecular targets are needed to develop a more universally effective antiplatelet drug with lower adverse events such as bleeding. Physiologic

platelet inhibitors act by increasing cyclic nucleotide levels and result in broad inhibition of platelet functions by interfering with multiple platelet activating pathways. Our studies support that calcium mobilization and dense granule release are key targets for the antiplatelet actions of cyclic nucleotides on thrombin signaling pathways. Specifically, PAR4 inhibition is dependent on the regulation of both calcium mobilization and dense granule release, and PAR1 inhibition is dependent predominantly on the regulation of dense granule release. These data also suggest that several of the cyclic nucleotide actions in platelets exist in tightly regulated microdomains that facilitate the rapid and targeted inhibition of specific platelet functions. Further delineation of these specific spatiotemporal relationships could identify novel molecular targets for a more selective platelet antagonism with improved clinical efficacy and less risk.

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