

# Mechanism of Activation and Functional Role of Protein Kinase C $\eta$ in Human Platelets\*<sup>§</sup>

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The novel class of protein kinase C (nPKC) isoform  $\eta$  is expressed in platelets, but not much is known about its activation and function. In this study, we investigated the mechanism of activation and functional implications of nPKC $\eta$  using pharmacological and gene knock-out approaches. nPKC $\eta$  was phosphorylated (at Thr-512) in a time- and concentration-dependent manner by 2MeSADP. Pretreatment of platelets with MRS-2179, a P2Y<sub>1</sub> receptor antagonist, or YM-254890, a G<sub>q</sub> blocker, abolished 2MeSADP-induced phosphorylation of nPKC $\eta$ . Similarly, ADP failed to activate nPKC $\eta$  in platelets isolated from P2Y<sub>1</sub> and G<sub>q</sub> knock-out mice. However, pretreatment of platelets with P2Y<sub>12</sub> receptor antagonist, AR-C69331MX did not interfere with ADP-induced nPKC $\eta$  phosphorylation. In addition, when platelets were activated with 2MeSADP under stirring conditions, although nPKC $\eta$  was phosphorylated within 30 s by ADP receptors, it was also dephosphorylated by activated integrin  $\alpha_{IIb}\beta_3$  mediated outside-in signaling. Moreover, in the presence of SC-57101, a  $\alpha_{IIb}\beta_3$  receptor antagonist, nPKC $\eta$  dephosphorylation was inhibited. Furthermore, in murine platelets lacking PP1 $\gamma$ , a catalytic subunit of serine/threonine phosphatase,  $\alpha_{IIb}\beta_3$  failed to dephosphorylate nPKC $\eta$ . Thus, we conclude that ADP activates nPKC $\eta$  via P2Y<sub>1</sub> receptor and is subsequently dephosphorylated by PP1 $\gamma$  phosphatase activated by  $\alpha_{IIb}\beta_3$  integrin. In addition, pretreatment of platelets with  $\eta$ -RACK antagonistic peptides, a specific inhibitor of nPKC $\eta$ , inhibited ADP-induced thromboxane generation. However, these peptides had no effect on ADP-induced aggregation when thromboxane generation was blocked. In summary, nPKC $\eta$  positively regulates agonist-induced thromboxane generation with no effects on platelet aggregation.

Platelets are the key cellular components in maintaining hemostasis (1). Vascular injury exposes subendothelial collagen that activates platelets to change shape, secrete contents of granules, generate thromboxane, and finally aggregate via activated  $\alpha_{IIb}\beta_3$  integrin, to prevent further bleeding (2, 3). ADP is a physiological agonist of platelets secreted from dense granules and is involved in feedback activation of platelets and hemo-

static plug stabilization (4). It activates two distinct G-protein-coupled receptors (GPCRs) on platelets, P2Y<sub>1</sub> and P2Y<sub>12</sub>, which couple to G<sub>q</sub> and G<sub>i</sub>, respectively (5–8). G<sub>q</sub> activates phospholipase C $\beta$  (PLC $\beta$ ), which leads to diacylglycerol (DAG)<sup>2</sup> generation and calcium mobilization (9, 10). On the other hand, G<sub>i</sub> is involved in inhibition of cAMP levels and PI 3-kinase activation (4, 6). Synergistic activation of G<sub>q</sub> and G<sub>i</sub> proteins leads to the activation of the fibrinogen receptor integrin  $\alpha_{IIb}\beta_3$ . Fibrinogen bound to activated integrin  $\alpha_{IIb}\beta_3$  further initiates feed back signaling (outside-in signaling) in platelets that contributes to the formation of a stable platelet plug (11).

Protein kinase Cs (PKCs) are serine/threonine kinases known to regulate various platelet functional responses such as dense granule secretion and integrin  $\alpha_{IIb}\beta_3$  activation (12, 13). Based on their structure and cofactor requirements, PKCs are divided into three classes: classical (cofactors: DAG, Ca<sup>2+</sup>), novel (cofactors: DAG) and atypical (cofactors: PIP<sub>3</sub>). PKC isoforms (14). All the members of the novel class of PKC isoforms (nPKC), viz. nPKC isoforms  $\delta$ ,  $\theta$ ,  $\eta$ , and  $\epsilon$ , are expressed in platelets (15), and they require DAG for activation. Among all the nPKCs, PKC $\delta$  (15, 16) and PKC $\theta$  (17–19) are fairly studied in platelets. Whereas nPKC $\delta$  is reported to regulate protease-activated receptor (PAR)-mediated dense granule secretion (15, 20), nPKC $\theta$  is activated by outside-in signaling and contributes to platelet spreading on fibrinogen (18). On the other hand, the mechanism of activation and functional role of nPKC $\eta$  is not addressed as yet.

PKCs are cytoplasmic enzymes. The enzyme activity of PKCs is modulated via three mechanisms (14, 21): 1) cofactor binding: upon cell stimulus, cytoplasmic PKCs mobilize to membrane, bind cofactors such as DAG, Ca<sup>2+</sup>, or PIP<sub>3</sub>, release autoinhibition, and attain an active conformation exposing catalytic domain of the enzyme. 2) phosphorylation: 3-phosphoinositide-dependent kinase 1 (PDK1) on the membrane phosphorylates conserved threonine residues on activation loop of catalytic domain; this is followed by autophosphorylation of serine/threonine residues on turn motif and hydrophobic region. These series of phosphorylations maintain an active conformation of the enzyme. 3) RACK binding: PKCs in active conformation bind receptors for activated C kinases (RACKs) and are lead to various subcellular locations to access the substrates (22, 23). Although various leading laboratories have elucidated the

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<sup>2</sup> The abbreviations used are: DAG, diacylglycerol; PI, phosphatidylinositol; PKC, protein kinase C; ERK, extracellular signal-regulated kinase.

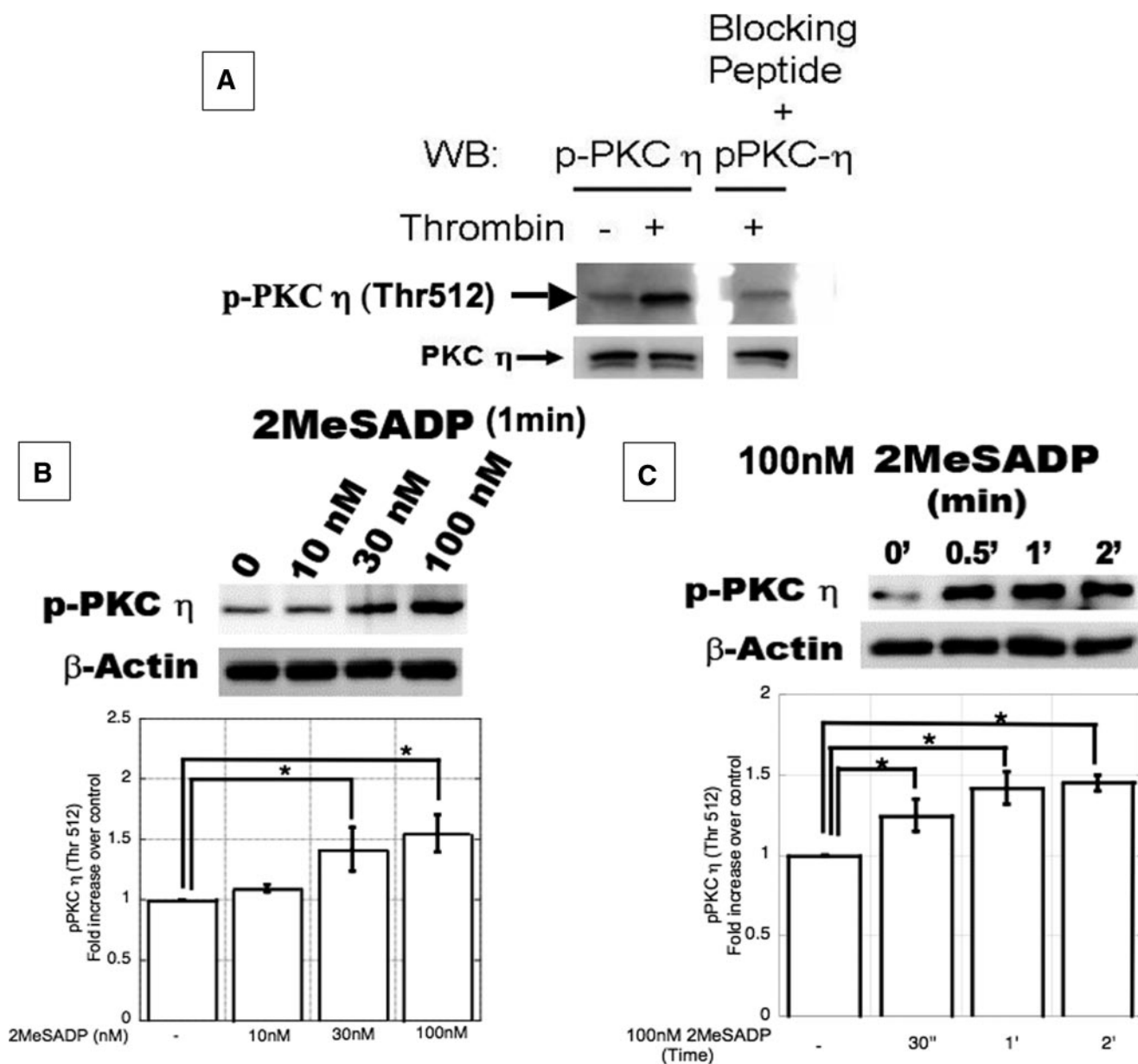


FIGURE 1. A, characterization of antiphospho-Thr-512 nPKC $\eta$  antibody. Washed and aspirin-treated human platelets were stimulated with 0.1 units/ml of thrombin for 30 s. The samples were subjected to SDS-PAGE in duplicates. Proteins were transferred on polyvinylidene difluoride membrane, and each membrane is subjected to immunoblotting with antiphospho-Thr-512 nPKC $\eta$  antibody or the same antibody was preincubated with the blocking peptide for 2 h.  $\beta$ -Actin was used to ensure equal protein concentrations in all lanes. B and C, ADP activates PKC $\eta$  in a concentration- and time-dependent manner. Washed and aspirin-treated human platelets were stimulated with increasing concentrations (B) of 2MeSADP for different time periods (C) under non-stirring conditions at 37 °C. The reaction was stopped by adding the Laemmli's buffer. The cell lysates were analyzed for Thr-512 phosphorylations on nPKC $\eta$  by Western blotting using phosphospecific antibodies as indicated.  $\beta$ -Actin was used to monitor protein concentrations in all lanes. The blot shown is representative of experiments performed using platelets from three different donors. Furthermore, data obtained from three different sets of experiments were quantified and expressed as mean  $\pm$  S.E.; \* indicates  $p < 0.05$ .

activation of PKCs, the mechanism of down-regulation of PKCs is not completely understood.

The premise of dynamic cell signaling, which involves protein phosphorylations by kinases and dephosphorylations by phosphatases has gained immense attention over recent years. PP1, PP2A, PP2B, PHLPP are a few of the serine/threonine phosphatases reported to date. Among them PP1 and PP2 phosphatases are known to regulate various platelet functional responses (24, 25). Furthermore, PP1c, is the catalytic unit of PP1 known to constitutively associate with  $\alpha_{IIb}$  and is activated upon integrin engagement with fibrinogen

and subsequent outside-in signaling (26). Among various PP1 isoforms, recently PP1 $\gamma$  is shown to positively regulate platelet functional responses (27). Thus, in this study we investigated if the above-mentioned phosphatases are involved in down-regulation of nPKC $\eta$ . Furthermore, reports from other cell systems suggest that nPKC $\eta$  regulates ERK/JNK pathways (28). In platelets ERK is known to regulate agonist induced thromboxane generation (29, 30). Thus, we also investigated if nPKC $\eta$  regulates ERK phosphorylation and thereby agonist-induced platelet functional responses.

In this study, we evaluated the activation of nPKC $\eta$  downstream of ADP receptors and its inactivation by an integrin-associated phosphatase PP1 $\gamma$ . We also studied if nPKC $\eta$  regulates functional responses in platelets and found that this isoform regulates ADP-induced thromboxane generation, but not fibrinogen receptor activation in platelets.

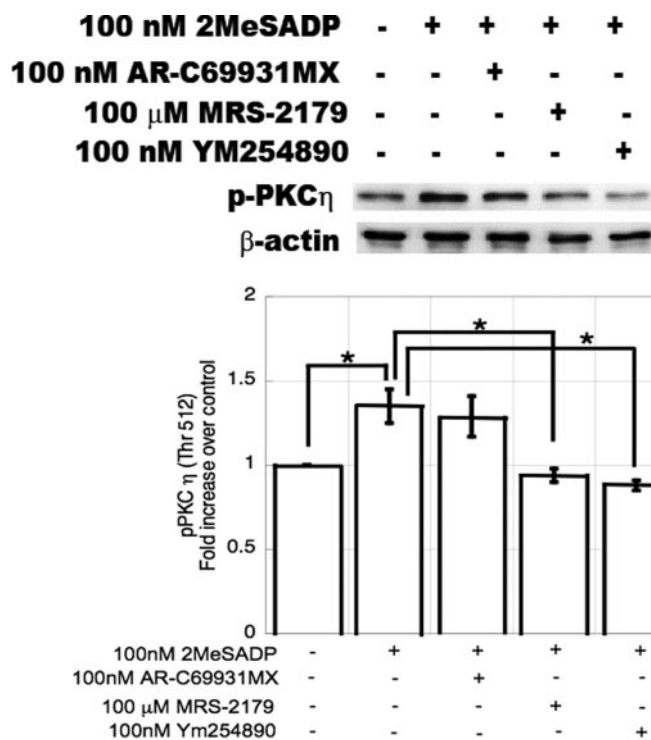
## EXPERIMENTAL PROCEDURES

Approval for this study was obtained from the Institutional Review Board of Temple University (Philadelphia, PA).

**Materials**—Apyrase (type VII), bovine serum albumin (fraction V), thrombin, 2MeSADP, MRS-2179 (*N*<sup>6</sup>-methyl-2'-deoxyadenosine-3',5'-bisphosphate) (tetra sodium salt), fibrinogen (type I), and acetylsalicylic acid were obtained from Sigma. Phospho-ERK antibodies against threonine 202 and tyrosine 204 residues and  $\beta$ -actin antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Alkaline phosphatase-labeled secondary antibody was from Kierkegaard & Perry Laboratories (Gaithersburg, MD). AR-C69931MX (*N*<sup>6</sup>-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)- $\beta$ , $\gamma$ -dichloromethylene ATP) (tetrasodium salt) was a kind gift from AstraZeneca (Loughborough, UK). YM-254890 was a generous gift from Yamanouchi Pharmaceutical (Ibaraki, Japan). SC-57101 was a gift from Searle and Co (Greenwich, CT). Phospho-PKC $\eta$  antibodies against p-Thr512 were custom made from 21<sup>st</sup> Century Biochemicals, Inc (Morlboro, MA).  $\eta$ -RACK antagonistic peptide and control peptide were a kind gift from Dr. Daria Mochly Rosen (Stanford University).

**Animals**—129/Sv mice carrying  $G\alpha_q$ -null mutation were obtained from Dr. T. Kent Gartner (31), with permission from Dr. Stefan Offermanns (University of Heidelberg, Heidelberg, Germany). CD-1 mice carrying PP1c $\gamma$ -null mutation were generated in the laboratory of Susannah Varmuza (University of Toronto) (32). C57/BL6 mice carrying P2Y<sub>1</sub>-null mutation were generated by subcontract with Lexicon Genetics Inc. (Woodlands, TX) through knock-out constructs as described previously (33). These P2Y<sub>1</sub> receptor-deficient mice were described previously by our group (29, 34–36).

**Isolation of Human Platelets**—All experiments using human subjects were performed in accordance with the Declaration of Helsinki. Whole blood was drawn from healthy, consenting human volunteers into tubes containing one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 ml of deionized water). Blood was centrifuged (Eppendorf 5810R centrifuge, Hamburg, Germany) at 230  $\times$  g for 20 min at room temperature to obtain platelet-rich plasma (PRP). If indicated, PRP was incubated with 1 mM acetylsalicylic acid (aspirin) for 30 min at 37  $^{\circ}$ C. The PRP was then centrifuged for 10 min at 980  $\times$  g at room temperature to pellet the platelets. Platelets were resuspended in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM glucose, 10 mM HEPES, pH 7.4, 0.2% bovine serum albumin) containing 0.1 units/ml apyrase. Cells were counted using the Coulter Z1 Particle Counter (Miami, FL), and concentration of cells was adjusted to 2  $\times$  10<sup>8</sup> platelets/ml. All experiments using washed platelets were performed in the absence of extracellular calcium unless otherwise mentioned.



**FIGURE 2. ADP activates nPKC $\eta$  via P2Y<sub>1</sub> receptor, which is coupled to  $G\alpha_q$ -pharmacological approach.** Washed and aspirin-treated human platelets were pretreated with 100 nM AR-C69931MX, a P2Y<sub>12</sub> antagonist, 100  $\mu$ M MRS-2179, a P2Y<sub>1</sub> antagonist or 100 nM YM-245890, a  $G_q$  blocker for 5 min (as indicated) and activated by 100 nM 2MeSADP under non-stirring at 37  $^{\circ}$ C. The reaction was stopped after 1 min by adding the Laemmli's buffer. The cell lysates were analyzed for Thr-512 phosphorylations on nPKC $\eta$  by Western blotting using phosphospecific antibodies as indicated. Total  $\beta$ -actin antibody was used to ensure equal protein concentrations in all lanes. The blot shown is representative of experiments performed using platelets from three different donors. Furthermore, data obtained from three different sets of experiments were quantified and expressed as mean  $\pm$  S.E.; \* indicates  $p < 0.05$ .

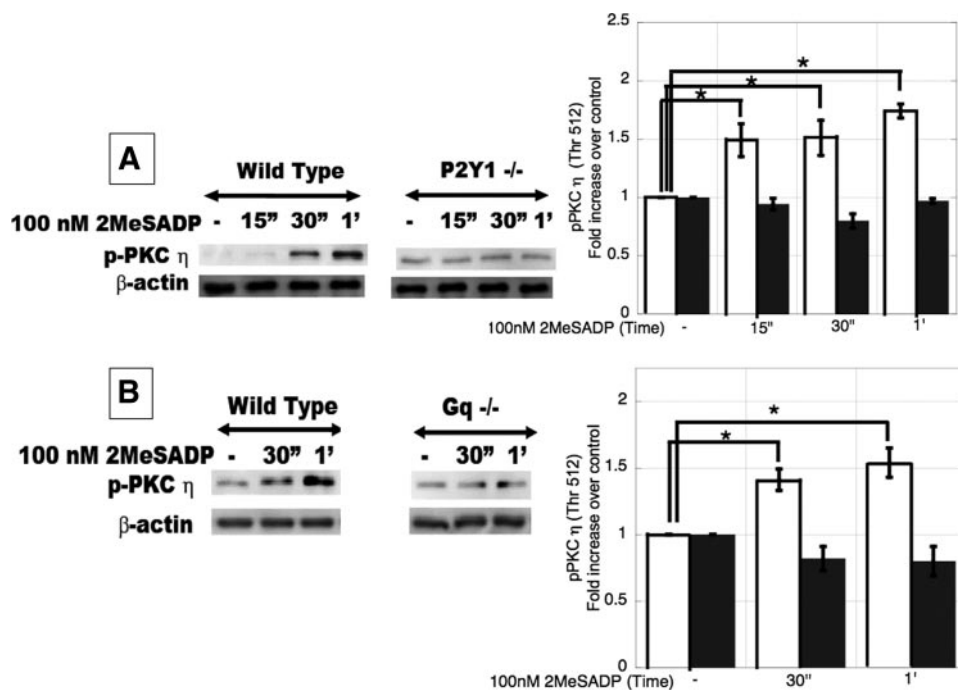
**Isolation of Mouse Platelets**—Blood was collected from the vena cava of anesthetized mice into syringes containing 1:10th blood volume of 3.8% sodium citrate as anticoagulant. Red blood cells were removed by centrifugation at 100  $\times$  g for 10 min. Platelet-rich plasma was recovered, and platelets were pelleted at 400  $\times$  g for 10 min. The platelet pellet was resuspended in Tyrode's buffer (pH 7.4) containing 0.01 units/ml apyrase. The washed platelets were subsequently used for experiments.

**Platelet Cell Lysates Preparation**—Platelets were stimulated with agonists for the appropriate time under non-stirring or stirring conditions at 37  $^{\circ}$ C. The reaction was stopped by the addition of 3 $\times$  SDS- Laemmli's buffer. Platelet lysates were boiled for 10 min and stored for Western blotting analysis.

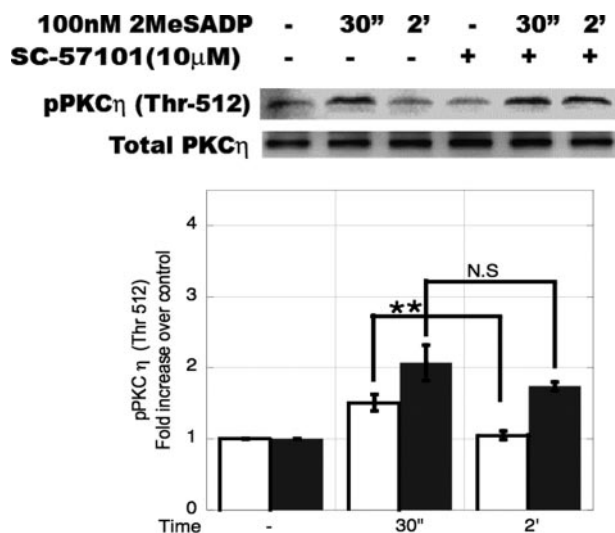
**Aggregometry**—Aggregation of 0.5 ml of washed platelets was analyzed using a P.I.C.A. lumiaggregometer (Chrono-log Corp. Havertown, PA). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37  $^{\circ}$ C. Each sample was allowed to aggregate for at least 3 min. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

**Measurement of Thromboxane A<sub>2</sub> Generation**—Washed human platelets without aspirin treatment were prepared as noted and brought to a concentration of 4  $\times$  10<sup>8</sup> platelets/ml. Stimulations were performed in a platelet aggregometer under stirring conditions (900 rpm) at 37  $^{\circ}$ C. The  $\eta$ -RACK antagonis-

## Regulation of nPKC $\eta$ Activation in Platelets



**FIGURE 3. ADP activates nPKC $\eta$  via P2Y $_1$  receptor coupled to G $\alpha_q$  protein-gene knock-out approach.** Washed platelets from P2Y $_1$  (Fig. 3A) or G $\alpha_q$  (Fig. 3B)-deficient mice (*dark bars*) and wild type littermates (*white bars*) were treated with 100 nM 2MeSADP under non-stirring conditions at 37 °C for different time periods as indicated. The reaction was stopped by adding the Laemmli's buffer. The cell lysates were analyzed for Thr-512 phosphorylations on nPKC $\eta$  by Western blotting using phosphospecific antibodies as indicated. Total  $\beta$ -actin antibody was used to ensure equal protein concentrations in all lanes. The blot shown is representative of experiments performed using platelets from three separate set of pooled blood from knock-out and wild type animals. Furthermore, data obtained from three different sets of experiments were quantified and expressed as mean  $\pm$  S.E.; \* indicates  $p < 0.05$ .



**FIGURE 4. nPKC $\eta$  is dephosphorylated by  $\alpha_{IIb}\beta_3$  mediated outside-in signaling.** Washed and aspirin-treated human platelets were treated with 100 nM 2MeSADP under stirring conditions, in the presence (*dark bars*) or absence (*white bars*) of 10  $\mu$ M SC-57101, an  $\alpha_{IIb}\beta_3$  antagonist at 37 °C for different time periods as indicated. The reaction was stopped by adding the Laemmli's buffer. The cell lysates were analyzed for Thr-512 phosphorylations on nPKC $\eta$  by Western blotting using phosphospecific antibodies as indicated. Total nPKC $\eta$  antibody was used to ensure equal protein concentrations in all lanes. The blot shown is representative of experiments performed using platelets from three different donors. Furthermore, data obtained from three different sets of experiments were quantified and expressed as mean  $\pm$  S.E.; \* indicates  $p < 0.05$ .

tic peptide and control peptide were added for 10 min before addition of the agonist. Stimulations were performed for 3.5 min, and the reaction was stopped by snap-freezing. Samples

were stored at  $-80$  °C until TXB $_2$  analysis was performed. Levels of TXB $_2$  were determined in duplicates using a Correlate-EIA Thromboxane B $_2$  Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI), according to the manufacturer's instructions. The mean  $\pm$  S.E. was derived from experiments performed in duplicate using platelets obtained from three independent donors.

**Western Blotting Analysis**—Lysates prepared from platelets were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites were blocked by incubation in Tris-buffered saline and Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% (v/v) Tween 20) containing 0.5% (w/v) milk protein and 3% (w/v) bovine serum albumin for 30 min at room temperature, and membranes were incubated overnight at 4 °C with the primary antibody (1:10,000 dilution in TBST with 2% bovine serum albumin) with gentle agitation.

After three washes for 5 min each with TBST, the membranes were probed with an alkaline phosphatase-labeled secondary antibody (1:5000 dilutions in TBST with 2% bovine serum albumin) for 1 h at room temperature. After additional washing steps, membranes were then incubated with CDP-Star chemiluminescent substrate (Tropix, Bedford, MA) for 10 min at room temperature, and immunoreactivity was detected using a Fuji Film Luminescent Image Analyzer (LAS-1000 CH, Japan).

**Statistical Analysis**—We have analyzed statistical significance of our data using paired Student's t-test or analysis of variance. Statistically significant data bearing  $p$  value  $< 0.05$  are annotated by an asterisk symbol. Data are expressed as mean  $\pm$  S.E.

## RESULTS

**Activation of nPKC $\eta$  Isoform by ADP**—Platelets express all the four nPKC isoforms, *viz.*  $\delta$ ,  $\theta$ ,  $\epsilon$ , and  $\eta$  (15). We have previously shown that ADP activates ERK and p38 MAP kinase in platelets (29, 37). It is also known that nPKC $\eta$  activates MAP kinases in keratinocytes (28) and other cell systems (38). Hence, we initiated our studies with evaluation of nPKC $\eta$  activation in platelets by ADP.

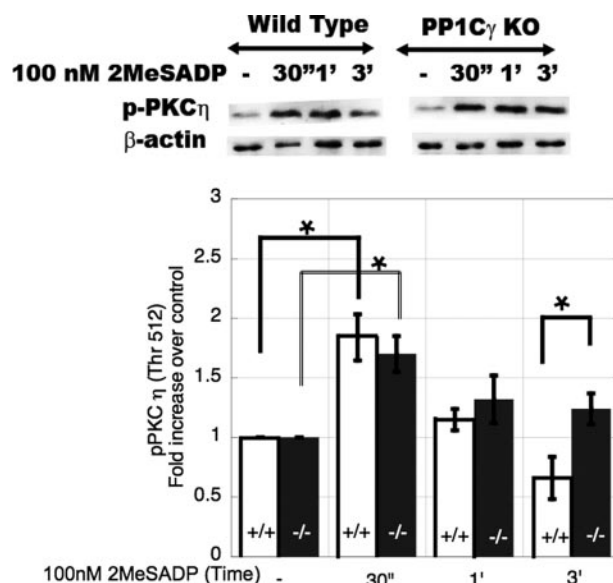
DAG binding to C1 domain followed by phosphorylation on conserved threonine residues in the activation loop primes activation of nPKC isoforms (14). As activation of nPKC $\eta$  is dependent on phosphorylation at Thr-512 (the consensus threonine residue (39)) in the activation loop, we first custom-synthesized and characterized the anti-phospho-Thr-512 nPKC $\eta$  antibody using the peptide GVTTA(pT)FCGTPD. Upon stimulation of aspirin-treated and washed platelets with thrombin

(0.1 units/ml), nPKC $\eta$  was activated as detected with the phosphospecific antibody (Fig. 1A). However, preincubation of the antibody with the peptide (Immunogen) used to generate antibodies in rabbits, the signal was blocked. These results confirm the specificity of the phosphospecific antibody against the Thr-512 of nPKC $\eta$ . The same antibody was used to study the activation of nPKC $\eta$  by ADP. When platelets were stimulated with different concentrations of 2MeSADP, an ADP analog, under non-stirring conditions for 1 min, nPKC $\eta$  was activated in a concentration-dependent manner (Fig. 1B). nPKC $\eta$  was also activated by 2MeSADP (100 nM) under non-stirring conditions in a time-dependent manner, with activation occurring as early as 30 s (Fig. 1C). Similar results were obtained with ADP as the agonist (data not shown).

**Role of P2Y<sub>1</sub> and P2Y<sub>12</sub> Receptors in Activation of nPKC $\eta$  by ADP**—ADP activates platelets via G<sub>q</sub>-coupled P2Y<sub>1</sub> receptor and G<sub>i</sub>-coupled P2Y<sub>12</sub> receptor (5, 6). The role of each of these receptors in activation of nPKC $\eta$  is evaluated using pharmacological and gene knock-out approaches. To evaluate the role of P2Y<sub>1</sub> and P2Y<sub>12</sub> receptor in activation of nPKC $\eta$ , we activated platelets with 2MeSADP in the presence of MRS-2179, a P2Y<sub>1</sub> receptor antagonist (40) and AR-C69331MX, a P2Y<sub>12</sub> antagonist (4) under non-stirring conditions. As shown in Fig. 2, pretreatment of platelets with MRS-2179 abolished 2MeSADP-induced nPKC $\eta$  activation. On the other hand, pretreatment of platelets with AR-C69331MX, had minimal effect on ADP-induced nPKC $\eta$  activation (Fig. 2). Furthermore, P2Y<sub>1</sub> receptor couples to G<sub>q</sub>, which leads to PLC activation, DAG generation, and calcium mobilization (6). To evaluate whether G<sub>q</sub> pathway, downstream of P2Y<sub>1</sub> receptor, causes nPKC $\eta$  activation, we pretreated platelets with YM-254890, which prevents G<sub>q</sub> coupling to GPCRs (41) and activated with 2MeSADP under non-stirring conditions. YM-254890 has been successfully used in platelets to block G<sub>q</sub> signaling pathways (42). As shown in Fig. 2, YM-254890 abolished ADP-induced nPKC $\eta$  activation. These results suggest that ADP activates PKC $\eta$  via P2Y<sub>1</sub> receptor coupled to G<sub>q</sub>.

Complementary to the pharmacological approach, we also evaluated the role of P2Y<sub>1</sub>-coupled G<sub>q</sub> pathway in activation of nPKC $\eta$  using P2Y<sub>1</sub> and G<sub>q</sub> knock-out mice. Platelets isolated from P2Y<sub>1</sub>, G<sub>q</sub> knock-out mice and wild type littermates were activated with 2MeSADP under non-stirring conditions for different time periods and phosphorylation of nPKC $\eta$  was studied using anti-phospho PKC $\eta$  antibody. As shown in Fig. 3A, while 2MeSADP activated nPKC $\eta$  in wild type murine platelets in a time-dependent manner, it failed to activate nPKC $\eta$  in P2Y<sub>1</sub> knock-out murine platelets. Furthermore, as shown in Fig. 3B, 2MeSADP also failed to activate nPKC $\eta$  in G<sub>q</sub> knock-out murine platelets, in comparison with wild type murine platelets (Fig. 3B). These data further confirm that ADP activates nPKC $\eta$  via P2Y<sub>1</sub> receptor coupled to G<sub>q</sub>.

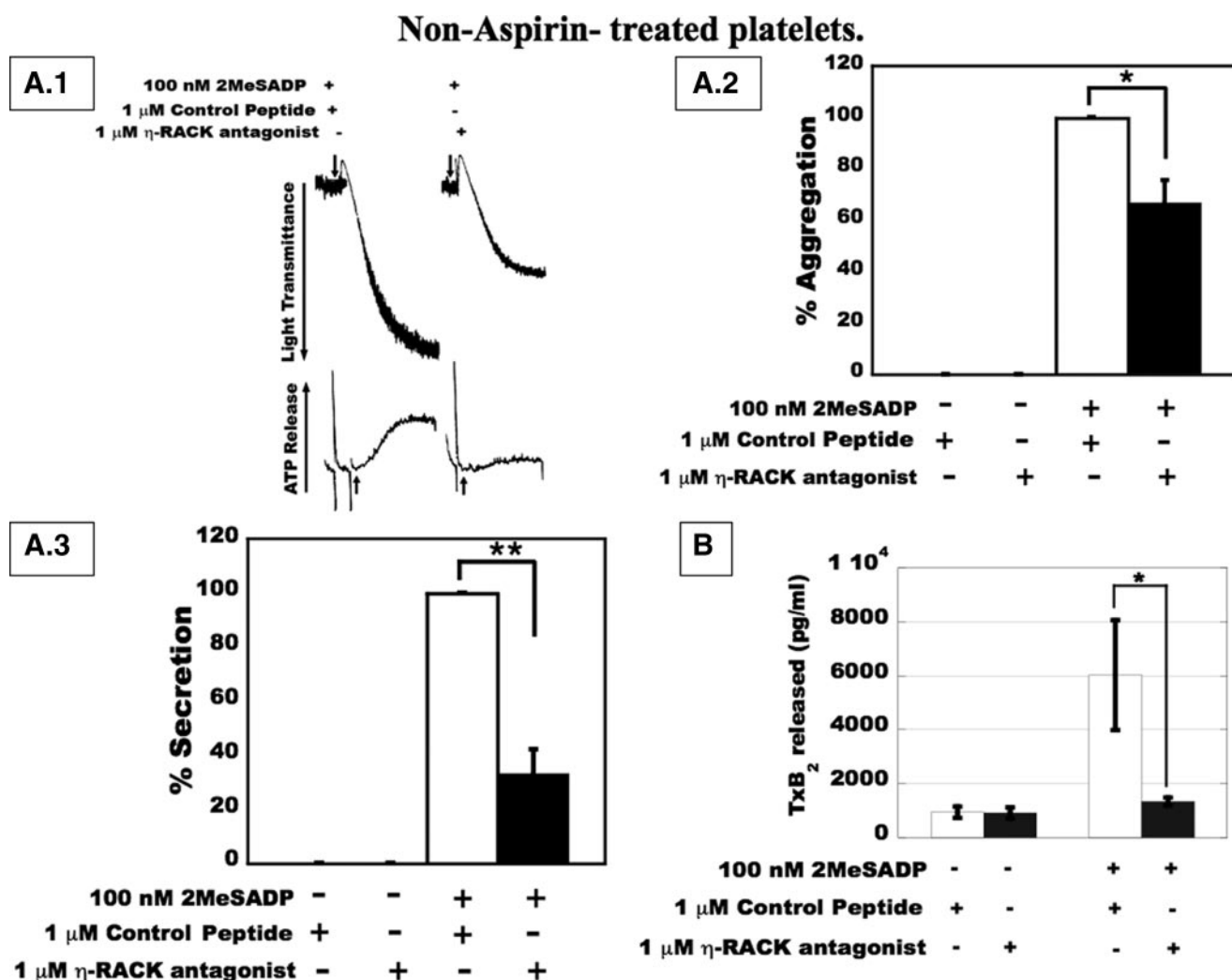
**Role of Activated  $\alpha_{IIb}\beta_3$  Integrin Signaling in Activation of nPKC $\eta$** —Signaling cascades initiated from P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors lead to integrin  $\alpha_{IIb}\beta_3$  activation (5, 6). Once activated,  $\alpha_{IIb}\beta_3$  binds its ligand, fibrinogen, and initiates outside-in signaling cascade, leading to PLC $\gamma$  activation, DAG generation, and Ca<sup>2+</sup> mobilization (11). To evaluate the role of outside-in signaling in activation of nPKC $\eta$ , platelets were activated by ADP with fibrinogen under stirring conditions. Under



**FIGURE 5.  $\alpha_{IIb}\beta_3$ -mediated integrin outside-in signaling dephosphorylates nPKC $\eta$  via PP1 $\gamma$  phosphatase.** Washed platelets from PP1 $\gamma$ -deficient mice (dark bars) and wild type littermates (white bars) were treated with 100 nM 2MeSADP under stirring conditions at 37 °C for different time periods as indicated. The reaction was stopped by adding the Laemmli's buffer. The cell lysates were analyzed for Thr-512 phosphorylations on nPKC $\eta$  by Western blotting using phosphospecific antibodies as indicated.  $\beta$ -actin was used to ensure equal protein concentrations in all lanes. The blot shown is representative of experiments performed using platelets from three separate set of pooled blood from knock-out animals. Furthermore, data obtained from three different sets of experiments were quantified and expressed as mean  $\pm$  S.E.; \* indicates  $p < .05$ .

stirring conditions, activated integrin  $\alpha_{IIb}\beta_3$  binds fibrinogen and initiates outside-in signaling. As shown in Fig. 4, 2MeSADP not only phosphorylated nPKC $\eta$  within 30 s, it also dephosphorylated nPKC $\eta$  by 2 min. These data indicate that nPKC $\eta$  is activated and also inactivated downstream of ADP receptors. To confirm whether the dephosphorylation of nPKC $\eta$  is a consequence of activated integrin  $\alpha_{IIb}\beta_3$ , we used SC-57101, an integrin  $\alpha_{IIb}\beta_3$  (fibrinogen receptor) antagonist. Pretreatment of platelets with SC-57101 inhibited ADP-induced dephosphorylation of nPKC $\eta$  at 2 min (Fig. 4) under stirring conditions. These data indicate a temporal phosphorylation pattern for nPKC $\eta$ , wherein, the initial phosphorylation of nPKC $\eta$  caused by ADP receptors is followed by a dephosphorylation that is mediated by integrin  $\alpha_{IIb}\beta_3$  mediated outside-in signals.

**Role of PP1 $\gamma$ , a Serine/Threonine Phosphatase in Dephosphorylation of nPKC $\eta$** —Serine/threonine phosphatases are reported to regulate PKCs in other cell systems. For example, PKC $\alpha$  is regulated by PP2B in endothelial cells (43), PP2A is reported to regulate atypical PKC isoforms in epithelial cells (44). PP1c is a catalytic subunit of PP1, a serine/threonine phosphatase that constitutively associates with  $\alpha_{IIb}$  tail (26) in integrin  $\alpha_{IIb}\beta_3$  complex. Upon activation and fibrinogen (ligand) binding to integrin  $\alpha_{IIb}\beta_3$ , PP1c dissociates from  $\alpha_{IIb}\beta_3$  and becomes catalytically active (26). Among different PP1 isoforms (PP1 $\alpha$ , PP1 $\beta$ , PP1 $\gamma$ ), PP1 $\gamma$  is known to regulate platelet functional responses (27). Because of the unavailability of specific pharmacological inhibitors for the above-mentioned phosphatases, we adapted gene knock-out approach to study the role of phosphatases in regulation of nPKC $\eta$  activation. We

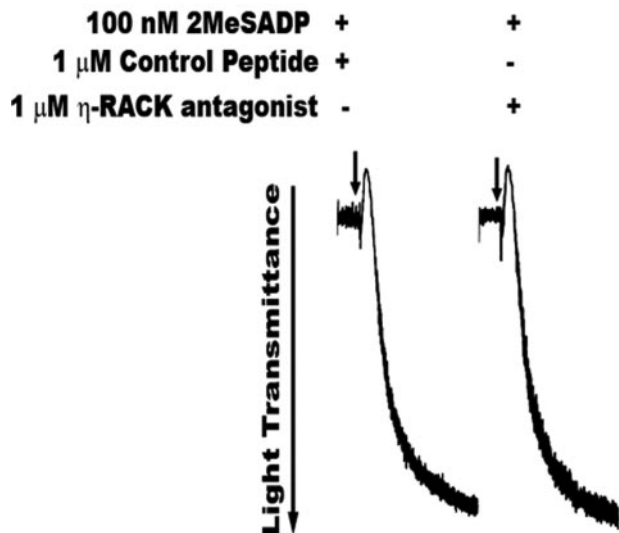


**FIGURE 6. nPKC $\eta$  positively regulates ADP-induced thromboxane generation.** Washed and non-aspirin-treated platelets were pretreated with 1  $\mu$ M  $\eta$ -RACK antagonistic peptide or control peptide for 10 min at 37 °C and were activated by 100 nM 2MeSADP. *A, A1*, representative aggregation (measured as deflections in light transmission using aggregometry), and secretion (measured as ATP release using lumichrome assay) tracings of non-aspirin-treated platelets activated by ADP. The % change in aggregation and secretion upon treatment with  $\eta$ -RACK antagonist compared with control is represented in *A2* and *A3*, respectively. *B*, graphical representation of thromboxane generated in non-aspirin-treated platelets activated by ADP, as measured using ELISA. The graphs are representative of data drawn from three separate experiments conducted using blood from three different donors. Data are expressed as mean  $\pm$  S.E.; \* indicates  $p$  value  $<0.05$ .

investigated whether integrin  $\alpha_{IIb}\beta_3$  engagement during stirring conditions, dephosphorylates nPKC $\eta$  via PP1 $\gamma$  phosphatase using PP1 $\gamma$  knock-out mice. Platelets isolated from PP1 $\gamma$  knock-out mice and wild type littermates were activated by 2MeSADP under stirring conditions. As shown in Fig. 5, ADP caused transient phosphorylation of nPKC $\eta$  in wild type murine platelets. However, in murine platelets lacking PP1 $\gamma$ , ADP caused sustained nPKC $\eta$  phosphorylation. These results suggest that binding of fibrinogen to integrin  $\alpha_{IIb}\beta_3$  dephosphorylates nPKC $\eta$  via PP1 $\gamma$ . Furthermore, it should also be noted that activated  $\alpha_{IIb}\beta_3$  integrin-induced dephosphorylation was only partially rescued in PP1 $\gamma$  knock-out mice. These data indicate that other isoforms of PP1 phosphatase may also be involved in dephosphorylation of nPKC $\eta$ . However, as PP1 $\alpha$  and PP1 $\beta$  knockouts are embryonic lethal, we could not evaluate the role of these phosphatases in dephosphorylation of nPKC $\eta$ .

*Role of nPKC $\eta$  in Agonist-induced Platelet Functional Responses*—Previous reports from other cell systems suggest that nPKC $\eta$  regulates MAP kinases such as ERK (28). We have previously demonstrated that ERK is involved in agonist-induced thromboxane generation (29). Thus, we evaluated the role of nPKC $\eta$  in ADP-induced thromboxane generation. We utilized  $\eta$ -RACK antagonistic peptides in our studies. These peptides are designed to bind intracellular RACKs, the proteins involved in transportation of activated PKC from the membrane to the intracellular substrate. Therefore, although the enzyme (nPKC $\eta$ ) is activated (phosphorylated), it cannot render its catalytic activity due to unavailability of its substrate and as a consequence corresponding signaling pathway is inhibited (45, 46). The peptides designed with similar strategy have been successfully used in various studies in other cell systems (46, 47). We evaluated the functional role of nPKC $\eta$  in agonist-induced platelet functional responses by pretreating

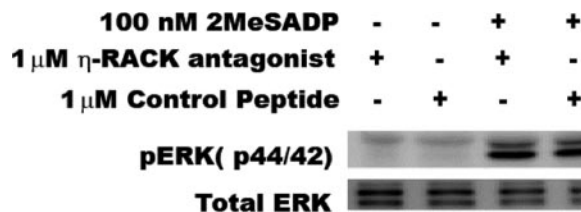
## Aspirin-treated platelets.



**FIGURE 7. nPKC $\eta$  has no effect on ADP-induced platelet aggregation and dense granule secretion.** Washed and aspirin-treated platelets were pretreated with 1  $\mu$ M  $\eta$ -RACK antagonistic peptide or control peptide for 10 min at 37 °C were activated by 100 nM 2MeSADP for 3 min. Shown are the representative aggregation tracings of three separate experiments conducted using blood drawn from three different donors.

non-aspirin-treated platelets with  $\eta$ -RACK antagonistic peptides and activating with 2MeSADP. As shown in Fig. 6, 2MeSADP-induced aggregation (Fig. 6A) and thromboxane generation (Fig. 6B) were inhibited in platelets pretreated with  $\eta$ -RACK antagonistic peptide compared with the platelets pretreated with equimolar control peptide. Furthermore, inhibition of thromboxane generation is also evident by the fact that ADP-induced dense granule secretion (measured as ATP release in Fig. 6A) in non-aspirin-treated platelets, which is solely dependent on thromboxane generation (48) is also inhibited. In addition, the peptides by itself had no effect on platelet aggregation and thromboxane generation. These data suggest that nPKC $\eta$  positively regulates ADP-induced thromboxane generation.

We further evaluated whether nPKC $\eta$  directly regulates ADP-induced platelet aggregation, independent of feedback effects from inhibited thromboxane, using aspirin-treated platelets. Aspirin is a well established cyclooxygenase (COX) inhibitor, which abolishes thromboxane generation, when used under the working conditions described under "Experimental Procedures" (37, 49). Aspirin-treated platelets were pretreated with  $\eta$ -RACK antagonistic peptides or control peptide and activated by 2MeSADP. As shown in Fig. 7, ADP-induced platelet aggregation was same in both platelets pretreated with  $\eta$ -RACK antagonistic peptides and control peptides. Thromboxane generation was completely inhibited in aspirin-treated platelets upon stimulation with 2MeSADP (data not shown). These results suggest that nPKC $\eta$  has no direct effect on ADP-induced aggregation. In addition, these data also confirm that the decrease in platelet aggregation observed in non-aspirin-treated platelets pretreated with  $\eta$ -RACK antagonist (Fig. 6A) is only due to decrease in thromboxane generation, which in turn results in decreased thromboxane-induced aggregation.



**FIGURE 8. nPKC $\eta$  does not regulate agonist-induced thromboxane generation via ERK pathway.** Washed and aspirin-treated platelets were pretreated with 1  $\mu$ M  $\eta$ -RACK antagonistic peptide or control peptide for 10 min at 37 °C were activated by 100 nM 2MeSADP for 1 min, and the reaction was stopped by adding the Laemmli's buffer. The cell lysates were subjected to Western blot analysis and ERK activation was analyzed using phospho-ERK antibody. Total ERK antibody was used to ensure equal protein concentrations in all lanes. The blot shown is representative of experiments performed using platelets from three separate donors.

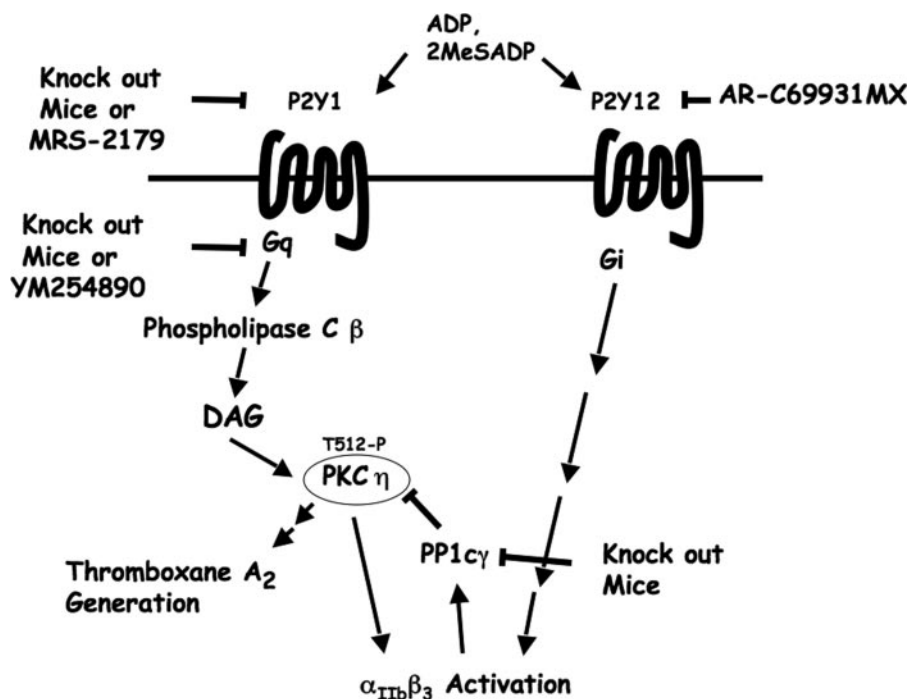
*Molecular Mechanism By Which nPKC $\eta$  Regulates Agonist-induced Functional Responses*—Previous reports suggest that agonist-induced thromboxane generation is regulated by ERK (29). Furthermore, results from Fig. 6A, suggest that nPKC $\eta$  positively regulates thromboxane generation. Thus, we evaluated if nPKC $\eta$  regulates thromboxane generation in platelets by regulating ERK. Aspirin-treated platelets pretreated with  $\eta$ -RACK antagonistic and control peptides were activated by 2MeSADP. The extent of activation of ERK was measured by Western blotting analysis using phospho-ERK antibody. As shown in Fig. 8, phosphorylation of ERK was not affected in platelets pretreated with  $\eta$ -RACK antagonist or control peptides. These data suggest that nPKC $\eta$  does not regulate thromboxane generation through ERK.

## DISCUSSION

The mechanism of activation of PKCs has been extensively studied in various cell systems including platelets. However, the mechanism by which they are inactivated is not completely understood. In this study, we demonstrate a novel mechanism of inactivation of nPKC $\eta$  isoform by integrin-associated serine/threonine phosphatase. Furthermore, although the role of some PKC isoforms in agonist-induced platelet functional responses have been previously studied, the role of nPKC $\eta$  in platelets has not been studied. In this study, we demonstrated that ADP activates nPKC $\eta$  via P2Y<sub>1</sub> receptor coupled to G<sub>q</sub>. As expected, G<sub>i</sub> pathway, which does not generate DAG or mobilize calcium, has no role in regulation of nPKC $\eta$ . Furthermore, nPKC $\eta$  positively regulates ADP-induced thromboxane generation without directly affecting ADP-induced aggregation. Finally, we show that upon activation of platelets,  $\alpha_{IIb}\beta_3$  mediated outside-in signaling dephosphorylates nPKC $\eta$  through PP1 $\gamma$  phosphatase.

Recent reports suggest that following activation, PKCs are subjected to lysosomal or proteasomal degradation involving ubiquitination (50–52). However, such proteasomal degradation is possibly not occurring in platelets, as ubiquitinated nPKC $\eta$  bands, which typically appear as a ladder, were not observed. (Figs. 4 and 5). Furthermore, total nPKC $\eta$  levels remains constant upon integrin signaling (as studied using anti-PKC $\eta$  antibody, Fig. 4). Hence, PKC $\eta$  is not inactivated by ubiquitin-mediated degradation in platelets. We have previously demonstrated that although Syk is ubiquitinated

## Regulation of nPKC $\eta$ Activation in Platelets



**FIGURE 9. Model depicting pathways involved in activation and inactivation of nPKC $\eta$ .** nPKC $\eta$  is activated by ADP via P2Y<sub>1</sub> receptor coupled to the G<sub>q</sub> pathway. Furthermore, nPKC $\eta$  is down-regulated by activated integrin  $\alpha_{IIb}\beta_3$  induced outside-in signaling via PP1 $\gamma$  phosphatase. Upon activation, nPKC $\eta$  positively regulates ADP-induced thromboxane generation without affecting platelet aggregation and dense granule secretion.

upon stimulation by collagen, it does not lead to its degradation in platelets (53).

We have also evaluated the role of nPKC $\eta$  using  $\eta$ -RACK antagonistic peptides that interfere with enzyme-substrate interaction. Similar antagonistic peptides have been successfully used in various cell systems such as cardiomyocytes (46) and neuronal cells (54). Using  $\eta$ -RACK antagonists we have demonstrated that nPKC $\eta$  positively regulates agonist-induced thromboxane generation (Fig. 6) with no effect on agonist-induced platelet aggregation (Fig. 7). The peptides were targeted in to the cell using TAT carrier protein, which is also used as a negative control for these experiments. The specificity of  $\eta$ -RACK antagonistic peptides is further elucidated by the fact that they do not affect the platelet aggregation (Fig. 7). Downstream of ADP receptors platelet aggregation is regulated by calcium and other PKCs such as cPKC $\alpha$ . Thus, as the antagonistic peptides did not affect platelet aggregation, its effect on other molecular events could be ruled out. Furthermore the nPKC $\delta$  and  $\theta$  are neither activated by ADP nor regulate ADP-induced functional responses (19, 20). Thus, the effects observed upon pretreatment with nPKC $\eta$  RACK antagonist could primarily be because of its interaction with nPKC $\eta$ .

In platelets, ADP-induced thromboxane generation is regulated by P2Y<sub>1</sub>, P2Y<sub>12</sub>, and  $\alpha_{IIb}\beta_3$  receptor-mediated signaling (55). Furthermore, in our previous studies we have shown that ERK is a positive regulator of agonist-induced thromboxane generation in platelets, and it requires signaling from both P2Y<sub>1</sub> and P2Y<sub>12</sub> receptor-mediated pathways for its activation (29, 30). As nPKC $\eta$  regulates ADP-induced thromboxane generation, we further investigated if the mech-

anism is by regulating P2Y<sub>1</sub> or P2Y<sub>12</sub> signaling. However, nPKC $\eta$  does not regulate thromboxane generation via ERK (Fig. 8). Furthermore, to evaluate if P2Y<sub>12</sub> receptor-mediated, G<sub>i</sub> pathway alone is regulated by nPKC $\eta$ , we studied Akt activation and measured decrease in cAMP levels. We chose to conduct these studies, as Akt activation and decrease in cAMP production are solely dependent on G<sub>i</sub> signaling (35). However, inhibition of nPKC $\eta$  by  $\eta$ -RACK antagonistic peptides did not affect cAMP levels or Akt activation in aspirin-treated platelets activated by ADP (data not shown). Thus nPKC $\eta$  appears to regulate thromboxane generation via an unknown mechanism possibly mediated by outside-in signaling, since the P2Y<sub>1</sub> and P2Y<sub>12</sub> receptor-mediated signaling is not affected.

In platelets, PP1c positively regulates agonist-induced platelet functional responses (27). Our data

show that nPKC $\eta$  also positively regulates ADP-induced thromboxane generation. In addition, PP1c dephosphorylates nPKC $\eta$ . We believe that PP1c regulation of platelet function is not through nPKC $\eta$ . We demonstrated that nPKC $\eta$  is activated within 30 s (Fig. 1C) of agonist-induced platelet stimulation. In addition, nPKC $\eta$  is dephosphorylated by PP1 $\gamma$  phosphatase, activated by  $\alpha_{IIb}\beta_3$  integrin by 2 min after agonist-induced platelet activation. Thus, as nPKC $\eta$  phosphorylation precedes PP1 $\gamma$  activation and, hence, we predict that the molecular mechanism by which PP1c regulates platelet functional responses is not via nPKC $\eta$ . Rather PP1 $\gamma$ -mediated dephosphorylation of nPKC $\eta$  is probably the mechanism by which catalytic activity of nPKC $\eta$  is regulated in platelets.

In addition, deletion of PP1 $\gamma$  does not completely rescue activated  $\alpha_{IIb}\beta_3$  integrin-induced dephosphorylation of nPKC $\eta$  (Fig. 5). These data leave us with the possibility that the other isoforms of PP1c such as PP1 $\alpha$  and PP1 $\beta$  might also be involved in dephosphorylation of nPKC $\eta$ .

In summary, nPKC $\eta$  is activated by ADP via P2Y<sub>1</sub> receptor. Once activated it is also dephosphorylated by integrin  $\alpha_{IIb}\beta_3$  via PP1 $\gamma$  phosphatase. Furthermore, activated nPKC $\eta$  positively regulates ADP-induced thromboxane generation with no effect on aggregation. In addition, nPKC $\eta$  possibly regulates thromboxane generation via an unknown pathway downstream of integrin  $\alpha_{IIb}\beta_3$  (Fig. 9).

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