G_q pathway regulates proximal C-type lectin-like receptor-2 (CLEC-2) signaling in platelets

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Platelets play a key role in the physiological hemostasis or pathological process of thrombosis. Rhodocytin, an agonist of the C-type lectin-like receptor-2 (CLEC-2), elicits powerful platelet activation signals in conjunction with Src family kinases (SFKs), spleen tyrosine kinase (Syk), and phospholipase γ2 (PLCγ2). Previous reports have shown that rhodocytin-induced platelet aggregation depends on secondary mediators such as thromboxane A2 (TxA2) and ADP, which are agonists for G-protein-coupled receptors (GPCRs) on platelets. How the secondary mediators regulate CLEC-2-mediated platelet activation in terms of signaling is not clearly defined. In this study, we report that CLEC-2-induced Syk and PLCγ2 phosphorylation is potentiated by TxA2 and that TxA2 plays a critical role in the most proximal event of CLEC-2 signaling, *i.e.* the CLEC-2 receptor tyrosine phosphorylation. We show that the activation of other GPCRs, such as the ADP receptors and protease-activated receptors, can also potentiate CLEC-2 signaling. By using the specific G_q inhibitor, UBO-QIC, or G_q knock-out murine platelets, we demonstrate that G_q signaling, but not other G-proteins, is essential for GPCR-induced potentiation of Syk phosphorylation downstream of CLEC-2. We further elucidated the signaling downstream of G_q and identified an important role for the PLCβ-PKCα pathway, possibly regulating activation of SFKs, which are crucial for initiation of CLEC-2 signaling. Together, these results provide evidence for novel G_q-PLCβ-PKCα-mediated regulation of proximal CLEC-2 signaling by G_q-coupled receptors.

Platelets play a key role in the physiological hemostasis or pathological process of thrombosis. The multiple cell-surface receptors that are capable of activating platelets fall into two main categories depending on whether they signal through activation of heterotrimeric G-proteins, such as $G\alpha_q$ and $G\alpha_i$, or non-receptor tyrosine kinases (NRTKs)[3] such as Src family kinases (SFKs) and spleen tyrosine kinase (Syk) (1). The major platelet G-protein-coupled receptors (GPCRs) include the $P2Y_1$ and $P2Y_{12}$ receptors for adenosine 5′-diphosphate (ADP), the PARs for thrombin (PAR1 and PAR3 or -4), and the thromboxane/prostaglandin endoperoxide receptor for thromboxane A2. The major NRTK-coupled platelet-activating receptors include the glycoprotein VI (GPVI)/Fc receptor γ-chain (GPVI-FcRγ) collagen receptor complex, the C-type lectin-like receptor-2 (CLEC-2) for podoplanin, and (in humans) the low affinity receptor for the Fc portion of the immunoglobulin γ heavy chain FcγRIIA (2).

GPCRs and NRTK-coupled receptors are transmembrane receptors that initiate intracellular signaling cascades in response to an array of ligands. It has been reported that the signal transduction initiated by these receptors is not organized in distinct signaling units in a linear manner (3, 4). In fact, signal integration and full activation of platelets result from a complex network involving cross-communication between separate signaling units. Signaling pathways regulate each other (cross-talk), and this is an established phenomenon. The cross-talk between the G_q pathways and G_i pathways has been studied in platelets, and it was shown that G_i pathways regulate G_q-mediated Rho kinase activation (5). There are also reports of the angiotensin receptor (a GPCR) trans-activating a receptor tyrosine kinase involving the release of reactive oxygen species and activation of PKCs and Src (6). GPCR cross-talk with hemITAM receptors in platelets has not been evaluated to date and the mechanism involved is not known.

Platelets release secondary mediators upon activation, which play a critical positive feedback role in mediating platelet activation by all agonists. Platelets are initially activated upon vascular injury by exposed subendothelial collagen through activation of GPVI. It is known that collagen-induced platelet aggregation activation depends on positive feedback by either generated agonists, such as thromboxane (7) and thrombin, or released agonists such as ADP (8, 9). The initial activation of platelets by GPVI is thus greatly amplified by the GPCR ago-

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[3] The abbreviations used are: NRTK, non-receptor tyrosine kinase; SFK, Src family kinase; Syk, spleen tyrosine kinase; PLCγ2, phospholipase γ2; TxA2, thromboxane A2; GPCR, G-protein-coupled receptor; PAR, protease-activated receptor; CLEC-2, C-type lectin-like receptor-2; PMA, phorbol 12-myristate 13-acetate; ITAM, immunoreceptor tyrosine activation motif; CRP, collagen-related peptide; 2MeSADP, 2-methylthio-ADP.

nists that are known to activate and recruit other circulating platelets to the site of injury. Similar dependence on secondary mediators was also reported for CLEC-2-mediated platelet aggregation (9).

CLEC-2 is a type II transmembrane protein that is highly expressed on the surface of platelets and megakaryocytes as non-covalent homodimers and at lower levels on several other hematopoietic lineages, including monocytes and dendritic cells (10). CLEC-2 is a hemITAM (immunoreceptor tyrosine activation motif)-containing receptor that has only one ITAM motif (Y*XX*(L/I)) in its cytoplasmic domain, unlike the GPVI receptor, which contains two such motifs. CLEC-2 was identified by affinity chromatography as a ligand for the snake venom protein rhodocytin, purified from the Malayan pit viper, *Calloselasma rhodostoma* (11). Independent crystal structures of rhodocytin show that it assembles as a tetramer, which leads to the suggestion that it mediates activation of CLEC-2 through clustering (12, 13). Consistent with this clustering model of activation, whole antibodies and $F(ab)_2$ fragments have been reported to activate CLEC-2. The activation of CLEC-2 by its physiological agonist podoplanin, expressed on lymphatic endothelial cells, is essential for the separation and maintenance of the lymphatic system and blood vasculature (14, 15). An interesting aspect of the CLEC-2 hemITAM is the presence of a single tyrosine motif in its cytoplasmic domain, which upon phosphorylation by SFKs activates Syk (16). We reported that the signaling downstream of CLEC-2 is distinct from GPVI and that PI3K/Tec kinases play an upstream role in Syk activation upon CLEC-2 stimulation (17).

In this study, we propose to investigate the mechanism of cross-talk between G-protein-coupled receptors and CLEC-2 during platelet activation induced by rhodocytin. We show that CLEC-2 signaling is dependent on thromboxane generation and is potentiated by co-stimulation with different GPCR agonists. We also report here that the G_q-PLCβ-PKC pathway, upon GPCRs stimulation, regulates the signaling events downstream of the CLEC-2 receptor via activation of Lyn, leading to enhanced CLEC-2 tyrosine phosphorylation and thus enhanced signaling.

Results

Non-aspirinated platelets respond more robustly to rhodocytin than aspirin-treated platelets

Platelets, when stimulated with the CLEC-2 agonist rhodocytin, results in a characteristic lag phase before they start aggregating. However, we observed that this lag phase was prolonged when washed human platelets were pre-treated with aspirin, as compared with platelets that were not treated with aspirin as shown in Fig. 1*A*. A similar delay in aggregation was observed when washed human platelets without aspirin were treated with indomethacin (Fig. 1*A*). Given that both aspirin and indomethacin inhibit thromboxane generation by inhibiting COX-1, this suggests that thromboxane plays an important role in rhodocytin-induced aggregation.

To study the possible differences in the signaling involved upon stimulation with 30 nM rhodocytin under aspirin and no-aspirin conditions, we evaluated the time course of Syk phosphorylation at tyrosine 525/526 and PLCγ2 phosphorylation at tyrosine 759, the important signaling molecules downstream of CLEC-2 activation. As observed in Fig. 1*B* (*panels i, iii,* and *iv*), Syk and PLCγ2 were phosphorylated as early as 2 min upon stimulation with rhodocytin in non-aspirinated human platelets. However, both Syk and PLCγ2 appear to get phosphorylated at a weaker rate only at 3 min, in platelets pre-treated with aspirin (Fig. 1*B* (*panels ii–iv*)).

Furthermore, we also compared the effect of indomethacin or aspirin on Syk and PLCγ2 phosphorylation upon stimulation of human platelets with rhodocytin at one time point (3 min). At this time point (3 min), non-aspirin platelets underwent shape change but not the aspirin-treated platelets. As shown in the Fig. 1*C*, rhodocytin-induced Syk and PLCγ2 phosphorylation were completely abolished in the presence of either aspirin or indomethacin as compared with platelets stimulated with rhodocytin in the absence of either of them. Similar results were also observed in the murine platelets treated with or without indomethacin; however, this effect was observed only at a low concentration of rhodocytin (5 nM) (Fig. 1*D*). Taken together, these results suggest that non-aspirin-treated platelets respond more robustly to rhodocytin as compared with platelets treated with aspirin.

Thromboxane potentiates the rhodocytin-induced aggregation in aspirin-treated platelets

As discussed in the above result, the inhibition of thromboxane generation by aspirin is responsible for the differences in rhodocytin-induced aggregation in aspirin or non-aspirin platelets. Thus, it is possible that in the aspirinated platelets, the delay in the rhodocytin-induced Syk and PLCγ2 phosphorylation could be rescued by co-stimulation of thromboxane receptors via exogenous addition of the TxA2 mimetic, U46619. We evaluated the effect of U46619 on aspirin-treated platelets co-stimulated with rhodocytin. As shown in Fig. 2*A* (*panels i, iii,* and *iv*), stimulation with both the agonists (rhodocytin and U46619) induced a rapid aggregation as well as potentiation of both Syk and PLCγ2 phosphorylation in aspirin-treated platelets as early as 30 s. However, U46619 alone does not cause any Syk or PLCγ2 phosphorylation (Fig. 2*A* (*panels ii–iv*)). Effect of co-stimulation of indomethacin-treated murine platelets with U46619 and rhodocytin further showed that both Syk and PLCγ2 were phosphorylated at the 1-min time point, but phosphorylation does not occur under conditions of stimulation with rhodocytin alone (Fig. 2*B*). This suggests that a secondary mediator, *i.e.* TxA2, downstream of CLEC-2 receptor activation, is responsible for potentiation of CLEC-2-mediated signaling.

Furthermore, non-aspirinated human platelets were pre-incubated with the TP receptor antagonist, BAY u3405, followed by stimulation with rhodocytin for a minute, to specifically delineate the role of TxA2. We observed that the Syk and PLCγ2 phosphorylation were inhibited, similar to treatment with indomethacin (Fig. 2*C*). Also, the potentiation of CLEC-2 signaling by U46619 was abolished in the presence of BAY u3405. These results further reinforced the importance of TxA2 in CLEC-2 signaling.

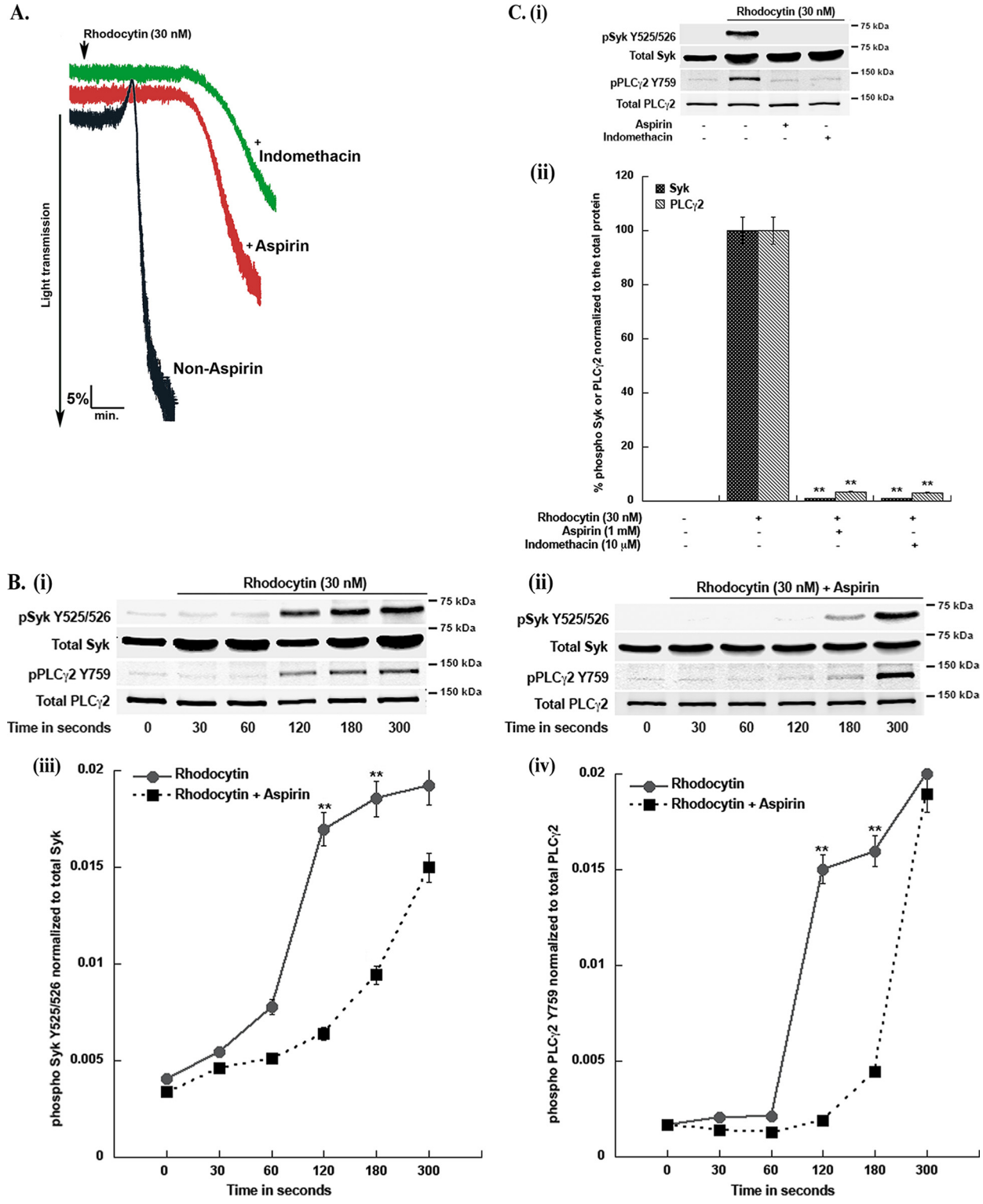

Figure 1. Non-aspirinated platelets respond more robustly to rhodocytin than aspirin-treated platelets. *A,* washed non-aspirin or aspirin (1 mM) or indomethacin (10 μM)-treated human platelets were stimulated with 30 nM rhodocytin for 5 min at 37 °C under stirred conditions in a lumi-aggregometer. The tracings are representative of data from at least three independent experiments. *B,* washed non-aspirin (*panel i*) or aspirin-treated human platelets (*panel ii*) were stimulated with 30 nM rhodocytin for the indicated time points, and the reaction was stopped by using 6.6 N perchloric acid. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and -PLCγ2 (Tyr-759). Statistical analysis of phospho-Syk (*panel iii*) and phospho-PLCγ2 (*panel iv*) of Western blottings are from *panels i* and *ii*. *C,* washed aspirin-treated or non-aspirin human platelets pre-treated with indomethacin (10 μM) were stimulated with rhodocytin (30 nM) for 3 min under stirred conditions. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526), Syk (Tyr-352), PLCγ2 (Tyr-759), and PLCγ2 (Tyr-1217) in *panel i* and statistical analysis of the Western blots in *panel ii*. *D, panel i,* washed murine wild-type platelets were stimulated with rhodocytin (5 nM) under stirred conditions in the presence or absence of indomethacin (10 μM). Platelet aggregation was measured by aggregometry. The tracings are representative of data from at least three independent experiments. *Panel ii,* washed murine platelets were stimulated with 5 nM rhodocytin for 1 min. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and PLCγ2 (Tyr-759). *Panel iii,* statistical analysis of the Western blottings in *panel ii*. All the Western blot analyses shown are representative of three independent experiments. **, $p < 0.01$.

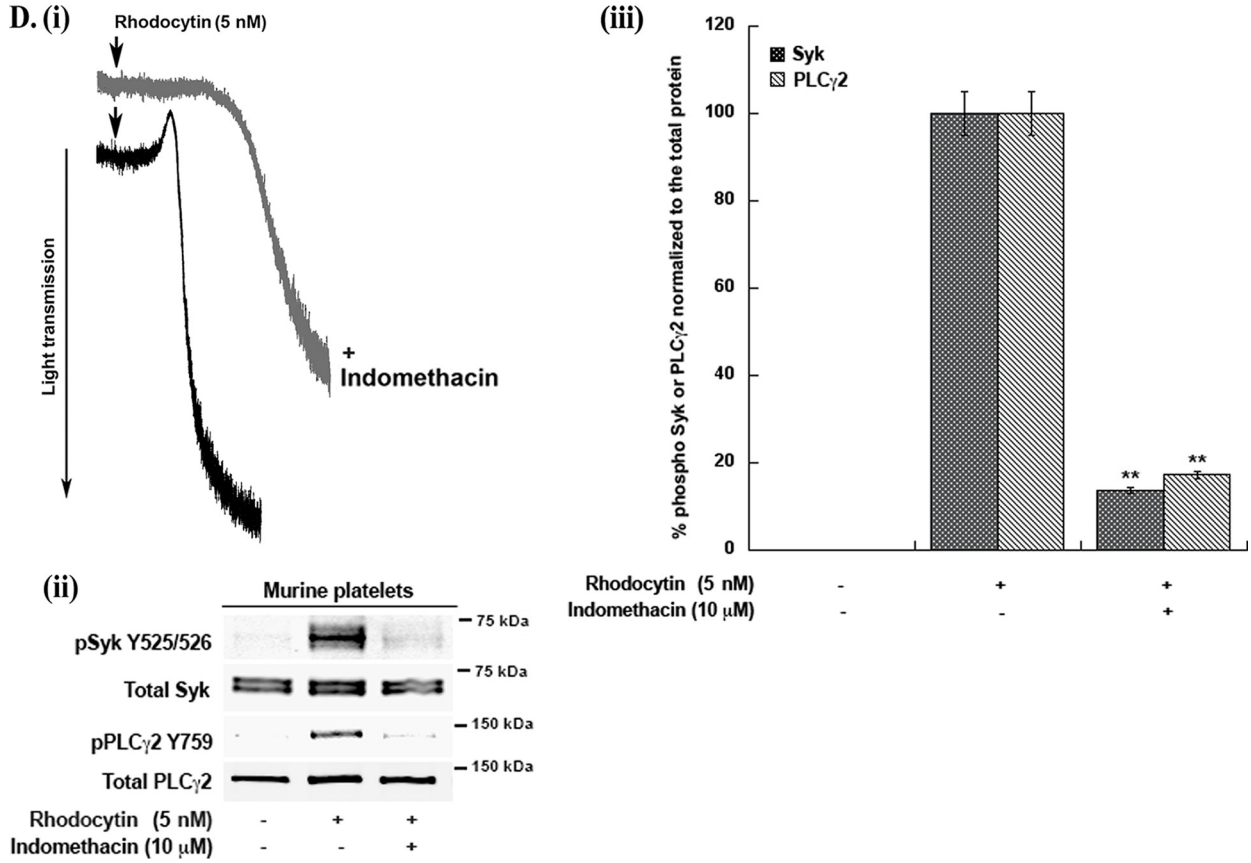

Figure 1—*continued*

It is possible that the potentiation of Syk phosphorylation observed upon stimulation with U46619 and rhodocytin together could be due to outside-in signaling initiated by activated integrin. To rule out this possibility, washed aspirin-treated human platelets were pre-incubated with the integrin $\alpha_{IIb}\beta_3$ antagonist, GR144053, and co-stimulated with rhodocytin and U46619 for a minute. As shown in Fig. 2*D*, in the presence of GR144053, the Syk phosphorylation was unaffected, suggesting that the potentiation of Syk phosphorylation by GPCRs is through inside-out signaling and not outside-in signaling.

G-protein-coupled receptors potentiate CLEC-2 signaling through G_q pathways

The TxA2 receptor, TP, is a G-protein-coupled receptor that is coupled to G_q and $G_{12/13}$ (18, 19). Platelets also express other GPCRs such as protease-activated receptors (PARs) and P2Y receptors. PARs are coupled to G_q and $G_{12/13}$, like TP receptors, and initiate similar signaling pathways (20). However, the P2Y receptors are coupled to G_i ($P2Y_{12}$) and G_q (P2Y1), which must co-activate for ADP-induced aggregation (20, 21). To evaluate whether co-activation of PARs and ADP receptors also leads to potentiation of Syk phosphorylation downstream of CLEC-2, we stimulated washed aspirin-treated human platelets with 2MeSADP (an ADP analogue), AYPGKF (PAR-4 agonist), or U46619 (as a positive control) with or without co-stimulation with rhodocytin. As shown in Fig. 3*A*, all three GPCR agonists caused robust phosphorylation of Syk and PLCγ2 upon co-stimulation with the CLEC-2 agonist at a time point when there was no phosphorylation observed in only the rhodocytin-treated platelet, suggesting a cross-talk mechanism between GPCRs and CLEC-2.

Because G_q is the common signaling pathway for all the three agonists, we speculated that G_q played a role in this potentiating effect on CLEC-2 signaling. Washed non-aspirinated human platelets were stimulated with rhodocytin for a minute in the presence or absence of UBO-QIC, a G_q inhibitor. Pre-treatment of platelets with UBO-QIC resulted in absence of rhodocytin-induced Syk and PLCγ2 phosphorylation at that time point as compared with the control without UBO-QIC, where there was robust phosphorylation observed (Fig. 3*B*). Additionally, washed aspirin-treated human platelets were also co-stimulated with rhodocytin and U46619 in the presence or absence of UBO-QIC. As shown in Fig. 3*C*, the robust Syk phosphorylation, upon co-stimulation with both the agonists for 1 min, was absent in the presence of UBO-QIC. These results suggest that the G_q pathways play an important role in mediating the potentiating effect on CLEC-2 signaling. Similar results were also observed upon co-stimulation of PAR-4 and CLEC-2 (Fig. 3*D*).

To elucidate whether the G_i or G_q pathway, activated downstream of $P2Y_{12}$ and $P2Y_1$, respectively, is involved in the potentiation of Syk phosphorylation upon CLEC-2 stimulation, we used specific antagonists for ADP receptors. Pre-incubation of platelets with the $P2Y_{12}$ antagonist, ARC-69931MX, resulted in similar levels of Syk phosphorylation as obtained in the absence

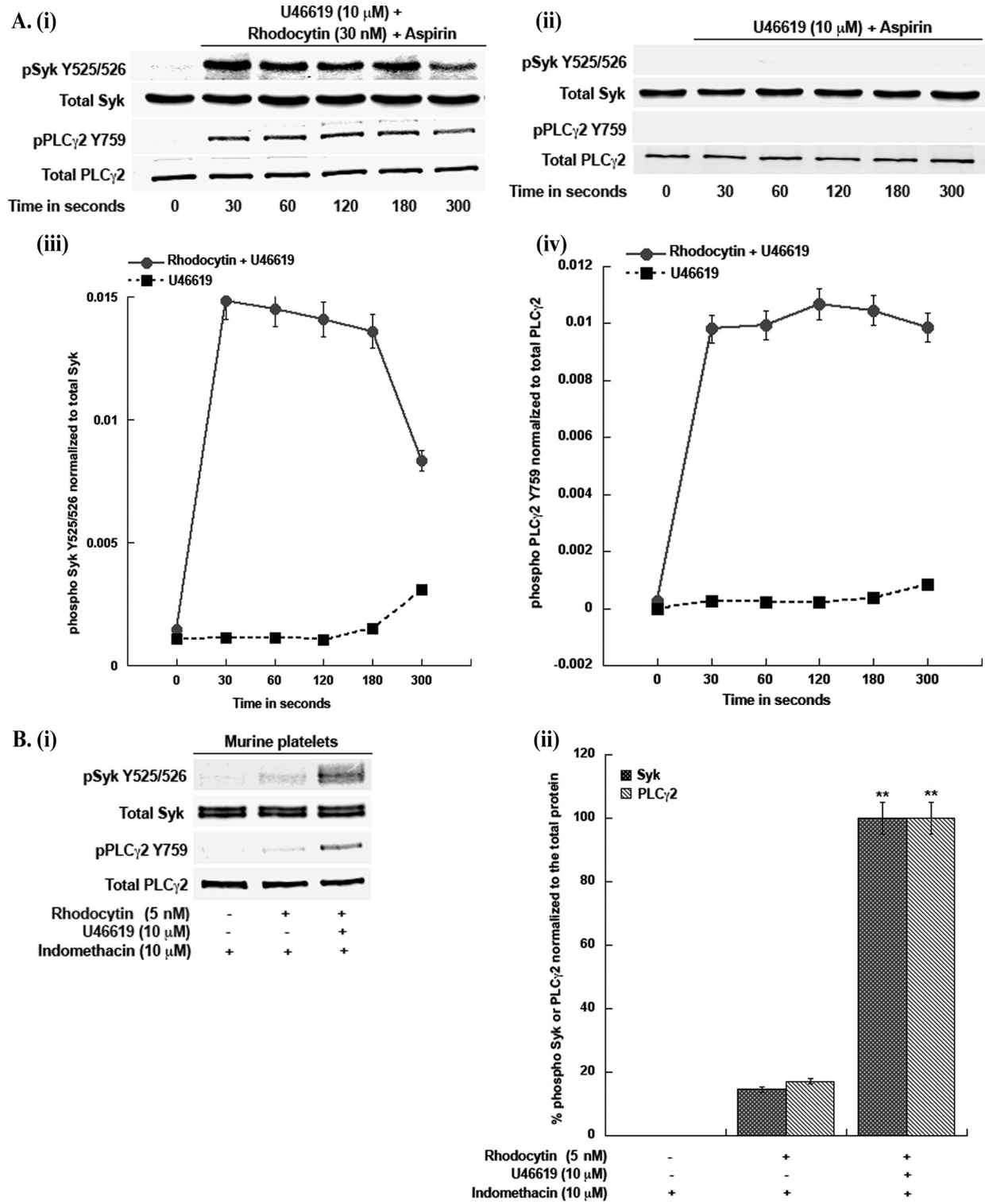

Figure 2. Thromboxane potentiates the rhodocytin-induced platelet activation in the aspirin treated platelets. *A,* washed aspirin-treated human platelets were co-stimulated with rhodocytin (30 nM) and U46619 (10 μM) (*panel i*) or U46619 (10 μM) alone (*panel ii*) for the indicated time points under stirred conditions, and the reaction was stopped by using 6.6 N perchloric acid. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and PLCγ2 (Tyr-759). Statistical analysis of phospho-Syk (*panel iii*) and phospho-PLCγ2 (*panel iv*) of Western blottings from *panels i* and *ii*. *B,* Western blotting (*panel i*) and statistical analysis (*panel ii*) of washed murine platelets stimulated with 5 nM rhodocytin for 1 min in the presence or absence of indomethacin (10 μM). *C, panel i,* washed human platelets without aspirin were pre-treated with either indomethacin (10 μM) or TP receptor antagonist, BAY u3405 (10 μM) for 5 min and stimulated with rhodocytin (30 nM) for 1 min. Also, aspirin-treated platelets were stimulated with rhodocytin or co-stimulated with rhodocytin and U46619 (10 μM) for 1 min in the presence or absence of BAY u3405 (10 μM); *panel ii* shows statistical analysis of the Western blots. *D, panel i,* washed human platelets were pre-incubated with αIIbβ_3 antagonist, GR 144053 (*GR*) (200 nM) for 5 min followed by stimulation with rhodocytin (30 nM) and U46619 (10 μM) for 1 min under stirred conditions; *panel ii* shows statistical analysis of the Western blots. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and PLCγ2 (Tyr-759). *N.S.*, not significant. **, $p < 0.01$. All the Western blot analyses shown are representative of three independent experiments.

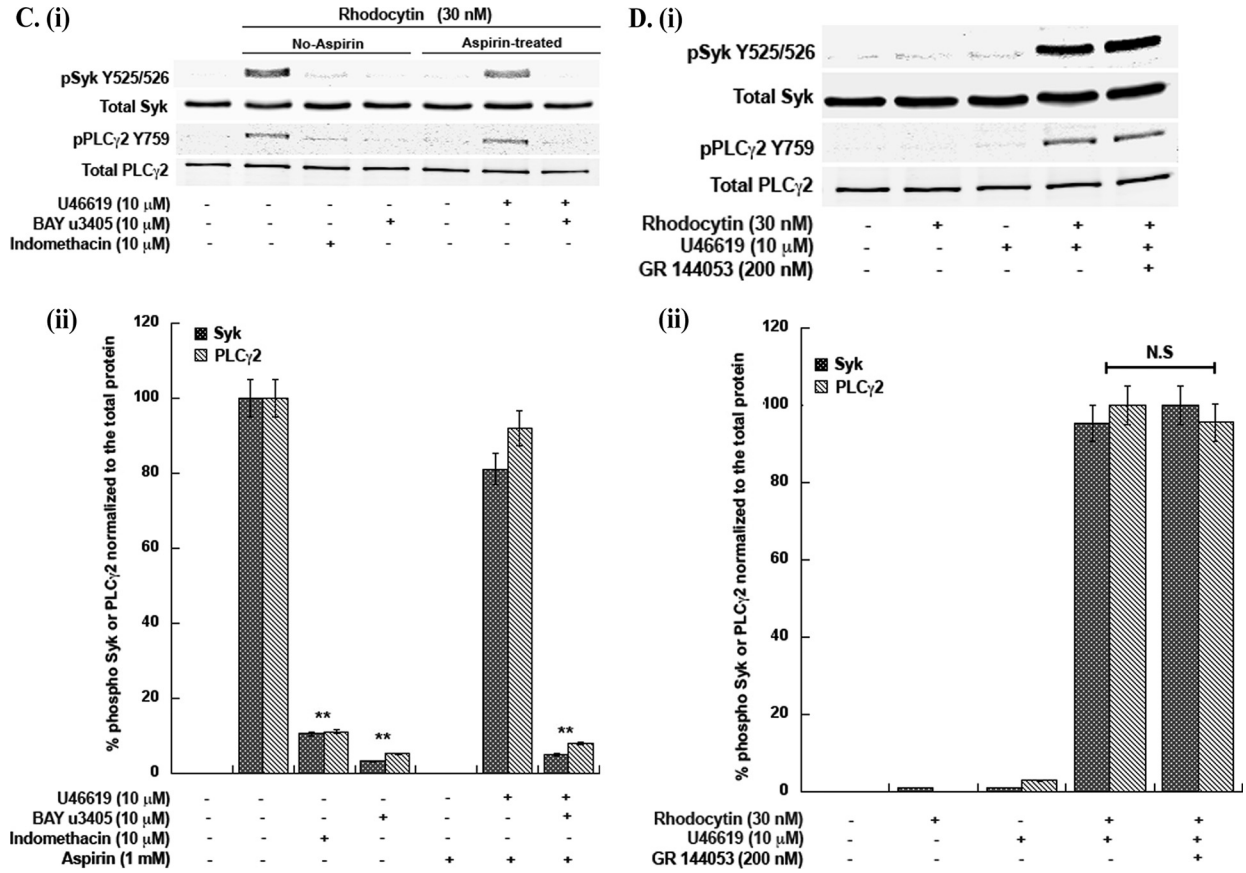

Figure 2—*continued*

of it upon co-stimulation with 2MeSADP and rhodocytin. However, the potentiation of Syk phosphorylation was absent in the presence of the $P2Y_1$ antagonist MRS2179 (Fig. 3*E*).

Additionally, in G_q knock-out murine platelets, there was no Syk/PLCγ2 phosphorylation observed when stimulated with rhodocytin (5 nM) for 1 min as compared with wild types (Fig. 3*F*). Together, these results suggest that the G_q pathways downstream of different GPCRs play an essential role in regulating Syk phosphorylation upon CLEC-2 receptor activation.

G_q potentiates CLEC-2 signaling through PLCβ-PKC pathway

G_q-coupled receptors signal by activating PLCβ and inducing Ca^{2+} mobilization and activation of PKCs (20). To determine the mechanism by which G_q mediates the potentiating effect on Syk phosphorylation upon CLEC-2 stimulation, we measured the effect of PLCβ and PKC inhibition on Syk phosphorylation. As shown in Fig. 4*A*, pre-incubation of washed human platelets with the PLCβ inhibitor, U73122, or the pan-PKC inhibitor, GF109203X, caused significant inhibition of Syk phosphorylation upon co-stimulation with rhodocytin and U46619. This suggests that the G_q-PLCβ-PKC pathway plays an essential role in regulating Syk phosphorylation by rhodocytin.

Platelets express seven isoforms of PKC (α, β, δ, θ, ϵ, η, and ζ) (22). To identify the PKC isoform responsible for mediating the potentiation of Syk phosphorylation, we used a pharmacological approach. We inhibited the activity of PKCδ by using rottlerin; novel PKCs by using the PKCδ/θ inhibitor; classical PKCs by using LY33531; and PKCβ by using PKCβ inhibitor. As shown in Fig. 4*B* (*panels i* and *ii*), only the pan-PKC inhibitor and the PKCα/β inhibitor, LY33531, resulted in significant inhibition of Syk hyper-phosphorylation. The PKCβ inhibitor by itself caused slightly more Syk phosphorylation. This result suggests that PKCα could possibly be the isoform responsible for regulating Syk phosphorylation downstream of GPCRs.

PKC activation by PMA potentiates CLEC-2 signaling

PMA is a well-known PKC activator (23, 24). To determine whether direct PKC activation by PMA can rescue Syk phosphorylation by rhodocytin in the presence of the G_q inhibitor, UBO-QIC (Fig. 3), we stimulated non-aspirinated human platelets with rhodocytin for 2 min in the presence and absence of UBO-QIC. As shown in Fig. 5*A*, there was no Syk and PLCγ2 phosphorylation in the presence of UBO-QIC. However, addition of PMA under these conditions rescued both Syk and PLCγ2 phosphorylations, suggesting that direct activation PKCs can lead to potentiation of CLEC-2 signaling. PMA by itself does not result in any Syk or PLCγ2 phosphorylation. Similar results were obtained with murine platelets (Fig. 5*B*) stimulated with rhodocytin and PMA, in the presence of UBO-QIC (100 nM).

SFK activation by PKC downstream of G_q regulates CLEC-2 tyrosine phosphorylation and CLEC-2 signaling

To elucidate the potential mechanism behind the G_q/PKC-mediated regulation of Syk phosphorylation, we exam-

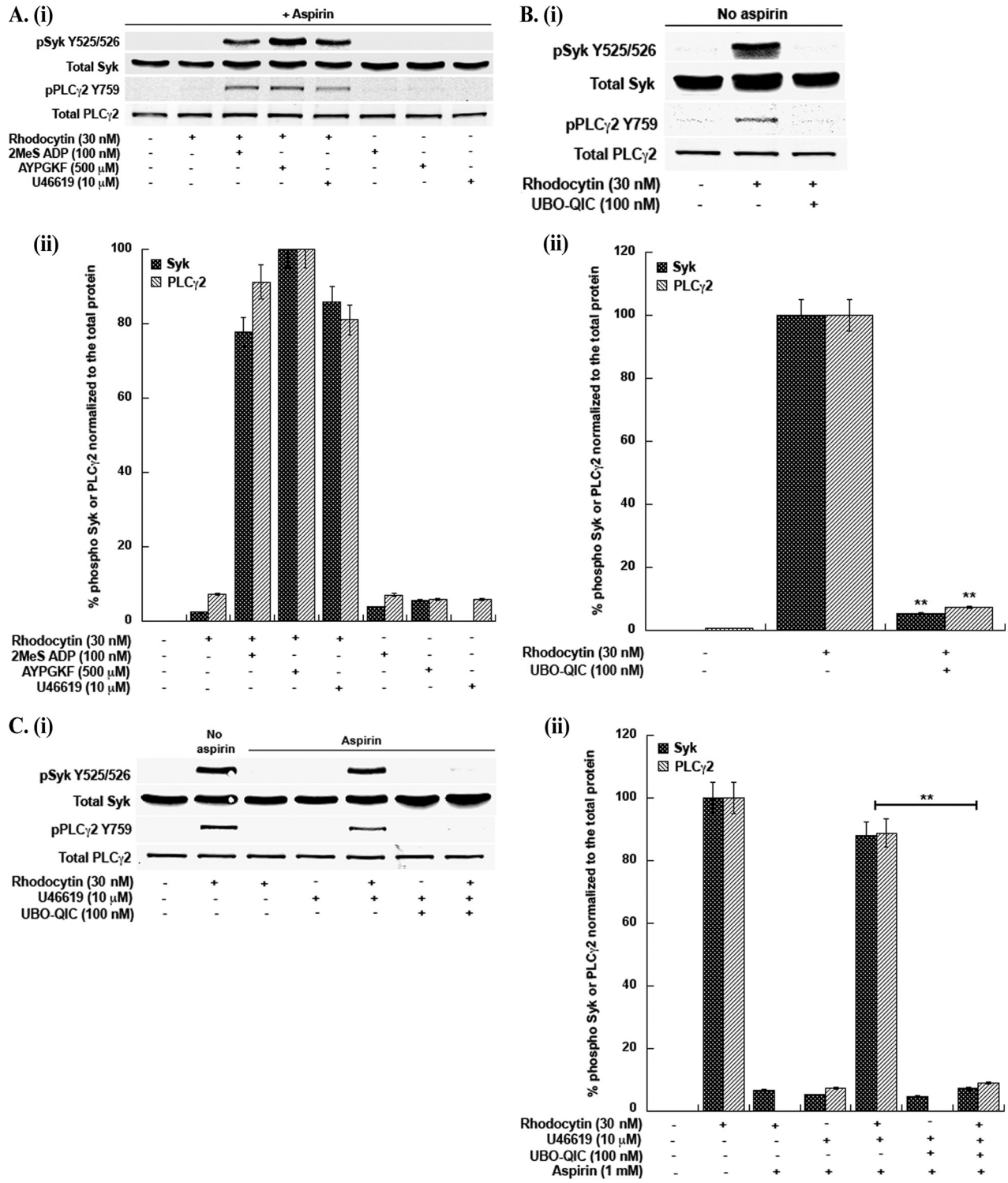

Figure 3. G-protein-coupled receptors potentiate CLEC-2 signaling through G_q pathways. *A, panel i,* washed aspirin-treated human platelets were co-stimulated with rhodocytin (30 nM) and 2MeSADP (100 nM) or AYPGKF (500 μM) or U46619 (10 μM) or all the agonists alone for 1 min at 37 °C under stirred conditions in lumi-aggregometer. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526), and PLCγ2 (Tyr-759); *panel ii* statistical analysis of the Western blots. *B, panel i,* washed non-aspirin human platelets, incubated with specific G_q inhibitor, UBO-QIC (100 nM), were stimulated with rhodocytin (30 nM). Western blottings of the separated proteins by SDS-PAGE were probed for phospho-Syk (Tyr-525/526) and PLCγ2 (Tyr-759); *panel ii* statistical analysis of the Western blots. *C, panel i,* washed aspirin-treated human platelets, incubated with specific G_q inhibitor, UBO-QIC (100 nM), were co-stimulated with rhodocytin (30 nM) and U46619 (10 μM) or *D, panel i,* AYPGKF (500 μM) for 1 min under stirred conditions. *C, panel ii,* and *D, panel ii,* statistical analysis of the respective Western blots. *E, panel i,* washed aspirin-treated human platelets pre-incubated for 5 min with either P2Y1 antagonist, MRS2179 (100 μM), or $P2Y_{12}$ antagonist, ARC 69931MX (100 nM), were co-stimulated with rhodocytin (30 nM) and 2MeSADP (100 nM); *panel ii,* statistical analysis of the Western blots. *F, panel i,* washed wild-type (*WT*) and $G\alpha_q$-deficient (knock-out (KO)) murine platelets were stimulated at 37 °C for 1 min with rhodocytin (5 nM). Western blottings of the proteins separated by SDS-PAGE were probed for phospho-Syk (Tyr-525/526) and phospho-PLCγ2 (Tyr-759); *panel ii,* statistical analysis of the Western blots. All the Western blot analysis shown is a representative of three independent experiments. *N.S.,* not significant. **, $p < 0.01$.

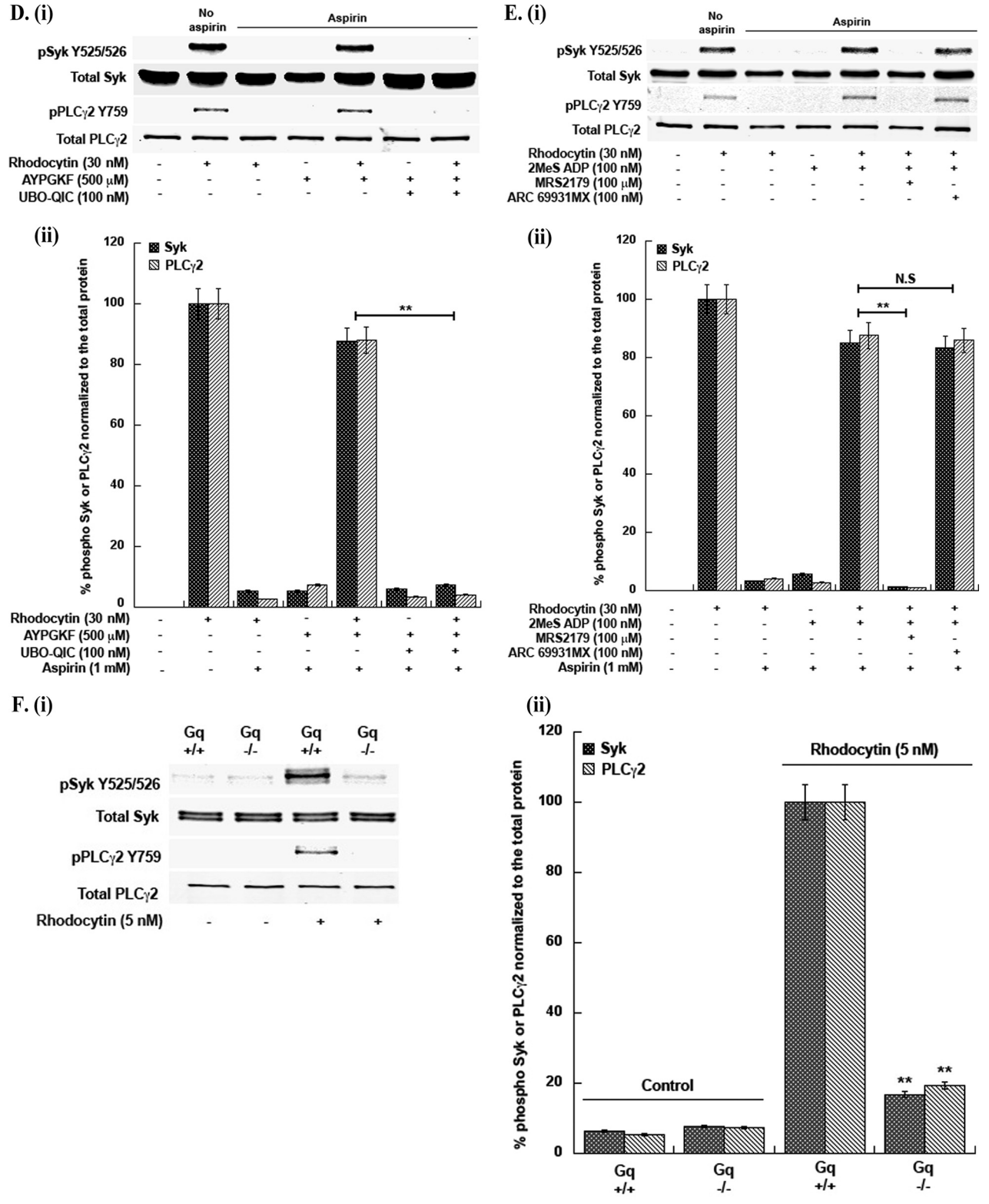

Figure 3—*continued*

ined whether Btk, a tyrosine kinase upstream of Syk in the CLEC-2 signaling pathway, is also potentiated upon co-stimulation with U46619 and rhodocytin. Fig. 6*A* shows that Btk Y223 phosphorylation is also potentiated upon stimulation with U46619 and rhodocytin as compared with rhodocytin or U46619 alone, suggesting that the G_q-dependent potentiation of CLEC-2 signaling is upstream of Syk and possibly upstream of Btk as well.

SFKs are the most proximal molecules involved in CLEC-2 signaling, as inhibition of SFKs completely abolishes CLEC-2 tyrosine phosphorylation and subsequent signaling (17). Based on this work and on previous literature that describes the inter-

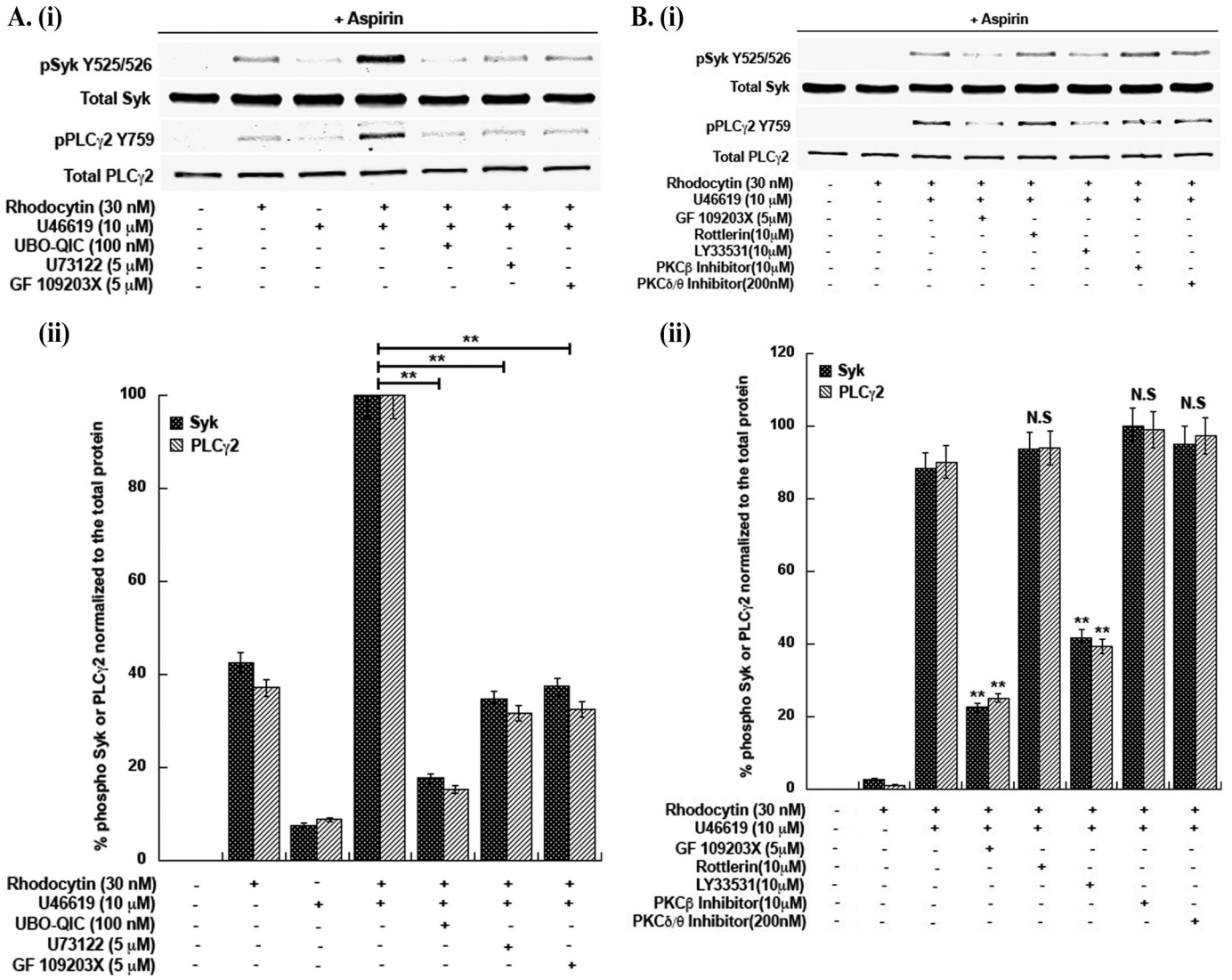

Figure 4. G_q potentiates CLEC-2 signaling through PLCβ-PKC pathway. *A,* washed aspirin-treated human platelets were pre-incubated with either G_q inhibitor, UBO-QIC (100 nM), or PLCβ inhibitor, U73122 (5 μM), or GF 109203X (5 μM) for 5 min followed by stimulation with rhodocytin (30 nM) and U46619 (10 μM) for 1 min under stirred conditions. Western blot (*panel i*) and statistical analysis (*panel ii*) of the proteins were obtained after subjecting platelet lysates to separation by SDS-PAGE. *B,* washed aspirin-treated human platelets were pre-incubated with GF 109203X (5 μM), or rottlerin (10 μM), or LY33531 (10 μM), or PKCβ inhibitor (10 μM), or PKCδ/θ inhibitor (200 nM) followed by co-stimulation with rhodocytin (30 nM) and U46619 (10 μM) for 1 min. Western blot (*panel i*) and statistical analysis (*panel ii*) of the same are shown. In all the cases, platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and PLCγ2 (Tyr-759). Western blot analysis shown is a representative of three independent experiments. *N.S.*, not significant. **, $p < 0.01$.

play between PKCs and SFKs (26–28), we hypothesized that PKCs, activated by G_q pathways, regulate SFK activity downstream of CLEC-2. To confirm that SFKs are activated downstream of G_q and that they are regulated by PKCs, we stimulated washed human platelets with U46619 in the absence or presence of either the G_q inhibitor, UBO-QIC, or the pan-PKC inhibitor, GF109203X, and probed for SFK tyrosine phosphorylation at Tyr-416. As shown in Fig. 6*B*, there is enhanced phosphorylation of SFKs by U46619 over control. However, this phosphorylation is significantly inhibited in the presence of either UBO-QIC or GF109203X, suggesting that the G_q-PKC pathway downstream of GPCRs regulates the SFKs.

Furthermore, to study the effect of enhanced SFK activation on the CLEC-2 tyrosine phosphorylation, we co-stimulated platelets with rhodocytin and U46619, and looked at the CLEC-2 tyrosine phosphorylation, which is mediated by SFKs. Fig. 6*C* shows that the CLEC-2 phosphorylation is dramatically enhanced when the platelets are stimulated with both the agonists as compared with rhodocytin alone. In the same experiment, we also tested the effect of G_q inhibition or PKC inhibition on CLEC-2 phosphorylation. As expected, CLEC-2 phosphorylation was significantly reduced in the presence of either a G_q or PKC inhibitor, suggesting that the G_q-PKC pathway regulates the SFKs leading to enhanced CLEC-2 tyrosine phosphorylation and therefore enhanced signaling.

Platelets are known to express several members of Src family kinases such as Lyn, Fyn, and Src (29). Thus, to determine the member of SFKs regulated by GPCRs and mediating CLEC-2 tyrosine phosphorylation, we used available knock-out murine models for Fyn and Lyn. Washed murine platelets from Lyn knock-out were stimulated with rhodocytin in the presence or absence of indomethacin and also co-stimulated with U46619

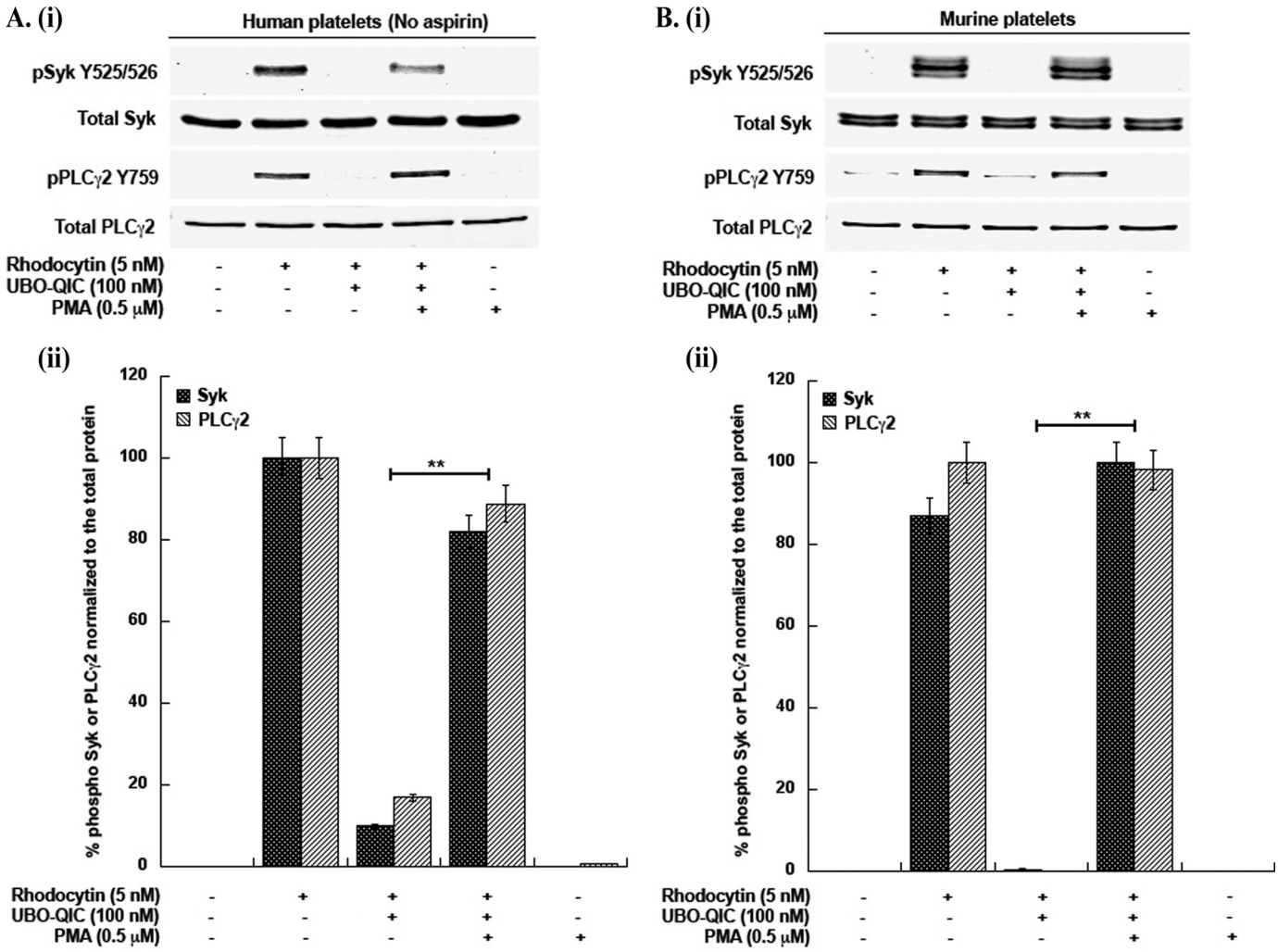

Figure 5. PKC activation by PMA potentiates CLEC-2 signaling. *A,* washed non-aspirin human platelets were pre-incubated with DMSO or G_q inhibitor, UBO-QIC (100 nM), for 5 min followed by stimulation with rhodocytin (30 nM) and PMA (0.5 μM) for 2 min under stirred conditions. Western blot (*panel i*) and statistical analysis (*panel ii*) of the proteins obtained after subjecting platelet lysates to separation by SDS-PAGE are shown. *B,* washed wild-type murine platelets were stimulated for 2 min with rhodocytin (5 nM) and PMA (0.5 μM) in the presence or absence of UBO-QIC (100 nM) or stimulated with rhodocytin or PMA alone. Western blots (*panel i*) and statistical analysis (*panel ii*) of the proteins separated by SDS-PAGE were probed for phospho-Syk (Tyr-525/526) and phospho-PLCγ2 (Tyr-759). All the Western blot analyses shown are representative of three independent experiments. **, $p < 0.01$.

in the presence of indomethacin. The amount of Syk/PLCγ2 phosphorylation in Lyn knock-out platelets, under all conditions, was comparable with wild-type littermates (Fig. 6*D*). This result ruled out the possibility of Lyn as the SFK activated downstream of GPCRs involved in the cross-talk. We next performed the similar experiments with the platelets obtained from Fyn knock-out murine platelets. As shown in Fig. 6*E*, there is no difference in Syk phosphorylation in Fyn-deficient platelets as compared with wild-type littermates when platelets are stimulated with both rhodocytin and U46619, although rhodocytin alone (without indomethacin) has reduced Syk phosphorylation in Fyn-deficient platelets. This suggests that Fyn is not the SFK downstream of G_q pathways regulating CLEC-2 tyrosine phosphorylation. It also implicates that some other SFK is involved in the cross-talk.

It was shown that PKCs activate SFKs through activation of the proline-rich tyrosine kinase, Pyk2, a member of focal adhesion kinase in hippocampus (30, 31). In platelets, Pyk2 is rapidly activated upon stimulation with PAR agonists (32, 33). However, we ruled out the possibility of Pyk2 in mediating SFK activation by PKCs, as Pyk2 inhibition did not affect the potentiation of CLEC-2 signaling by U46619 (Fig. 6*F*). The activity of the inhibitor was tested using known controls (data not shown).

Discussion

G-protein-coupled receptors (TxA_2 or ADP receptors or PARs) and tyrosine kinase pathway-activating receptors (CLEC-2) initiate complex intracellular signaling cascades which are not organized in distinct signaling units in a linear manner (3, 4). Platelets release secondary mediators upon activation, which play a critical positive feedback role in mediating platelet activation by all agonists (1). Similar dependence on secondary mediators for platelet aggregation was also shown to exist for the CLEC-2 agonist, rhodocytin (9). However, the mechanism by which secondary mediators contribute to CLEC-2 signaling is not clearly understood, and the cross-talk between CLEC-2 and GPCRs in platelets has not been evaluated until now.

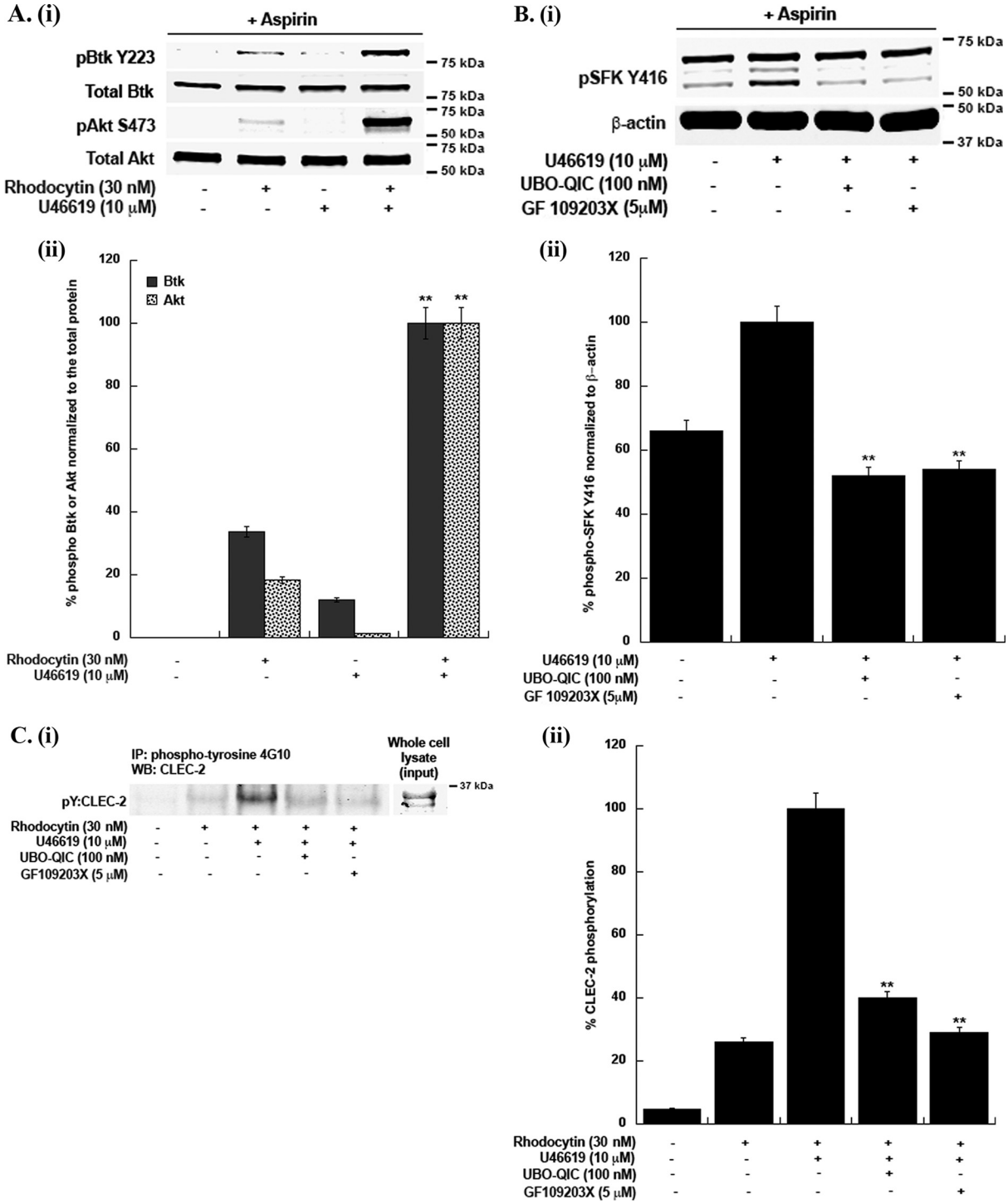

Figure 6. SFK activation by PKC downstream of G_q regulates CLEC-2 tyrosine phosphorylation and CLEC-2 signaling. *A, panel i,* washed non-aspirin and aspirin-treated human platelets were stimulated with rhodocytin (30 nM) alone or co-stimulated with rhodocytin (30 nM) and U46619 (10 μM) for 1 min under stirred conditions. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Btk (Tyr-223); *panel ii,* statistical analysis of the Western blots. *B, panel i,* washed aspirin-treated human platelets were pre-incubated with either UBO-QIC (100 nM) or GF 109203X (5 μM) and stimulated with U46619 (10 μM) for 1 min. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Src family kinases (Tyr-416). β-Actin was used as the lane loading control; *panel ii,* statistical analysis of the Western blots. *C, panel i,* washed aspirin-treated human platelets were pre-incubated with either UBO-QIC (100 nM) or GF 109203X (5 μM) followed by co-stimulation with rhodocytin (30 nM) and U46619 (10 μM). Platelet lysates were immunoprecipitated with agarose-conjugated Tyr(P) 4G10 antibody, and samples were probed for immunoprecipitating CLEC-2 using anti-CLEC-2 goat antibody; *panel ii,* statistical analysis of the Western blots obtained. Washed murine platelets from Lyn-deficient mice ($Lyn^{-/-}$) (*D*) or Fyn-deficient mice ($Fyn^{-/-}$) and wild-type controls ($Lyn^{+/+}$ or $Fyn^{+/+}$) (*E*) were either stimulated with rhodocytin (5 nM) alone in the presence or in the absence of indomethacin (10 μM) or co-stimulated with rhodocytin (5 nM) and U46619 (10 μM) in the presence of indomethacin for 1 min. *Panel i,* Western blot and statistical analysis (*panel ii*) of the respective figures. *F,* washed human platelets were pre-incubated with Pyk2 inhibitor, AG-17 (1 μM), or DMSO for 5 min followed by stimulation with rhodocytin (30 nM) and U46619 (10 μM) for 1 min under stirred conditions. In all the cases, platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and PLCγ2 (Tyr-759). *Panel i,* Western blot, and *panel ii*, statistical analysis of the same. Western blot analysis shown is a representative of three independent experiments. *N.S.,* not significant. **, $p < 0.01$.

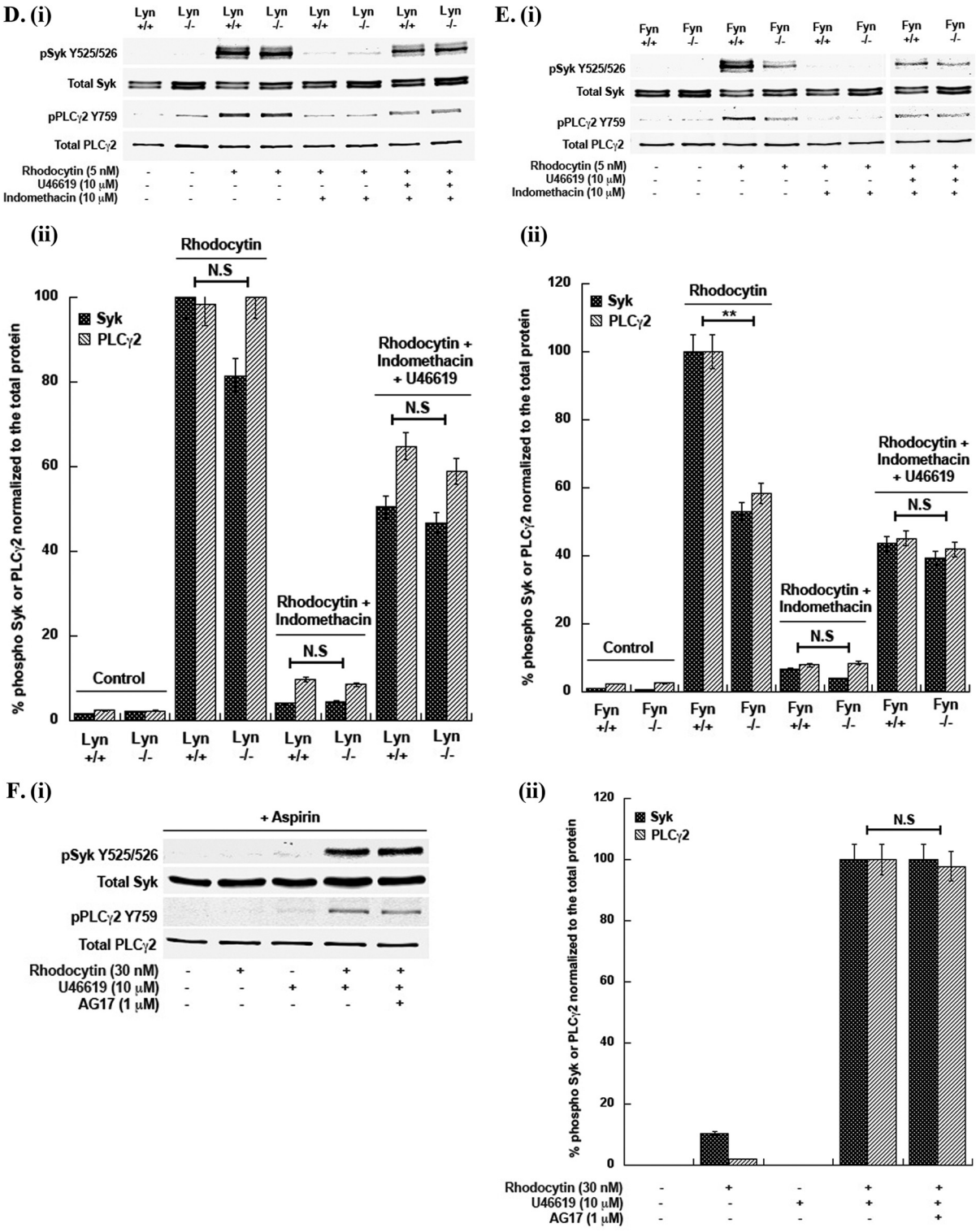

Figure 6—*continued*

The study presented here provides insight into the role of secondary mediators, such as thromboxane, in the amplification of CLEC-2-mediated signaling as has been reported earlier. While using the CLEC-2 agonist rhodocytin, we observed a dramatic difference in the activation of human platelets treated with aspirin and without treatment of aspirin. Unlike other agonists in similar conditions, rhodocytin responses are much delayed in the presence of aspirin. Such discrepancy can only be explained by the generation of thromboxane by rhodocytin-induced platelet activation. It also indicated that rhodocytin could generate thromboxane (in non-aspirin treated platelets) at a concentration that does not cause aggregation in aspirin-treated platelets. This result suggested a positive feedback loop that is distinct from the other platelet agonists. Using the TxA2

receptor antagonist, BAY u3405, we could antagonize this effect of the generated thromboxane in non-aspirinated platelets. Supplementing aspirin-treated platelets with the exogenous addition of the TxA2 mimetic, U46619, resulted in robust Syk phosphorylation. U46619 does not cause any Syk phosphorylation by itself at the early time points; however, minimal Syk phosphorylation could be observed at a later time point, which could be downstream of αIIbβ_3 activation and not due to primary TP receptor signaling. We report here that inhibiting the TxA2 generation by using aspirin or indomethacin and by blocking the TP receptor using an antagonist results in delay of the Syk phosphorylation by rhodocytin. These results add to the earlier finding by Pollitt *et al.* (9), where they showed that rhodocytin-induced platelet aggregation is dependent on secondary mediators. They also reported in the same study that CLEC-2 tyrosine phosphorylation is inhibited upon pre-treatment of platelets with indomethacin or apyrase (9). In support of this, we report that co-stimulation of platelets with rhodocytin and the TxA2 mimetic, U46619, results in potentiation of CLEC-2 tyrosine phosphorylation, indicating an important proximal role of TxA2 in CLEC-2 signaling.

The major finding from this study is the observation that G_q pathways potentiate CLEC-2 signaling via the PLCβ–PKCα–SFK pathway downstream of GPCRs. Collagen-induced platelet aggregation is particularly dependent on secondary mediators, because they intracellularly activate the collagen receptor $\alpha_2\beta_1$, thereby facilitating collagen binding to the $\alpha_2\beta_1$ (34). Because collagen is a macromolecule, it was suggested that collagen binding to the adhesion receptor $\alpha_2\beta_1$ might be required for an effective association between the activation receptor GPVI and collagen (34, 35). In contrast, platelet aggregation induced by specific GPVI agonists such as collagen-related peptide (CRP) or convulxin is much less dependent on secondary mediators. It was previously reported that rhodocytin-induced platelet aggregation is highly dependent on TxA2 generation compared with platelet aggregation induced by CRP (9). We also show that CLEC-2 signaling is highly dependent on other secondary mediators as well. Because rhodocytin also binds to integrin $\alpha_2\beta_1$ (36), it may be that activated $\alpha_2\beta_1$ binds to rhodocytin via the secondary mediators, which may facilitate rhodocytin binding to CLEC-2, as is in the case for collagen. Alternatively, Pollitt *et al.* (9) suggested secondary mediators may stimulate Rac1 activation, contributing to CLEC-2 tyrosine phosphorylation. However, this result was obtained by using the Rac1 inhibitor, EHT1864, which is highly nonspecific (37), and thus the results observed cannot be validated.

Our study clearly reveals that the potentiation of CLEC-2 signaling by secondary mediators, acting through GPCRs, is via the activation of PKCs (specifically PKCα) downstream of the G_q pathways. Inhibition of G_q using a G_q-specific inhibitor or in G_q-deficient murine platelets, suggested that G_q is essential for potentiating the CLEC-2 signaling. This also suggested that the $G_{12/13}$ pathway downstream of the TP receptor/PARs does not contribute to CLEC-2 signaling as in the absence of G_q pathway the activated $G_{12/13}$ could not rescue the potentiation of CLEC-2 signaling. We report here that PKCs activated downstream of the G_q pathway, particularly PKCα, are important for mediating CLEC-2 signaling. However, the specificity of the inhibitors can be a problem when using pharmacological inhibitors of PKCs in platelets, as these inhibitors can exhibit off-target effects. Hence, future studies using PKC isoform-specific knock-out murine models will confirm these results. The rescue of CLEC-2 signaling by PMA in presence of the G_q inhibitor (Fig. 5) further confirms our finding that PKCs can lead to the activation of Syk. Whether this effect is direct or indirect cannot be deduced through this. PMA by itself does not lead to any Syk/PLCγ2 phosphorylation, suggesting that PKCs require the Syk to be in a certain structural confirmation, which is most probably achieved through the docking of Syk to the hemITAM motifs of the agonist-bound CLEC-2 receptor. We show that not only the CLEC-2 signaling but also the CLEC-2 hemITAM tyrosine phosphorylation is significantly inhibited by G_q/PKC inhibition, suggesting that GPCRs mediate CLEC-2 signaling at the most proximal event of CLEC-2 receptor activation.

CLEC-2 is phosphorylated by Src family kinases on a single Y*XX*L motif, after which the tandem Src homology 2 domains of Syk bind to the phosphorylated hemITAM motif (11). It was also reported that SFKs are activated downstream of G_q and play an auxiliary role in G_q-coupled receptor signaling, lying downstream of PLCβ and Ca^{2+} mobilization (26). We hypothesized that SFKs, activated by G_q/PKC pathway, mediate CLEC-2 phosphorylation, thereby enhancing CLEC-2 phosphorylation. We showed that stimulation of platelets with U46619, a TxA2 mimetic, resulted in SFK Tyr-416 phosphorylation, which was inhibited by G_q or pan-PKC inhibitor, suggesting that SFKs are activated downstream of G_q-PKC pathway. However, we do not rule out that SFKs are also activated downstream of $G_{12/13}$ or G_i, but their role in potentiating the CLEC-2 signaling remains to be defined. Both Lyn and Fyn are not involved in cross-talk between G_q and CLEC-2, as was observed by using isoform-specific knock-out murine platelets. This indicates either the role of other SFKs, such as Fgr, or could also indicate a possible redundancy with other SFKs present in platelets of single isoform-specific knock-out. In the future, studies using double knock-out mouse models for different SFK isoforms might address this question. The inhibition of Syk phosphorylation in Fyn knock-out platelets when stimulated with rhodocytin indicates that Fyn is important for primary CLEC-2 signaling (Fig. 6*E*). Because there was no difference observed when both the agonists were added (rhodocytin and U46619), however, this suggests that there is another SFK downstream of GPCRs that could rescue the Syk phosphorylation.

There is considerable evidence of the interplay between SFKs and PKCs. For example, PKC interacts directly with Fyn and is tyrosine-phosphorylated at positions Tyr-311 and Tyr-565 in an SFK-dependent manner that potentiates PKC activity in response to thrombin (27). This is consistent with the earlier finding that phosphorylation of Ser-12 in the membrane-binding domain of Src by PKC induces cytoskeletal association and an increase in substrate affinity (28). This finding can explain the important role of PKCs in SFK activation and hence enhanced CLEC-2 tyrosine phosphorylation and signaling. We could not test this hypothesis, as the phosphoserine 12 SFK antibody is not commercially available. However, we cannot rule out other possible mechanisms of SFK activation by PKCs.

It was shown that PKCs activated downstream of G_q-coupled muscarinic receptors potentiate NMDA-evoked responses in hippocampal neurons (30). In the same study, it was shown that PKCs activate SFKs through activation of the proline-rich tyrosine kinase, Pyk2, a member of focal adhesion kinase (30, 31). In platelets, Pyk2 is rapidly activated upon stimulation with PAR agonists, and Pyk2 phosphorylation is mostly dependent on $G_{12/13}$ or the integrin $\alpha IIb\beta_3$ pathway (32, 33). However, we ruled out the possibility of Pyk2 in mediating SFK activation by PKCs, as the Pyk2 inhibition did not affect the potentiation of CLEC-2 signaling by U46619.

One possible mechanism for GPCR-mediated potentiation of CLEC-2 signaling could be by regulation of a phosphatase downstream of G_q, which in turn is involved in regulating key signaling molecules in the CLEC-2 pathway such as Syk. Recent studies in platelets have shown that deficiency of the dual-specificity phosphatase 3 (DUSP3) results in the inhibition of Syk phosphorylation at low concentrations of CLEC-2 agonists, suggesting a positive role of DUSP3 in CLEC-2 signaling (38). Although the activation mechanism of DUSP3 and the pathways regulating it are not known, Syk was suggested as the target of DUSP3 directly or indirectly (38). Because CLEC-2 signaling is dependent on thromboxane, DUSP3 is likely regulating the TxA2 generation. Thus, DUSP3 deficiency leads to reduced TxA_2 and hence diminished feedback signaling by G_q-coupled TP receptors resulting in inhibition of G_q-mediated potentiation of CLEC-2 signaling. Given that DUSP3 positively regulates Syk and hence CLEC-2 signaling, it might be positively regulated by GPCRs. Another possible phosphatase could be T-cell ubiquitin ligand-2 (TULA-2), which dephosphorylates Syk at Tyr-346, thereby inactivating Syk (39, 40). Hence, in a possible scenario, the G_q–PLCβ–PKC pathway might lead to inhibition of TULA-2 by a PKC-dependent phosphorylation, thereby blocking its negative regulatory activity on Syk. However, the possibility of other phosphatases or kinases still exists and remains to be defined in future studies.

It is also possible that the potentiation of CLEC-2 tyrosine phosphorylation is downstream of enhanced Syk phosphorylation and not vice versa, as it was reported in a previous study by Severin *et al.* (41) that CLEC-2 hemITAM is mainly phosphorylated by Syk itself upon binding to the single hemITAM motif in the CLEC-2 dimer phosphorylated by SFKs. R406, a specific Syk inhibitor, inhibits CLEC-2 hemi-ITAM phosphorylation induced by rhodocytin, suggesting that CLEC-2 is phosphorylated by Syk and SFKs in human platelets. In murine platelets, Severin *et al.* (41) reported that phosphorylation of CLEC-2 by rhodocytin is abolished in Syk-deficient mice, whereas phosphorylation is not altered in mice deficient in the major platelet SFKs Fyn, Lyn, or Src or in the tyrosine phosphatase CD148, which regulates the basal activity of the SFKs. The same group also reported that PP2 does not inhibit phosphorylation of mouse CLEC-2 by rhodocytin, suggesting that Syk phosphorylates CLEC-2 independently of the SFKs in mice. Interestingly, however, downstream signaling, including phosphorylation of PLCγ2, and platelet aggregation are abolished by PP2 in murine platelets, indicating that the SFKs play a crucial role further downstream in CLEC-2 signaling, through activation of the PI3K–Btk pathway. Given this, the G_q pathways might also regulate Syk directly via PKCs. A direct interaction between PKCα and Syk has been reported in platelets by Pula *et al.* (42). In a separate study, phosphorylation of serine 297 site on Syk by the PKCs was shown to enhance the ability of Syk to interact with the B-cell receptor, thereby enhancing downstream signaling (43). The importance of this phosphorylation could be addressed by developing a knock-in murine model in the future.

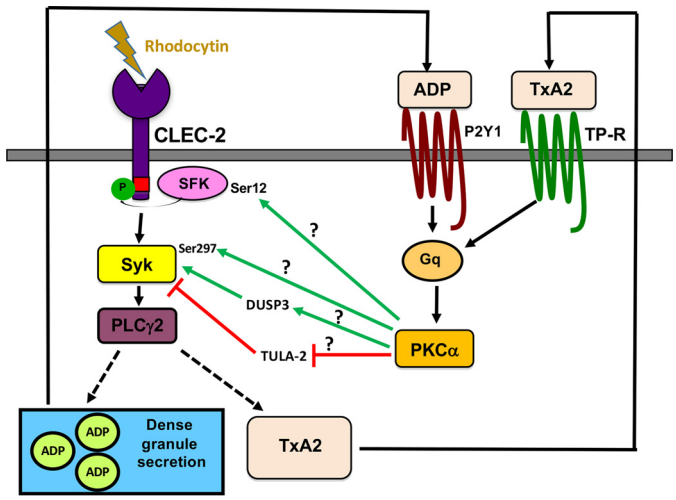

Figure 7. Proposed model depicting cross-talk between G_q-coupled GPCRs and CLEC-2 pathways. CLEC-2 activation by rhodocytin leads to initial signaling downstream of CLEC-2 resulting in thromboxane generation and secretion of ADP. TxA2 and ADP activate the G_q pathway, resulting in the activation of PKCα, which in turn can activate SFKs possibly through direct phosphorylation at Ser-12. PKCα can also regulate Syk directly, via phosphorylation on Ser-297 site, or indirectly, via activation of DUSP3 and/or inhibition of TULA-2 phosphatase. The activated SFKs or Syk can contribute and thereby potentiate the initial CLEC-2 signaling. The *green arrows* represent positive regulation, and *red lines* represent negative regulation.

In summary, this study suggests a novel mechanism of cross-talk between GPCRs and hemITAM-bearing receptors (outlined in Fig. 7). Specifically, we show that activation of the G_q-coupled receptor–PLCβ–PKC pathway leads to enhanced CLEC-2 tyrosine phosphorylation and thus enhanced signaling, possibly through PKCα-mediated Ser-12 phosphorylation of SFKs thereby enhancing SFK activity.

Experimental procedures

Materials

Apyrase (type VII) and MRS2179 were obtained from Sigma. Rhodocytin is gift from Dr. Johannes A Elbe. Whatman protein nitrocellulose transfer membrane was obtained from Thermo Fisher Scientific; LI-COR Odyssey blocking buffer and goat anti-mouse or anti-rabbit secondary antibodies was purchased from LI-COR Biosciences (Lincoln, NE). Antibodies to phospho-Syk (Tyr-525/526) (2711S), phospho-PLCγ2 (Tyr-759) (3874S), phospho-Btk (Tyr-223) (5082P), phospho-Src family (Tyr-416) (2101S), phospho-Akt (Ser-473) (4060S), and β-actin (4970S) were bought from Cell Signaling Technology (Beverly, MA); total Syk (sc1240), PLCγ2 (sc5283), Akt (sc5298), and Btk (sc28387) antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-hCLEC-2 goat IgG (AF1718) was purchased from R&D Systems, and 4G10-anti-phosphotyrosine-agarose conjugate (16-638) was from Millipore (Temecula, CA). 3,5-Di-*t*-butyl-4-hydroxyben-

zylidenemalononitrile (AG17) (10010248) and BAY u3405 (10156) were from Cayman Biochemicals. Pan-PKC inhibitor GF109203X and U73122 were from ENZO Life Sciences Inc. (Plymouth Meeting, PA). PKCδ/θ and PKCβ and inhibitors were from EMD Millipore (Billerica, MA). LY333531 was purchased from AG Scientific. PMA (P8139) was from Sigma. GR144053 was obtained from Tocris. ARC-69331MX was a gift from The Medicines Co. (Parsippany, NJ). UBO-QIC was purchased from University of Bonn, Bonn, Germany.

Preparation of human platelets

Blood was collected from informed healthy volunteers into one-sixth volume of acid/citrate/dextrose (2.5 g of sodium citrate, 2 g of glucose, and 1.5 g of citric acid in 100 ml de-ionized water). Platelet-rich plasma was obtained by centrifugation at 230 × *g* for 20 min at ambient temperature and incubated with 1 mM aspirin for 30 min at 37 °C. Platelets were isolated from plasma by centrifugation at 980 × *g* for 10 min at ambient temperature and resuspended in Tyrode's buffer, pH 7.4 (138 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, and 0.2 units/ml apyrase, pH 7.4). The platelet count was adjusted to 2×10^8 cells/ml for whole-cell lysate samples and 1×10^9 cells/ml for co-immunoprecipitation. Approval was obtained from the Institutional Review Board of Temple University for these studies. Informed consent was provided prior to blood donation.

Preparation of murine platelets

All mice were maintained and housed in a specific pathogen-free facility, and animal procedures were carried out in accordance with institutional guidelines after the Temple University Animal Care and Use Committee approved the study protocol. Age- and gender-matched wild-type littermates were used as controls. Blood was drawn via cardiac puncture into 0.1 volume of 3.8% sodium citrate. Blood was then spun at 100 × *g* for 10 min, and the platelet-rich plasma was separated. Red blood cells were mixed with 400 μl of 3.8% sodium citrate and spun for 10 min at 100 × *g*. Resulting platelet-rich plasma were combined; 1 μM PGE1 was added and centrifuged for 10 min at 400 × *g*. The platelet pellet was resuspended in Tyrode's buffer, pH 7.4, containing 0.2 units/ml apyrase. Platelet counts were determined using a Hemavet 950FS blood cell counter (Drew Scientific Inc., Dallas, TX). For this study a density of $1.5–2 \times 10^8$ platelets/ml was used.

Platelet aggregation

Platelet aggregation was measured using a lumi-aggregometer (Chrono-Log, Havertown, PA) at 37 °C under stirring conditions. A 0.5-ml sample of aspirin-treated or non-aspirinated washed platelets was stimulated with different agonists, and a change in light transmission was measured. Platelets were pre-incubated with different inhibitors/antagonists where noted before agonist stimulation. The chart recorder was set for 0.2 mm/s.

Western blot analysis

Platelets were stimulated with agonists in the presence of inhibitors or vehicles for the appropriate time under stirring conditions at 37 °C. Samples were prepared, and SDS-PAGE and Western blotting were performed as described previously (25).

Co-immunoprecipitation

Washed platelet (1×10^9 cells/ml) activation was stopped using equal volumes of chilled 2× Nonidet P-40 Lysis buffer, pH 7.4 (2× = 50 mM HEPES, 100 mM NaCl, 2% Nonidet P-40, 2 mM EGTA, and Halt Protease and Phosphatase mixture solution (Pierce)). Samples were rocked for 30 min at 4 °C and centrifuged at 15,000 × *g* for 10 min at 4 °C to remove cytoskeleton. 10 μl of normal mouse agarose-conjugated IgG or 4G10 agarose-conjugated anti-phosphotyrosine was added to the samples and incubated overnight on rocker at 4 °C. Samples were centrifuged at 5000 × *g* for 30 s at 4 °C to pellet down agarose beads. Agarose beads were then washed three times using 1× Nonidet P-40 lysis buffer and once using PBS. Proteins were solubilized in 2× sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane.

Statistical analysis

Each experiment was repeated at least three times. Results are expressed as means ± S.E. with number of observations, *n*. Data were analyzed, and graphs were plotted using KaleidaGraph software. The phospho-Syk or PLCγ2 band density was first normalized using total Syk or PLCγ2 as the lane loading control. The unstimulated value was then subtracted from all other values. The highest value thus obtained was then taken as 100%, and the rest of the samples were converted into % values of the highest value. Significant differences were determined using Student's *t* test. *p* value <0.05 was considered significant.

Author contributions—R. B. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. V. I. and B. K. M. performed the experiments. C. D. analyzed the data and edited the manuscript. J. E. provided rhodocytin, purified from the snake venom, for the studies. S. P. K. designed the experiments and analyzed and interpreted the data.

Note added in proof—Dr. Johannes Eble was inadvertently omitted from the version of this article that was published as a Paper in Press on July 13, 2017. This error has now been corrected.

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