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Arrestin-2 Differentially Regulates PAR4 and ADP Receptor Signaling in Platelets*[S]

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Arrestins can facilitate desensitization or signaling by G protein-coupled receptors (GPCR) in many cells, but their roles in platelets remain uncharacterized. Because of recent reports that arrestins can serve as scaffolds to recruit phosphatidylinositol-3 kinases (PI3K)s to GPCRs, we sought to determine whether arrestins regulate PI3K-dependent Akt signaling in platelets, with consequences for thrombosis. Co-immunoprecipitation experiments demonstrate that arrestin-2 associates with p85 PI3Kα/β subunits in thrombin-stimulated platelets, but not resting cells. The association is inhibited by inhibitors of P2Y12 and Src family kinases (SFKs). The function of arrestin-2 in platelets is agonist-specific, as PAR4-dependent Akt phosphorylation and fibrinogen binding were reduced in arrestin-2 knock-out platelets compared with WT controls, but ADP-stimulated signaling to Akt and fibrinogen binding were unaffected. ADP receptors regulate arrestin recruitment to PAR4, because co-immunoprecipitates of arrestin-2 with PAR4 are disrupted by inhibitors of P2Y1 or P2Y12. P2Y1 may regulate arrestin-2 recruitment to PAR4 through protein kinase C (PKC) activation, whereas P2Y12 directly interacts with PAR4 and therefore, may help to recruit arrestin-2 to PAR4. Finally, arrestin2$^{-/-}$ mice are less sensitive to ferric chloride-induced thrombosis than WT mice, suggesting that arrestin-2 can regulate thrombus formation *in vivo*. In conclusion, arrestin-2 regulates PAR4-dependent signaling pathways, but not responses to ADP alone, and contributes to thrombus formation *in vivo*.

Arrestins are cytoplasmic proteins that were originally characterized by their ability to associate with agonist-activated G protein-coupled receptors (GPCRs),[2] mediating their internalization and desensitization (1). More recent studies suggest that arrestins play additional roles in GPCR signaling, by serving as scaffolds to recruit signaling complexes to the receptor, thereby facilitating activation of G protein-dependent and -independent pathways (2, 3). One such arrestin-mediated pathway is the PI3K-dependent activation of the Ser-Thr kinase, Akt (4, 5). In fibroblasts, colorectal, and gastric carcinoma cells, arrestins have been found to play a critical role in localizing PI3K to GPCR complexes through an interaction with Src family kinases (SFKs) (6–8). Perhaps most relevant for platelet agonists, thrombin-stimulated Akt phosphorylation involved activation of both G_i and G_q: G_i-dependent signaling to Akt required ras activation, while G_q-dependent Akt activation required arrestin-2 (9).

Previous work from our laboratory and others has demonstrated that Akt-dependent pathways contribute to platelet activation by G protein-coupled receptors (10, 11). Yet, the mechanisms leading to Akt activation in platelets remain incompletely defined. Multiple laboratories have demonstrated that thrombin-dependent Akt phosphorylation in platelets is reduced by about 90% in the presence of inhibitors for the G_i-coupled ADP receptor, P2Y12, and is blocked by inhibitors of PKC (12, 13). These data have been interpreted to mean that Akt activation by thrombin is wholly dependent on the PKC-stimulated release of ADP. Yet, the amount of Akt phosphorylation induced by ADP reaches only a fraction of the magnitude of that induced by thrombin. In other words, P2Y12 activation is necessary, but not sufficient, to achieve maximal Akt stimulation by thrombin or PAR4 agonist. Studies to evaluate the contribution of specific G protein α-subunits to thrombin *versus* ADP-dependent signaling in mouse platelets provided data consistent with this view: specifically, while G_q was required for Akt phoshorylation induced by thrombin or ADP, G_{i2} was required solely for ADP signaling (10). These results suggested that a secondary role of PAR4 activation was required that was not induced by ADP alone. Furthermore, a recent study shows that PAR4 is capable of stimulating Akt phosphorylation in P2Y12 knock-out platelets (14). Taken together, these results suggest that the mechanisms of Akt activation induced by thrombin receptors *versus* P2Y12 are different, but synergistic.

Because studies in fibroblasts suggest that Akt phosphorylation depends in part on the ability of arrestin-2 to form complexes with PI3Ks (9), we evaluated the formation of arrestin2-PI3K complexes in thrombin-stimulated human platelets. Results from immunoprecipitation experiments suggest that arrestin-2 facilitates the recruitment of signaling complexes containing PI3K subunits and the SFK Lyn to the PAR4 receptor for thrombin. To determine whether arrestin-2 is important for Akt activation, Akt phosphorylation induced by PAR4 agonists or ADP was assessed in arrestin-2 knock-out (−/−) *versus* wild type (WT) mouse platelets. The functional responses of platelets from arrestin-2$^{-/-}$ mice were also tested *in vitro*. The results show that Akt phosphor-

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; SFK, Src family kinase.

ylation stimulated by PAR4 agonist is arrestin-2-dependent, whereas ADP-dependent Akt phosphorylation is not. Fibrinogen binding induced by PAR4 agonists is also arrestin-dependent, while ADP-induced fibrinogen binding is not. The role of arrestin-2 in supporting platelet signaling by PAR4 appears to contribute to platelet function *in vivo*, because arrestin-2 knock-out mice have a mild defect in thrombus formation following carotid artery injury *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified, reagents were from Sigma-Aldrich. ARL66096 and ARC69931MX were kind gifts of Astra Zeneca (Wilmington, DE). ADP was from Chronolog Corp (Havertown, PA). MRS2179 was from Sigma Aldrich. LY294002 was from Calbiochem-Novabiochem Corp. (San Diego, CA). PAR1 TRAP agonist peptide SFLLRN was synthesized by New England Peptide (Gardner, MA) and AYPGKF by Kimmel Cancer Center of Thomas Jefferson University (Philadelphia, PA). Antibodies were from Cell Signaling (Boston, MA) (anti-Akt, phospho-Akt-Ser-473, actin), Santa Cruz Biotechnology (Santa Cruz, CA) (for anti-arrestin-2, arrestin-3, PAR4, and P2Y12), and Upstate (Temecula, CA) for antibody to PI3K. Alexafluor-488-labeled fibrinogen was from Molecular Probes (Invitrogen, Carlsbad, CA).

Animals—Arrestin-2 knock-out (−/−) mice were generated as described (15) and kindly provided by the laboratory of Dr. Robert Lefkowitz. All animal procedures were approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University.

Platelet Isolation and Preparation of Human Blood—Blood for biochemical studies of human platelets was collected by venipuncture from adult human volunteers after providing written informed consent as approved by the Institutional Review Board at Thomas Jefferson University. Blood was collected into a 60-cc syringe containing ACD (trisodium citrate, 65 mM; citric acid, 70 mM; dextrose, 100 mM; pH 4.4) at a ratio of 1:6 parts ACD/blood. Anticoagulated blood was spun by centrifugation at 250 × g to remove red cells. Platelets from the resulting platelet rich plasma (PRP) were pelleted at 750 × g (10 mins), washed once in HEN buffer (10 mM HEPES, pH 6.5, 1 mM EDTA, 150 mM NaCl) containing 0.05 units/ml apyrase and resuspended with HEPES-Tyrode's buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/liter BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4) at a concentration of from 4–10 × 10^8 platelets/ml in HEPES-Tyrode's buffer containing 0.05 units/ml apyrase, for immunoblotting, immunoprecipitation, or fibrinogen binding.

Platelet Isolation from Mice—Blood was isolated from the inferior vena cava of anesthetized mice (100 mg/kg pentobarbital) using a syringe containing 150 units/ml heparin (1:9 dilution with blood), diluted 50% with HEPES-Tyrode's buffer, and spun at 250 × *g* for 4 min to remove red cells. Generally, blood from two mice of each genotype was used for experiments. Platelets from the resulting platelet-rich plasma (PRP) were pelleted at 750 × *g* (10 min), washed once in HEN buffer, and resuspended with HEPES-Tyrode's buffer. Platelets were counted on a Coulter counter (Beckman-Coulter Z1) and the final platelet count adjusted with Tyrode's buffer.

Immunoblotting—Samples (4 × 10^8 platelets/ml) were treated with antagonist for 10 min at room temperature. Agonist was added in a 2 μl volume to 100 μl platelets per sample; platelets were incubated for 0–10 min at 37 °C and were lysed by addition of 5× Laemmli buffer containing a mixture of protease inhibitors (Sigma-Aldrich). Lysates were resolved on 10% SDS-PAGE and immunoblotted with an antibody to Akt phospho-Ser-473 (Cell Signaling Technology, Beverly, MA), arrestin-2, or arrestin-3 (Santa Cruz Biotechnology) at a 1:1000 dilution, then anti-rabbit alexafluor680 (LiCor) or anti-Goat AlexaFluor680 (LiCor) in blotting buffer (LiCor) in TBS and exposed on a LiCor fluorescence imager.

Immunoprecipitation (IP)—Samples (8–10 × 10^8 platelets/ml) were treated with antagonist for 10 min at room temperature. Agonist was added in a 5 μl volume to 500 μl platelets per sample; platelets were incubated for 0–10 min at 37 °C and were lysed by addition of 2xIP buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM Na_3V0_4, 5 mM EDTA, 0.5 mM PMSF, pH 7.4) containing a mixture of protease inhibitors (Sigma-Aldrich); rotated at 4 °C for 30 min and spun 30 min at 12,000 × *g*. Antibodies or control IgG were added to lysates (2 μg/per samples) and rotated at 4 °C for 3 h or overnight followed by protein A/G-agarose 15 μl/ml at 4 °C for 2 h. Samples were washed with 1× IP buffer three times and applied in Laemmli buffer to 10% SDS-PAGE for immunoblotting.

Fibrinogen Binding—Washed mouse platelets (0.4 × 10^8/ml) were incubated simultaneously with 12.5 μg/ml AlexaFluor488-labeled Fibrinogen (Molecular Probes) and indicated concentrations of agonist peptide AYPGKF at 37 °C for 10 min, then fixed in 1% paraformaldehyde-containing Tyrode's buffer for 10 min at 37 °C, diluted five times with Tyrode's buffer, and analyzed by flow cytometry.

Megakaryocyte Differentiation—Megakaryocytes were differentiated from mouse embryonic stem (ES) cells in culture, essentially as described by Eto et al. (16). Mouse ES cells were seeded onto confluent OP9 cells and cultured in MEM medium supplemented with 20% fetal bovine serum (FBS). In 5 days, the ES cells were differentiated into hematopoietic progenitors without formation of embryonic bodies. For differentiation into megakaryocytes, the cells were trypsinized on day 5 and passed over fresh mitomycin C-treated OP9 cells in the same culture medium containing 20 ng/ml TPO. Then, on day 8, cells were seeded in a fresh OP9 feeder layer in the same culture medium containing 10 ng/ml TPO, IL-6 10 ng/ml, and IL-11 10 ng/ml for harvest at day 12. Differentiation was evaluated by immunostaining, Wright-Giemsa staining, and flow cytometry.

Immunofluorescence—Mouse ES cells were grown on Fluorodishes as described above. Cells were fixed with 4% paraformadehyde and washed with PBS, incubated with primary antibody for 1~2 h, and incubated with rhodamine or fluorescein-conjugated secondary antibodies for 30~60 min. Stained cells were observed on an Olympus confocal microscope (40×).

FeCl3-induced Carotid Artery Thrombosis—The right carotid artery of an anesthetized adult mouse (6–10 weeks of age, 18–30 kg treated with 100 mg/kg pentobarbital) was ex-

posed to a strip of filter paper saturated with either 10% FeCl3 for 2 min 15 s or 5% FeCl3 for 3 min, then rinsed with PBS, essentially as described (10). Arterial flow rate was recorded for 30 min with a Doppler flow probe. Stable occlusive thrombi were scored as complete cessation of blood flow which remained for the 30 min duration of the assay. Thrombi were scored as unstable if flow resumed before the end of the 30 min time period or decreased by at least 80% from the initial flow rate, but remained incomplete. The animal was scored as having no occlusive thrombus if the flow rate never decreased by 80% of the initial flow rate during the term of the assay. The mice were sacrificed at the end of the procedure. Statistical significance was calculated using Fisher's test of exact probability.

RESULTS

Arrestin-2 Forms Agonist-dependent Complexes with PI3K and Lyn in Human Platelets—Given that the Ser-Thr kinases Akt1 and Akt2 have been shown to play important roles in platelet aggregation and thrombosis (10, 11), we sought to uncover additional signaling proteins that may regulate Akt activation in platelets and also play important roles in thrombus formation. We and others have previously shown that PAR4-dependent activation of Akt is dependent on activation of SFKs (13, 17). SFKs are incorporated into signaling complexes containing PI3K subunits and arrestins in other cells (6); therefore, we reasoned that arrestins may contribute to Akt activation in platelets. We show in Fig. 1*A* that arrestin-2 is present in platelets isolated from mice and humans and immunodetection of arrestin-2 expression is lost in platelets genetically deleted for arrestin-2 (arrestin-$2^{-/-}$). To determine whether thrombin stimulates the association of arrestin-2 with the p85 subunit of PI3Kα/β, platelets were stimulated with thrombin, lysed, and immunoprecipitated with an antibody recognizing p85 PI3Ks α or β. Immunoprecipitates were then immunoblotted for arrestin-2. Formation of signaling complexes containing p85-PI3K and arrestin-2 were stimulated by thrombin and inhibited in the presence of the SFK inhibitor PP2, or ARL66096, an inhibitor of the P2Y12 receptor for ADP (Fig. 1*B*). Complex formation was also detected in thrombin-stimulated platelets immunoprecipitated with antibodies to arrestin-2 and immunoblotted for p85-PI3K, and blocked by apyrase, an enzyme which hydrolyzes ADP (Fig. 1*C*). PI3K-arrestin-2 complexes were detected in platelets stimulated with thrombin, PAR4 agonist peptide, and to a lesser extent, PAR1 agonist peptide (Fig. 1*C*). Given that thrombin-dependent PI3K-arrestin association is inhibited by SFK inhibitors, we also tested whether SFKs were incorporated into complexes with PI3K and arrestin-2. Fig. 1*D* shows that Lyn co-precipitates with arrestin-2 and PI3K upon thrombin stimulation and that thrombin-dependent association of Lyn with PI3K was inhibited by antagonists of P2Y12 and SFKs. Fyn and Src were not detected as part of the complexes (additional data not shown).

Deletion of Arrestin-2 Reduces Platelet Sensitivity to Thrombin, but Not ADP, Stimulation—The thrombin-stimulated association of PI3K with arrestin-2 suggests that arrestin-2 may regulate PI3K-dependent signaling events. Therefore, to determine whether arrestin-2 regulates Akt phosphorylation induced by thrombin receptor activation, Akt phosphorylation induced by the PAR4-activating peptide AYPGKF was evaluated in mouse platelets lacking arrestin-2 compared with WT control mice. The results show that arrestin$2^{-/-}$ platelets have a reduced sensitivity (right shift in dose-response curve) to PAR4 peptide- or thrombin-mediated Akt phosphorylation relative to their WT counterparts (Fig. 2, *A* and *B*). To

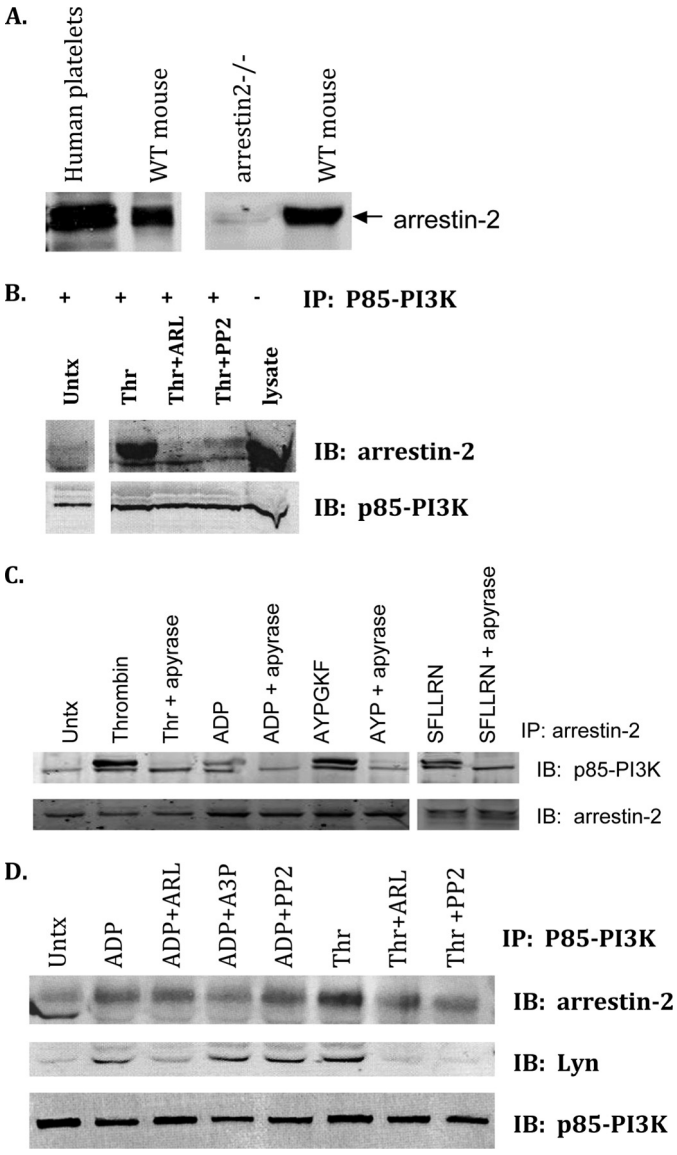

FIGURE 1. **Arrestin-2 expression and complex formation in mouse and human platelets.** *A*, 2×10^7 mouse or human platelets were loaded per lane and immunoblotted with antibody to arrestin-2. *B*, human platelets were left untreated or stimulated by thrombin (0.1U/ml) for 10 min with or without ARL66096(300 nM) or PP2(50uM), lysed, immunoprecipitated with antibodies to p85-PI3K(Upstate, Temecula CA; 2 μg/ml) and immunoblotted with antibodies to arrestin-2 (Santa Cruz Biotechnology, 1:000). *C*, platelets were stimulated for 10 min with ADP(10 μM), thrombin(0.1 units/ml), peptides AYPGKF(150uM), or SFLLRN(5 μM), with or without apyrase (1 unit/ml); then lysed and immunoprecipitated with antibody to arrestin-2 and immunoblotted with anti-p85-PI3K. *D*, human platelets treated with ADP or thrombin as in *C*, with or without ARL66096 (300 nM), A3P5PS(300 μM), or PP2(50uM) were immunoprecipitated with antibody to p85-PI3K (2 μg/ml) and immunoblotted with antibodies to arrestin-2, Lyn kinase, or p85-PI3K. Each of the figures shown is representative of results from a minimum of three separate experiments.

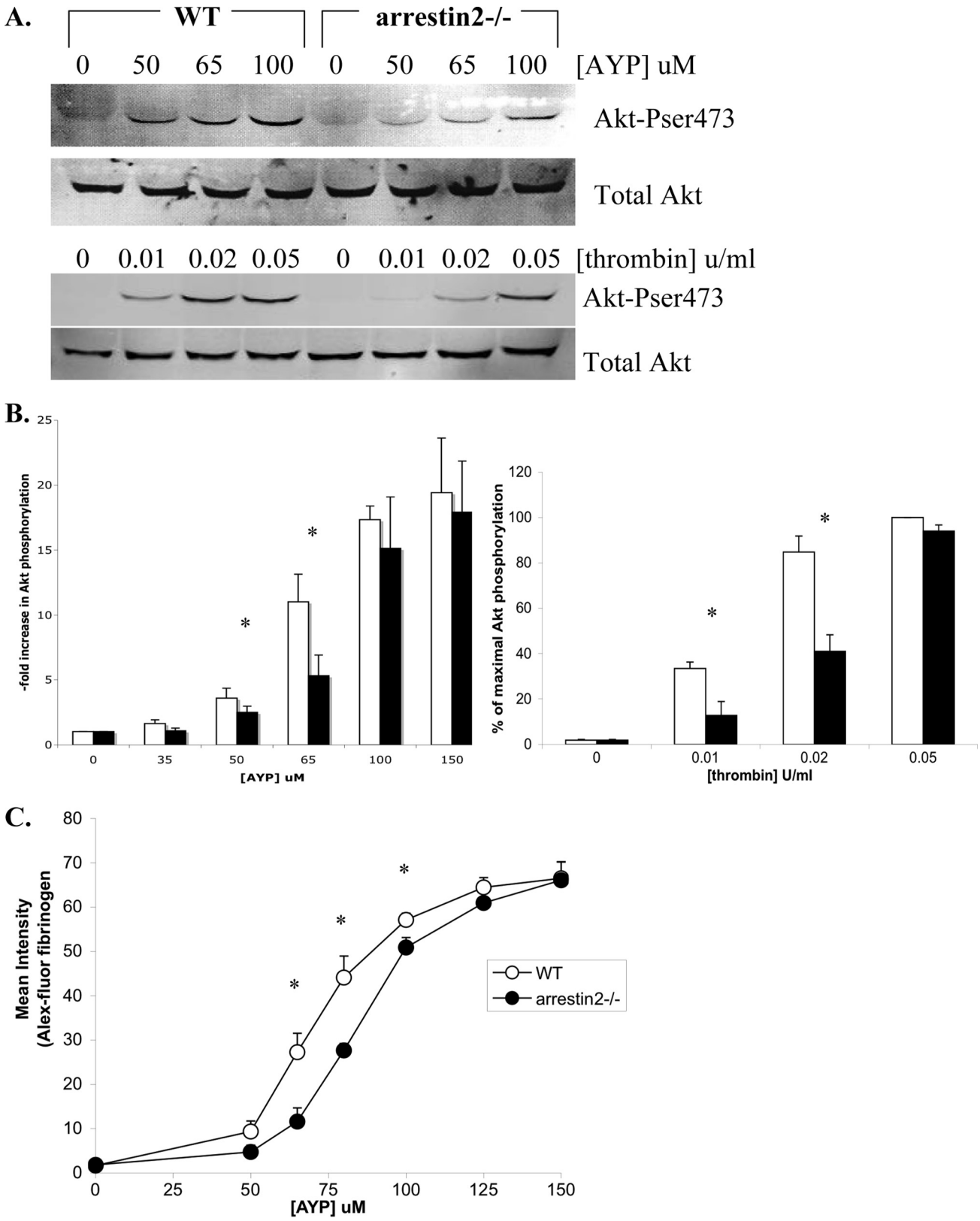

FIGURE 2. **Akt phosphorylation and fibrinogen binding in response to PAR4 agonist or thrombin in WT and arrestin2$^{-/-}$ platelets.** *A*, platelets (2×10^7/lane) from WT or arrestin-2$^{-/-}$ mice were stimulated for 5 min at 37 °C with the indicated concentration of AYPGKF, lysed, resolved by SDS-PAGE, and immunoblotted with phosphospecific antibody to p-Akt473 or total Akt. *B*, average ± S.E. of three or more experiments at each concentration as in *A*, quantified by densitometry, is shown. *White bars* are WT, *black* are arrestin-2$^{-/-}$. * indicates a significant difference between arrestin-2$^{-/-}$ and WT platelets is detected by 2-tailed, paired Student's *t* test, with $p \leq 0.05$. *C*, platelets from WT or arrestin-2$^{-/-}$ mice (4×10^7/ml) were stimulated with the indicated concentration of AYPGKF together with AlexaFluor488-conjugated fibrinogen, then fixed and analyzed by flow cytometry. Shown is the mean fluorescence intensity, averaged over three experiments ± S.E. * indicates significant difference between arrestin-2$^{-/-}$ and WT platelets is detected by 2-tailed, paired Student's *t* test, with $p \leq 0.05$.

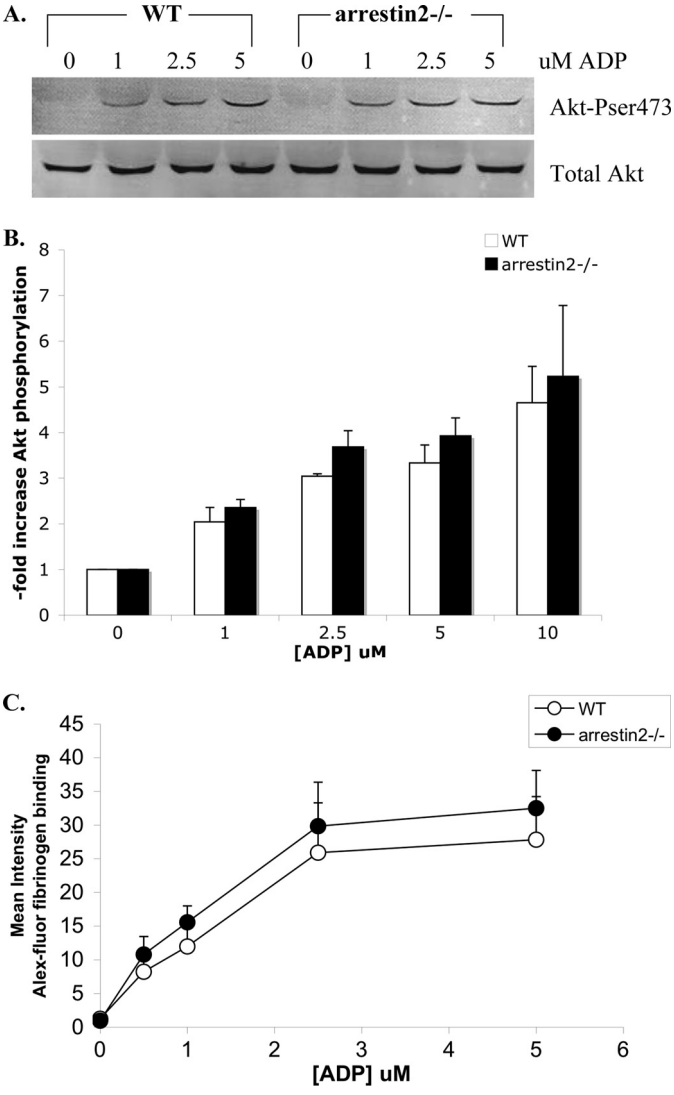

FIGURE 3. **Akt phosphorylation and fibrinogen binding in response to ADP in WT and arrestin-2$^{-/-}$ platelets.** *A*, platelets from WT or arrestin-2$^{-/-}$ mice were stimulated with the indicated concentration of ADP and immunoblotted for p-Akt473 or total Akt as in Fig. 2. *B*, average ± S.E. of three experiments as in *A*, quantified by densitometry is shown. *C*, platelets from WT or arrestin-2$^{-/-}$ mice were stimulated with the indicated concentration of ADP and analyzed for fibrinogen binding by flow cytometry as in Fig. 2. Shown is the mean fluorescence intensity, averaged over three experiments ± S.E.

determine whether Akt phosphorylation might affect platelet function, fibrinogen binding was evaluated in arrestin2$^{-/-}$ platelets. Arrestin2$^{-/-}$ platelets also displayed reduced sensitivity to fibrinogen binding relative to wild-type control platelets (Fig. 2*C*), likely reflecting the reduced sensitivity to Akt phosphorylation, which has been shown to promote fibrinogen binding (10).

PAR4-dependent Akt phosphorylation has been demonstrated to be largely dependent on the presence of ADP (12). Therefore, we considered that arrestin might be required for ADP signaling to Akt and influence PAR4 signaling as a secondary consequence. To our surprise, the concentration-response curves for Akt phosphorylation stimulated by ADP did not differ between arrestin2$^{-/-}$ mice and WT control mice (Fig. 3, *A* and *B*). There was also no difference in the concentration response curves for fibrinogen binding between arrestin2$^{-/-}$ platelets and WT mice (Fig. 3*C*). Taken together, the results in Figs. 2 and 3 suggest that PAR4 and P2Y12-dependent signaling to Akt occur through different mechanisms: PAR4-dependent Akt phosphorylation is partially dependent on arrestin-2, whereas P2Y12 signaling to Akt is not.

PAR4 Co-localizes and Associates with Arrestin-2 and PI3K upon Thrombin Stimulation—Our co-immunoprecipitation studies show that thrombin stimulates association of arrestin-2 and PI3Ks (Fig. 1). Because arrestins commonly interact directly with G protein-coupled receptors, we wondered whether arrestin-2 was recruited to the PAR4 thrombin receptor upon its activation, in turn recruiting PI3K to the receptor. We first tested this hypothesis using immunofluorescence microscopy of megakaryocytes differentiated in culture from ES cells (see Ref. 16). Megakaryocytic cells were incubated in the presence or absence of thrombin, then permeablized and immunostained with antibodies to PAR4, arrestin-2 or p85-PI3K. Although diffuse staining of PAR4 is seen in unstimulated cells, PAR4 co-localizes in discrete domains with arrestin-2 (Fig. 4*A*) and PI3K (Fig. 4*B*) upon thrombin stimulation. Little or no immunostaining of PAR4 or arrestin-2 was evident in the absence of Triton X-100 to permeablize the cells (additional data not shown). The PAR4 antibody used for immunostaining was directed against amino acids 180–300, spanning transmembrane domains 4 through 6, including the 3rd and 4th intracellular loops of PAR4. These data suggest that PAR4, PI3K, and arrestin-2 are co-localizing within an endocytic compartment upon thrombin stimulation.

To verify that arrestin-2 is recruited to PAR4 in platelets, rather than solely in a megakaryocyte model system, we also evaluated their association using a co-immunoprecipitation approach from human platelets. Our co-immunoprecipitation studies show that thrombin-induced association of PI3K with arrestin-2 is dependent on ADP; therefore, we tested whether the association was dependent on the P2Y1 or P2Y12 ADP receptors. Human platelets were stimulated with PAR4 agonist peptide or thrombin in the presence or absence of P2Y1 or P2Y12 antagonists, then immunoprecipitated with antibody to arrestin-2 and immunoblotted for PAR4. Stimulation of platelets with either PAR4 agonist (upper blot) or thrombin (lower blot) induces association of arrestin-2 with PAR4. The association is blocked by two different antagonists for P2Y12 (MeSAMP or ARC69931MX) or P2Y1 (A3P5PS or MRS2179) (Fig. 4*C*). Association of arrestin-2 with PAR4 is also evident in thrombin-stimulated cells immunoprecipitated with PAR4 and immunoblotted for arrestin-2 (Fig. 4*D*).

Arrestin Recruitment to PAR4 Is Dependent upon P2Y1-stimulated PKC Activation—Whereas a role for P2Y12 in arrestin association with PAR4 is not unexpected given that both P2Y12 and arrestin are required for maximal Akt phosphorylation by thrombin, the requirement for P2Y1 in arrestin recruitment was unforeseen. P2Y1 is a G_q-coupled receptor, activation of which stimulates phospholipase Cβ2, leading to protein kinase C (PKC) activation and release of calcium from the dense tubular system. To determine whether PKC was important for arrestin association with

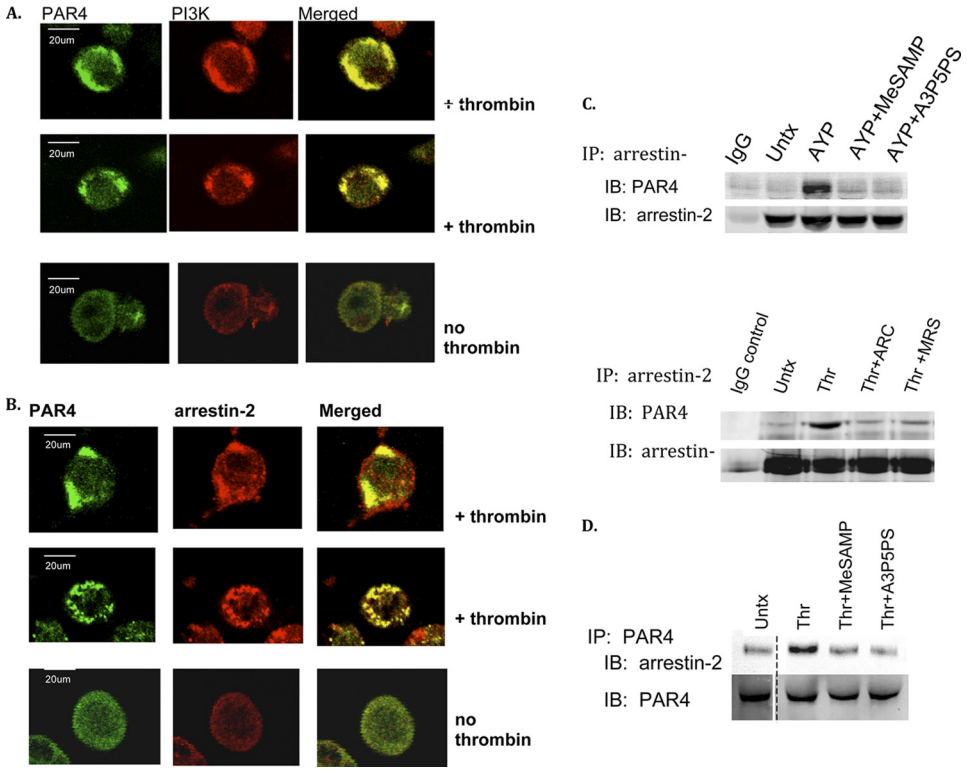

FIGURE 4. **Immunofluorescence localization of PAR4, arrestin-2, and p85-PI3K (*A* and *B*) and co-immunoprecipitation of PAR4 and arrestin-2 (*C* and *D*).** *A*, mouse megakaryocytes were differentiated in culture, grown on Fluorodishes, and incubated in the presence (*upper panels*) *versus* absence (*lower panels*) of thrombin (2 units/ml) for 10 min at 37 °C. Cells were then incubated with FITC-conjugated antibody to PAR4 and rhodamine-conjugated antibody to p85-PI3K (*A*) or rhodamin-conjugated antibody to arrestin-2 (*B*), fixed, and slides evaluated at 40× magnification on an Olympus Confocal microscope. 2-Color merge is shown in *yellow*. *C*, human platelets (4×10^8/lane) were treated with AYPGKF (150uM) for 5 min at 37 °C with or without 2MeSAMP (100 μM), A3P5PS (300 μM), ARC69931MX (300 nM), or MRS2179 (100 μM), then immunoprecipitated with either IgG control or antibody to arrestin-2. Precipitates were immunoblotted with anti-PAR4 or -arrestin-2 antibodies. *D*, human platelets were incubated with/without thrombin (0.1 unit/ml) for 5 min at 37 °C, then immunoprecipitated with antibody to PAR4 and immunoblotted with antibodies to arrestin-2 or PAR4.

PAR4, PAR4-stimulated co-immunoprecipitation of PAR4 and arrestin-2 was tested in the presence of various PKC inhibitors. Akt phosphorylation was also tested under the same conditions. The broad spectrum PKC inhibitor staurosporine blocks arrestin association with PAR4, as well as PAR4-dependent Akt phosphorylation (Fig. 5, *A* and *B*). Similarly, the broad-spectrum inhibitor Go6983, which inhibits both classical PKC isoforms (α, β, and γ) and atypical, non-Ca^{2+}-dependent isoforms (δ and ζ), also decreased PAR4-arrestin association and Akt phosphorylation. In contrast, the PKC inhibitor Go6976, selective for classical isoforms PKCα and PKCβ, did not. These data suggest that arrestin recruitment to PAR4 is dependent upon the non-Ca^{2+} dependent, atypical class of PKCs. However, incubation with PMA did not stimulate Akt phosphorylation, implying that PKC is required, but not sufficient for PAR4-arrestin association and Akt phosphorylation. Interestingly, P2Y12 has been shown to enhance PKC phosphorylation through inhibition of DAGkinase (18). Therefore, P2Y12 and P2Y1 may both contribute to arrestin recruitment via PKC-dependent phosphorylation of PAR4.

Maximal PAR4-induced Akt Phosphorylation Requires both P2Y1 and P2Y12—The results shown in Fig. 4*B* implicate a role for P2Y12, in addition to P2Y1, in arrestin-2 recruitment to PAR4. To understand the relative roles of P2Y1 and P2Y12 in arrestin signaling to Akt, Akt phosphorylation was compared at 1, 3 and 5 min after PAR4 stimulation in the presence and absence of P2Y1 and P2Y12 inhibitors in WT and arrestin-2 knock-out mice. An average of three experiments using the P2Y12 inhibitor MeSAMP and P2Y1 inhibitor A3P5PS is shown in Fig. 6*A*, while single representative ex-

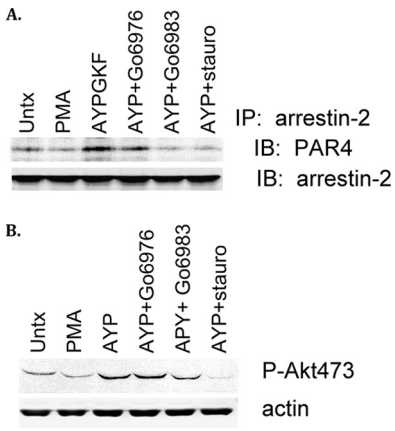

FIGURE 5. **Effect of PKC inhibition on arrestin-2 association with PAR4 and Akt phosphorylation.** *A*, human platelets (4×10^8/lane) were preincubated with or without PKC inhibitors Go6983 (1 μM), Go6976(1 μM), or staurosporine (1 μM), then stimulated by PMA (1 μM) or AYPGKF (120 μM) and lysed. Arrestin-2 was immunoprecipitated as in Fig. 4*B*, then precipitates were immunoblotted for PAR4 or arrestin-2. *B*, human platelets (2×10^7/lane) treated with inhibitors as in *A* were lysed and immunoblotted with phosphospecific antibody to Akt-Ser-473 or actin. Results shown are representative of two experiments with similar results.

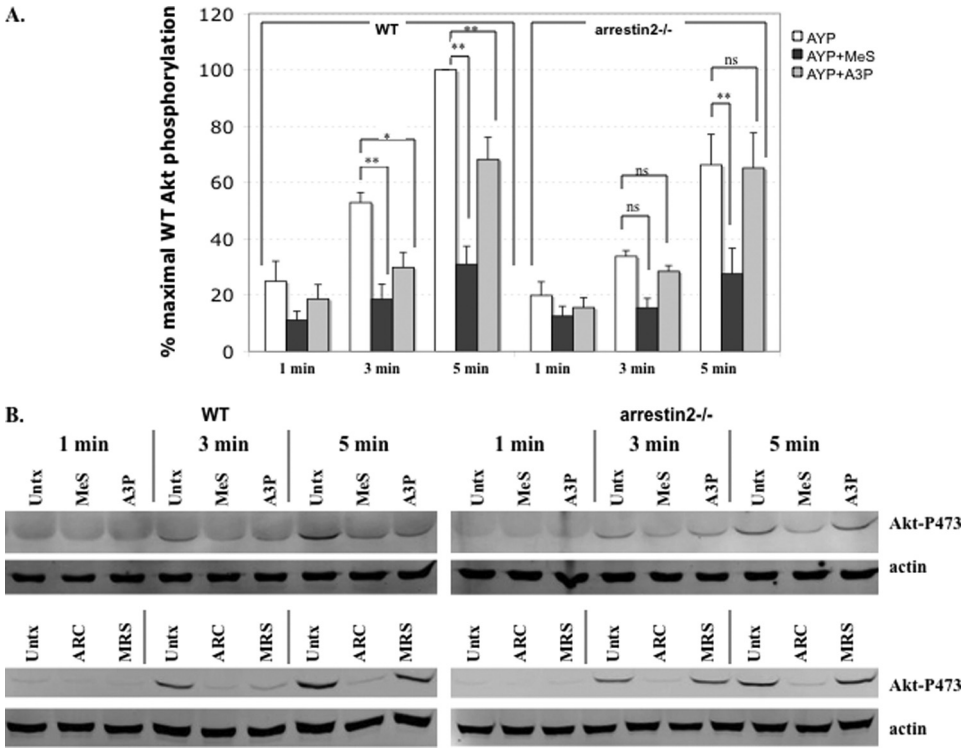

FIGURE 6. **Effect of P2Y1 and P2Y12 inhibition on Akt phosphorylation in WT *versus* arrestin-2$^{-/-}$ platelets.** Platelets from WT or arrestin-2$^{-/-}$ mice (2×10^7/lane) were incubated for the indicated amount of time with 75 μM AYPGKF without inhibitor (*white bars*), with 2MeSAMP (100 μM) (*black bars*) or with A3P5PS(300 μM) (*gray bars*), then lysed and immunoblotted for Akt phosphorylation of Ser-473 as in Fig. 2*A*. Immunoblots were scanned by densitometry and expressed as % maximal Akt phosphorylation detected in WT platelets stimulated for 5 min (*A*). The average of four experiments ± S.E. is shown. A significant difference between drug-treated and untreated sample at that time point is denoted by * with $p \leq 0.05$ or ** with $p \leq 0.01$ using 2-way ANOVA with Bonferroni post-test analysis. Representative immunoblots probed with phospho-Akt antibody (*upper blots*) and re-probed with antibody to actin (*lower blots*) are shown in *B*. Also shown are representative immunoblots of AYPGKF-stimulated platelets in the presence or absence of ARC69931MX (300 nM), or MRS2179 (100 μM) to inhibit P2Y12 and P2Y1, respectively. The same results were obtained in two additional experiments using these inhibitors.

periments using inhibitors MeSAMP and A3P5PS, or ARC69931MX (P2Y12 inhibitor) and MRS2179 (P2Y1 inhibitor), are shown in Fig. 6*B*. Consistent with the unforeseen role of P2Y1 in arrestin recruitment to PAR4, a role for P2Y1 in Akt phosphorylation is evident at both 3 and 5 min, as Akt phosphorylation is inhibited by A3P5PS or MRS2179 at these time points ($p < 0.05$ at 3 min, $p < 0.001$ at 5 min ANOVA with Bonferroni post-test analysis). In arrestin-2 knock-out mice, the degree of Akt phosphorylation at 3 or 5 min is comparable to that of WT platelets treated with A3P5PS. In addition, no inhibition of Akt phosphorylation by A3P5PS or MRS2179 was seen in arrestin2$^{-/-}$ platelets at these time points, suggesting that the role of P2Y1 in Akt phosphorylation is mediated by arrestin-2 (difference is not significant by Bonferroni post-test). In contrast, P2Y12 appears to play some arrestin-independent roles in Akt phosphorylation, since the inhibition of P2Y12 reduces Akt phosphorylation even in the absence of arrestin-2 ($p < 0.001$ at 5 min)(Fig. 6). This reveals an arrestin-independent role for P2Y12 in addition to the role in arrestin recruitment evident from Fig. 4.

P2Y12 Directly Associates with PAR4 after Thrombin Stimulation of Human Platelets—To address the mechanism by which P2Y12 contributes to arrestin recruitment to PAR4, we considered recent evidence demonstrating oligomerization of P2Y12 receptors in platelets (19). We hypothesized that P2Y12 may physically associate with PAR4 and that the heterodimer or oligomer may present a site that facilitates arrestin binding. Previous work has shown that arrestin-2 facilitates the internalization of P2Y12 (20); therefore, the association of P2Y12 with PAR4 may simply recruit the P2Y12-associated arrestin-2 to the same complex. To determine whether PAR4 and P2Y12 physically associate in platelets after agonist stimulation, human platelets were stimulated with thrombin or PAR4 agonist, lysed, then immunoprecipitated with antibody to P2Y12 (Fig. 7*A*) or PAR4 (Fig. 7*B*) (the entire blot is shown in the supplemental Fig. S1). The precipitates were immunoblotted for PAR4 or P2Y12, respectively. Fig. 7 shows that PAR4 associates with P2Y12 after thrombin or PAR4 stimulation of human platelets. The association is reduced by P2Y12 antagonist. A slight association is detected in platelets stimulated with ADP. These results suggest that P2Y12 and PAR4 form agonist-dependent heteromers in platelets, consistent with the idea that the physical association of P2Y12 with PAR4 helps to recruit arrestin-2 to PAR4.

Arrestin-2 Is Important for Thrombus Formation in a Carotid Artery Injury Model—Akt is important for the formation and maintenance of stable occlusive thrombi in mice (10). To determine whether arrestin-2 contributes to thrombus formation in mice, a ferric chloride-induced carotid artery injury model was used. Ferric chloride was applied for 2 min, 15 s to carotid arteries of wild type or arrestin2$^{-/-}$ mice and the number of mice forming stable thrombi that impeded flow rate for 30 min was recorded. A graph of the results is shown in Fig. 8*A*. Those mice forming thrombi that resolved before

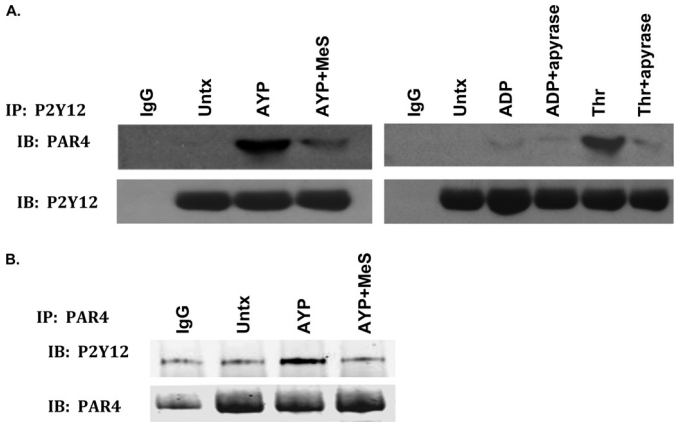

FIGURE 7. **Agonist-dependent association of PAR4 and P2Y12.** *A*, human platelets (4×10^8/lane) were treated with AYPGKF (150uM), ADP (10 μM), or Thrombin (0.1 unit) for 5 min at 37 °C with/without 2MeSAMP (100 μM) or Apyrase (1 unit/ml), then immunoprecipitated with either IgG control or antibody to P2Y12 (2 μg/ml). Precipitates were immunoblotted with anti-PAR4 antibodies (1:1000). *B*, human platelets treated as above were immunoprecipitated with antibody to PAR4 (2 μg/ml), then immunoblotted with antibody to P2Y12 (1:1000).

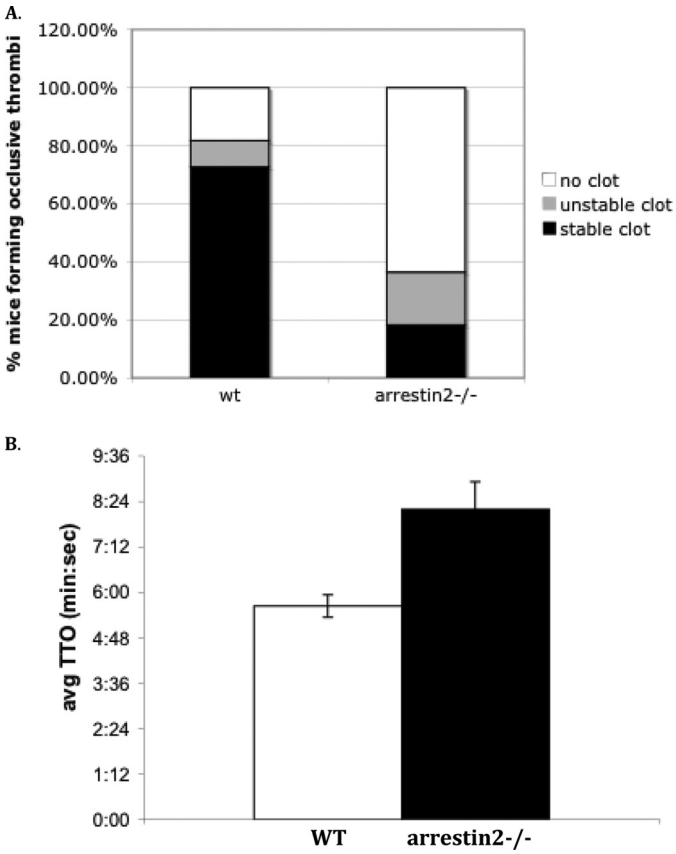

FIGURE 8. **Arterial thrombus formation in WT and arrestin-$2^{-/-}$ mice.** *A*, 10% ferric chloride-soaked filter paper was applied for 2 min and 15 s (2′15″) to carotid arteries of pentobarbitol-sedated wild type or arrestin-$2^{-/-}$ mice and the carotid arterial flow rate was measured using a Doppler flow probe. The percentage of each genotype forming stable thrombi that completely impeded flow rate for 30 min is shown in *black*, the percentage forming unstable thrombi are shown in *gray*, and the percentage with no occlusion is shown in *white*. The number of stable occlusions formed differs between WT and arrestin-$2^{-/-}$ mice, with $p = 0.03$ (two-tailed Fisher's exact probability test). The results of 11 WT and 11 arrestin-$2^{-/-}$ mice are shown. *B*, 5% ferric chloride-soaked filter paper was applied for 3 min to carotid arteries of sedated mice and flow rate was recorded as described: time to occlusive thrombus formation was recorded for 10 mice of each genotype. The mean time to occlusions differ between the two genotypes of mice with $p = 0.005$ (unpaired 2-tailed Student's *t* test).

the end of the 30 min assay period were scored as having unstable thrombi. In wild-type mice, 73% of mice assayed formed stable occlusions compared with 18% of arrestin$2^{-/-}$ mice, indicating that arrestin$2^{-/-}$ mice have a statistically significant reduction in stable occlusive thrombus formation under these conditions ($p = 0.03$, two-tailed Fisher's exact probability test). Because this is a milder difference in total occlusion than that we have previously reported for mice lacking Akt2, we also assessed whether time to thrombus formation differed between wild type and arrestin$2^{-/-}$ mice when mice were exposed to a lower concentration of ferric chloride (5%) for a longer time (3 min). The results show a significant difference in time to occlusive thrombus formation in 10 mice forming thrombi of each genotype (Fig. 8*B*; $p = 0.005$, unpaired two-tailed Student's *t* test).

DISCUSSION

Arrestins can positively or negatively regulate distinct aspects of cellular function (2, 21), but the roles of arrestins in platelet function remain uncharacterized. Of the two non-visual arrestins, arrestin-2 is more easily detected via immunoblot analysis (Fig. 1*A* and additional data not shown), and its mRNA is more readily detectable in the platelet transcriptome (22). Small amounts of arrestin-3 may also be present and may provide some compensatory regulation in the absence of arrestin-2. Both single knock-out mice are viable (15, 23), but arrestin-2/arrestin-3 double knock-out mice die *in utero* (24). Arrestin-2 knock-out mice have few physiological defects, but display increased sensitivity to β-adrenergic stimulation in the heart, suggesting a role in desensitization of cardiac responses to β-agonists (15). To determine whether arrestin-2 might regulate platelet signaling or function, we evaluated arrestin complex formation in human and mouse platelets and the effects of arrestin-2 loss on mouse platelet function *in vitro* and *in vivo*.

The results show that activation of PAR4 stimulates association of the p85 regulatory subunit of PI3K with arrestin in a manner dependent on P2Y12 and SFKs. Lyn is incorporated into the complexes, suggesting that this is the relevant Src kinase that contributes to arrestin-dependent signaling downstream of PAR4 and likely explains the role of Lyn in contributing to thrombin-dependent Akt phosphorylation and secretion noted by Cho *et al.* (17). We propose a mechanism in which arrestin-2 is recruited to activated PAR4 and in turn helps to recruit Lyn complexed with PI3K (see Fig. 9 for diagrammatic summary of the signaling mechanism). ADP contributes to arrestin recruitment to PAR4, since inhibition of either P2Y12 or P2Y1 reduces arrestin association with PAR4. This work has uncovered a unique and surprising role for P2Y1 in contributing to arrestin recruitment to PAR4, which may partially explain the unexpected effect of P2Y1 inhibition on aggregation induced by low concentrations of thrombin (25). These data would suggest that P2Y1 should also affect Akt phosphorylation, which has not been reported previously. In fact, a role for P2Y1 in Akt phosphorylation is evident at 3

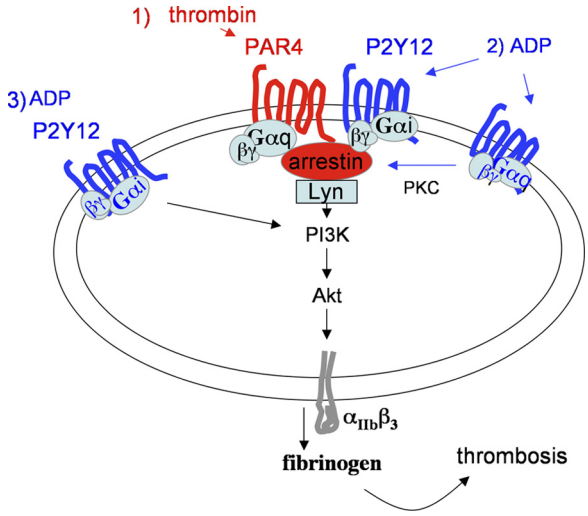

FIGURE 9. **Thrombin-dependent signaling in platelets (1) requires coincident signaling by ADP(2) for maximal Akt phosphorylation and fibrinogen binding.** Part of the requirement for coincident activation of ADP receptors is due to P2Y1-mediated recruitment of arrestin-2 to the PAR4 receptor complex. P2Y12 activation is also required for arrestin recruitment to the PAR4 complex. However, Akt phosphorylation mediated by ADP stimulation of P2Y12 alone (in the absence of thrombin-dependent PAR4 activation) is not dependent on arrestin-2 (3).

and 5 min post-PAR4 stimulation (Fig. 6). The reduction in PAR4-mediated Akt phosphorylation due to P2Y1 antagonism is smaller than that due to blockade of P2Y12 and is overcome at higher agonist concentrations, as is the case with arrestin-2 deletion. This may explain why no effect of P2Y1 antagonist on Akt phosphorylation was observed previously (26, 27). Fig. 4 suggests that arrestin recruitment to PAR4 is reduced by broad-spectrum PKC inhibitors, but not by classical PKC inhibitors alone: these results may suggest that a unique non-Ca^{2+}-dependent PKC isoform is stimulated by P2Y1, which plays a role in PAR4 phosphorylation to allow arrestin recruitment.

The role of P2Y12 in Akt phosphorylation is not limited to arrestin-dependent signaling, since P2Y12 inhibition further reduces Akt phosphorylation in arrestin-$2^{-/-}$ platelets (Fig. 6). P2Y12 may play a direct role in Lyn activation, for example: Src family members have been found to associate with G_i family members (28–30) and G protein-coupled receptors (31, 32). P2Y12 is thus involved in both arrestin-independent and -dependent signaling, because P2Y12 also plays a role in arrestin recruitment to PAR4 (Fig. 4). The recent observation that P2Y12 receptors form homo-oligomers in platelets (19) suggested to us that P2Y12 may help to recruit arrestin-2 to PAR4 by forming a heterodimer or oligomer that facilitates arrestin binding. The idea that GPCR dimers may be required for arrestin-dependent signaling has precedent in both the muscarinic and α-adrenergic receptor systems (33, 34). In fact, we have detected the agonist-dependent association of PAR4 with P2Y12 in human platelets using an immunoprecipitation approach (Fig. 7). Taken together with a previous study showing that arrestin-2 facilitates internalization of P2Y12 (20), these data suggest a model in which agonist stimulation of PAR4 recruits P2Y12 pre-complexed with arrestin-2.

It is clear that PAR4-dependent signaling to Akt activation and fibrinogen binding is not solely due to ADP release, because PAR4 and ADP-induced signaling are differentially sensitive to arrestin-2. This study uncovers a unique role for arrestin-dependent PAR4 signaling to Akt, for which P2Y12 signaling alone is insufficient. It is worth noting that these experiments have been done primarily with thrombin and PAR4 peptide agonists, due to the capability to compare responses in arrestin-2 knock-out mice (PAR1 is not expressed in mouse platelets); whether arrestin-2 is required for signaling downstream of PAR1 in platelets is still unresolved.

Analysis of thrombus formation using a ferric chloride arterial injury model reveals that arrestin-2 positively regulates thrombus formation *in vivo*. This would seem to reflect its role in supporting PAR4 signaling to Akt, since arrestin-2 did not affect ADP-induced fibrinogen binding. The defect in thrombosis in arrestin-2 knock-out mice appears milder than that previously observed in Akt2$^{-/-}$ mice under similar conditions, which is consistent with the notion that arrestin-2 is only partially responsible for Akt phosphorylation by PAR4. While clearly arrestins can mediate desensitization of receptor signaling in some contexts, ADP-induced fibrinogen binding and Akt phosphorylation were not significantly affected by the loss of arrestin-2. Furthermore, the positive role played by arrestin-2 in the thrombosis model suggests that its role in recruiting PI3K complexes is more important in thrombus formation than any potential role in desensitizing platelet receptors for ADP or other agonists. Alternatively, these results may reflect the largely thrombin-dependent nature of the ferric chloride injury model, which may be particularly sensitive, and thus somewhat biased toward detecting defects in PAR4-dependent pathways. Despite this caveat, the model reveals that arrestin-dependent signaling can play important positive roles in regulating thrombus formation *in vivo*.

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