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Vascular inflammation and thrombosis require the concerted actions of several different agonists, many of which act on G protein-coupled receptors (GPCRs). GPCR dimerization is a well-established phenomenon that can alter protomer function. In platelets and other cell types, protease-activated receptor-4 (PAR4) has been shown to dimerize with the purinergic receptor P2Y12 to coordinate β-arrestin–mediated Akt signaling, an important mediator of integrin activation. However, the mechanism by which the PAR4-P2Y12 dimer controls β-arrestin–dependent Akt signaling is not known. We now report that PAR4 and P2Y12 heterodimer internalization is required for β-arrestin recruitment to endosomes and Akt signaling. Using bioluminescence resonance energy transfer, immunofluorescence microscopy, and co-immunoprecipitation in cells expressing receptors exogenously and endogenously, we demonstrate that PAR4 and P2Y12 specifically interact and form dimers expressed at the cell surface. We also found that activation of PAR4 but not of P2Y12 drives internalization of the PAR4-P2Y12 heterodimer. Remarkably, activated PAR4 internalization was required for recruitment of β-arrestin to endocytic vesicles, which was dependent on co-expression of P2Y12. Interestingly, stimulation of the PAR4-P2Y12 heterodimer promotes β-arrestin and Akt co-localization to intracellular vesicles. Moreover, activated PAR4-P2Y12 internalization is required for sustained Akt activation. Thus, internalization of the PAR4-P2Y12 heterodimer is necessary for β-arrestin recruitment to endosomes and Akt signaling and lays the foundation for examining whether blockade of PAR4 internalization reduces integrin and platelet activation.

The activation of platelets requires agonist stimulation of several G protein-coupled receptors (GPCRs)[4] and is essential for thrombotic events during vascular injury. Thrombin, the key effector protease of the coagulation cascade, is the most potent platelet agonist. Activation of platelets by thrombin occurs through protease-activated receptor-1 (PAR1) and -4 (PAR4), two distinct GPCRs (1). Thrombin binds to and cleaves the N-terminal domain of PAR1, generating a new N terminus that functions as a tethered ligand by binding intramolecularly to the receptor to promote activation (2, 3). Although PAR4 is a low-affinity receptor for thrombin, it appears to be activated through a similar mechanism (4, 5). Whereas PAR1 is clearly an important target for the development of drugs that can reduce thrombotic events (6, 7), recent work indicates that blockade of PAR4 also provides robust anti-thrombotic activity but without the bleeding risk associated with many antiplatelet drugs (8). Thus, the regulation of PAR4 signaling is important to understand.

In addition to PARs, platelets express two purinergic GPCRs, P2Y1 and P2Y12, that are activated by dense granule release of ADP (9, 10). P2Y12 has been linked to important aspects of platelet activation, including enhancement of dense granule secretion, recruitment of additional platelets to the site of vascular injury, and enhancement of the efficacy of other pro-coagulant agonists (11, 12). P2Y12 couples to $G\alpha_i$ and mediates phosphoinositide 3-kinase–dependent Akt activation, an important mediator of integrin activation in platelets (13). Integrins are heterodimeric transmembrane receptors that mediate cell-cell adhesion and cell adhesion to extracellular matrix components. In agonist-stimulated platelets, the integrin subunits glycoprotein IIb and IIIa heterodimerize to yield their active form, integrin $\alpha_{IIb}\beta_3$, which binds to fibrinogen with high affinity to facilitate platelet adhesion to the extracellular matrix (14, 15). Therefore, P2Y12 controls important aspects of platelet function through activation of Akt.

Previous studies using platelets and other cell types suggest that PAR4 and P2Y12 cross-talk is also important for proper Akt-mediated integrin activation (16). PAR4-stimulated Akt

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[4] The abbreviations used are: GPCR, G protein-coupled receptor; PAR, protease-activated receptor; β-arr, β-arrestin; BRET, bioluminescence resonance energy transfer; ERK1/2, extracellular-signal regulated kinase-1/2; IP, immunoprecipitation; PAR4 Y/A, PAR4 Y264A/L268A; Rluc, *Renilla* luciferase; TF, TFLLRNPNDK; ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid.

activation was diminished in platelets derived from arrestin-2 (also known as β-arrestin-1) knock-out mice (16). P2Y12 receptor antagonists blocked activated PAR4-induced recruitment of arrestin-2 and Akt activation, suggesting that P2Y12 plays a critical role. In more recent work, PAR4 and P2Y12 were shown to form dimers in an agonist-dependent manner, which is important for arrestin-2 recruitment and Akt activation (17). However, it remains unclear how activation of the PAR4-P2Y12 dimer regulates β-arrestin recruitment to promote Akt activation.

β-Arrestin-1 and -2 are widely expressed multifunctional adaptor proteins that mediate GPCR desensitization and internalization and promote signaling from the plasma membrane and endosomes (18). The P2Y12 receptor interacts with β-arrestins and requires β-arrestins for clathrin-mediated internalization (19). In contrast, PAR4 internalization occurs independent of β-arrestins and, rather, utilizes the clathrin adaptor-protein complex-2 for internalization through clathrin-coated pits (20). These findings are consistent with previously published work demonstrating that activated PAR4 is not phosphorylated, which is generally a requirement for β-arrestin recruitment to most activated GPCRs (21).

In this study we sought to determine how the PAR4-P2Y12 dimer complex functions to control β-arrestin–mediated Akt activation. Here, we demonstrate that PAR4-P2Y12 interaction occurs basally at the cell surface. Intriguingly, activation of PAR4 drives P2Y12 co-internalization but not vice versa. Remarkably, activated PAR4-P2Y12 internalization is necessary for β-arrestin recruitment to endosomes and association with Akt. Finally, activated PAR4-P2Y12–induced Akt signaling is dependent on internalization of the receptor heterodimer complex. Together these studies suggest that PAR4-P2Y12 exists as a dimer or higher order oligomer and that PAR4-P2Y12 internalization is required for β-arrestin endosomal recruitment and Akt signaling.

Results

PAR4-P2Y12 heterodimers exist at the cell surface

PAR4 and P2Y12 exhibit cross-talk to mediate β-arrestin–dependent Akt activation in platelets, but mechanistically how this occurs is not clear. To assess whether PAR4 and P2Y12 associate in cells, co-immunoprecipitation (co-IP) experiments were performed. HeLa cells were transfected with either N-terminal FLAG-tagged PAR4 alone, N-terminal HA-tagged P2Y12 alone, or both receptors together (Fig. 1*A*). In cells co-expressing both receptors, PAR4 and P2Y12 were detected in co-IPs using anti-FLAG antibody but not in IgG control co-IPs (Fig. 1*A*, *lanes 3* and *4*). However, neither the PAR4 peptide agonist AYPGKF nor the P2Y12 agonist ADP changed the extent of PAR4-P2Y12 co-association detected by co-IP in HeLa cells (Fig. 1*A*, *lanes 4–6*), suggesting that PAR4 and P2Y12 basally associate in cells.

To investigate if PAR4 and P2Y12 have the capacity to physically associate, we examined heterodimer formation in living cells using bioluminescence resonance energy transfer (BRET). BRET assays were conducted using full-length PAR4 and P2Y12 fused in-frame to either YFP or Rluc at the C terminus,

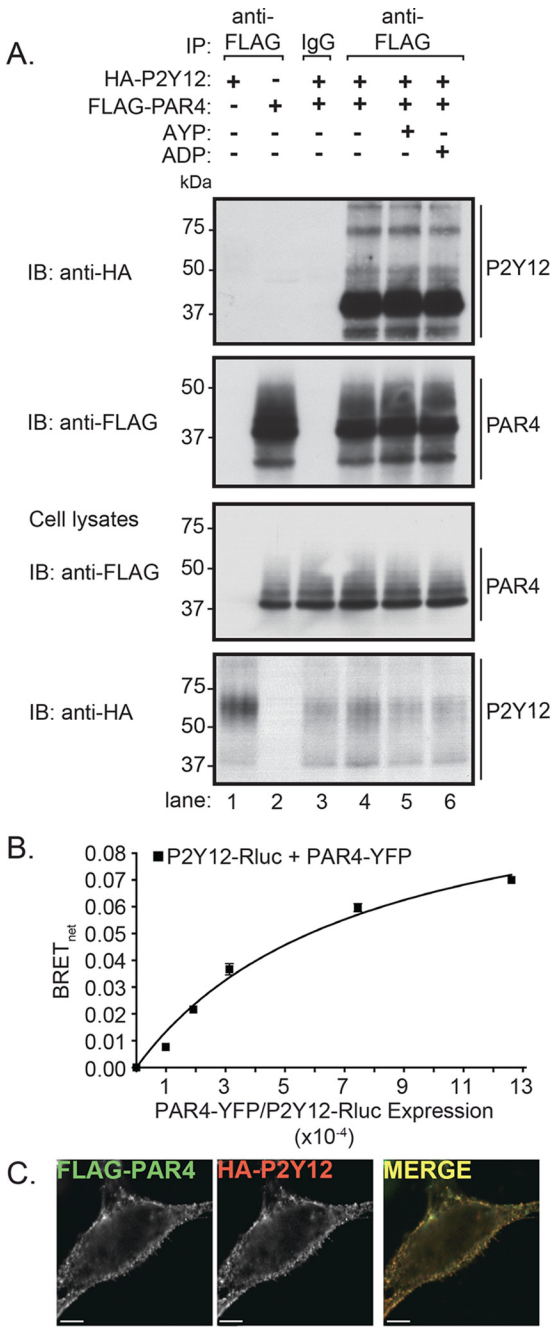

Figure 1. PAR4 and P2Y12 form a stable heterodimer. *A*, HeLa cells expressing FLAG-PAR4 and/or HA-P2Y12 were treated with 500 μM AYPGKF (*lane 5*) or 10 μM ADP (*lane 6*) for 15 min at 37 °C. Cells were lysed, immunoprecipitated (*IP*), resolved by SDS-PAGE, and immunoblotted (*IB*) with the indicated antibodies. Immunoblots shown are representative of three independent experiments. *B*, COS-7 cells were transfected with a fixed amount of P2Y12-Rluc and increasing amounts of PAR4-YFP. $BRET_{net}$ is plotted as a function of the ratio of PAR4-YFP/P2Y12-Rluc expression. *C*, COS-7 cells transfected with FLAG-PAR4 and HA-P2Y12 were immunostained with anti-FLAG and anti-HA antibodies, fixed, processed, and imaged by confocal microscopy. The *yellow* color in the merged image is indicative of colocalization of P2Y12 (*red*) and PAR4 (*green*). The image is representative of three independent experiments (*scale bars*, 10 μm).

respectively. COS-7 cells were transfected with a fixed amount of P2Y12-Rluc and increasing amounts of PAR4-YFP, and the net BRET signal was quantified. We observed a hyperbolic increase in the net BRET signal as the ratio of PAR4-YFP expression to P2Y12-Rluc expression increased (Fig. 1*B*). These

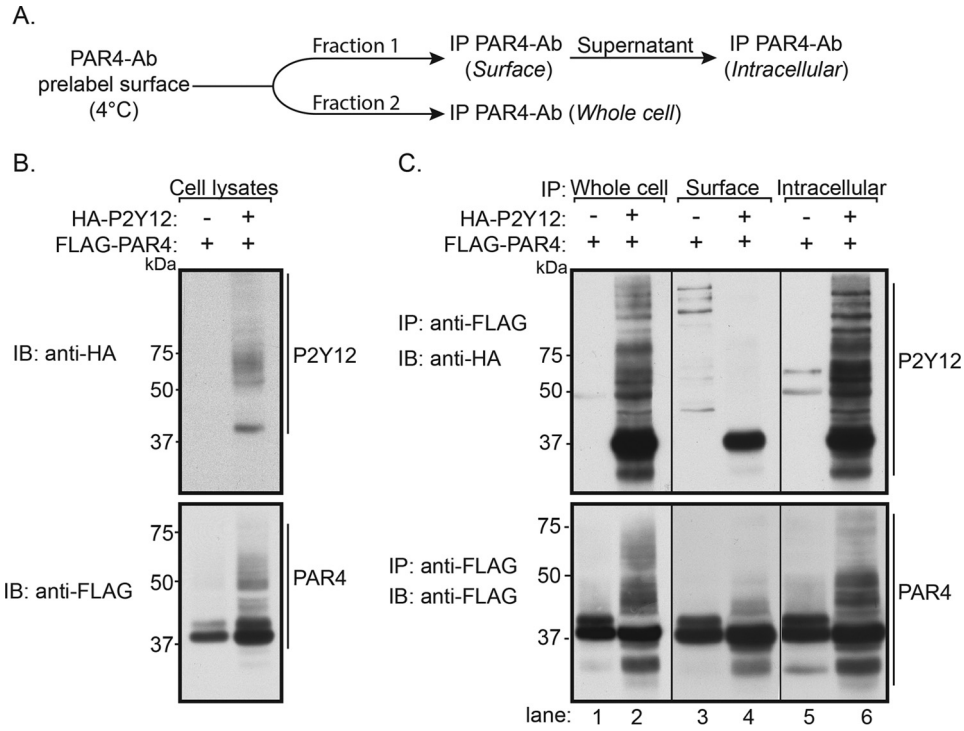

Figure 2. PAR4 and P2Y12 interact at the cell surface. *A*, schematic of sequential co-IP experimental work flow used to selectively immunoprecipitate PAR4 from the cell surface and intracellular compartments. COS-7 cells expressing FLAG-PAR4 and/or HA-P2Y12 were incubated with anti-FLAG antibody for 1.5 h at 4 °C to label surface PAR4, then lysed and evenly divided into two fractions: *Fraction 1* and *Fraction 2*. *Fraction 1* was immunoprecipitated to obtain the *Surface* IP (*C*, *lanes 3* and *4*), and the supernatant was collected, incubated with anti-FLAG antibody for 1.5 h at 4 °C, and immunoprecipitated to obtain the *Intracellular* IP (*C*, *lanes 5* and *6*). *Fraction 2* was incubated with anti-FLAG antibody for an additional 1.5 h at 4 °C and then immunoprecipitated to obtain the *Whole cell* IP (*C*, *lanes 1* and *2*). *Cell lysates* (*B*) were collected directly after lysis. *B* and *C*, cell lysates (*B*) and IP samples (*C*) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots are representative of three independent experiments.

results are indicative of a specific protein-protein interaction rather than a nonspecific bystander BRET effect, where the relationship would be linear (22). Together these studies suggest that PAR4 and P2Y12 form dimeric or higher-order oligomeric complexes in cells.

Immunofluorescence confocal microscopy studies further indicate that PAR4 and P2Y12 co-localize at the cell surface. COS-7 cells were co-transfected with FLAG-PAR4 and HA-P2Y12 and incubated with anti-FLAG and anti-HA antibodies for 1 h at 4 °C. Under these conditions only the cell-surface receptor cohorts are labeled with antibody. Confocal imaging revealed substantial co-localization of PAR4 and P2Y12 at the cell surface displayed as a *yellow* color in the merged image (Fig. 1*C*). To assess PAR4 and P2Y12 association in distinct subcellular compartments, we performed sequential co-IP experiments to separate the cell surface and intracellular populations of PAR4-P2Y12 complexes in COS-7 cells. In these experiments, COS-7 cells were incubated with anti-FLAG antibody on ice to specifically label the cell-surface population of PAR4. Cells were then lysed and incubated with protein A-Sepharose beads to capture the antibody-bound surface PAR4 cohort. A second IP was then performed on the supernatant to isolate the intracellular pool of PAR4 (Fig. 2*A*). Immunoblotting with receptor-specific antibodies revealed that both FLAG-PAR4 and HA-P2Y12 express as a low molecular weight band that is consistent with their predicted molecular weight and several higher molecular species that are likely due to glycosylated forms of the receptors (Fig. 2*B*) (23). As expected, PAR4 was detected in IPs from whole cell lysates using the anti-FLAG antibody (Fig. 2*C*, *lanes 1* and *2*). P2Y12 was also detected in anti-FLAG co-IPs when co-expressed with PAR4 (Fig. 2*C*, *lane 2*). PAR4 and P2Y12 were similarly detected in co-IPs of the isolated cell-surface cohort and intracellular compartment (Fig. 2*C*, *lanes 4* and *6*). However, the low molecular weight form of P2Y12 was the predominant species associated with surface PAR4 (Fig. 2*C*, *lane 4*), whereas low- and high-molecular weight P2Y12 was associated with intracellular localized PAR4 (Fig. 2*C*, *lane 6*). These findings indicate that PAR4 and P2Y12 form heterodimers that are localized at the cell surface and in intracellular compartments.

Activation of PAR4 but not P2Y12 induced changes in the heterodimer complex

To determine if the PAR4-P2Y12 heterodimer is functional at the cell surface, we next examined whether agonist stimulation results in changes in the molecular arrangement of the PAR4-P2Y12 complex by performing BRET saturation assays. BRET saturation curves are used to derive the affinity of protein-protein interactions ($BRET_{50}$) and changes in the molecular organization of protein-protein interactions ($BRET_{max}$) (22). COS-7 cells transfected with a fixed amount of P2Y12-Rluc and increasing amounts of PAR4-YFP were treated with thrombin or buffer control for 10 min at 37 °C. In both control and thrombin-treated cells, a hyperbolic increase in BRET signal was observed as the ratio of PAR4-YFP to P2Y12-Rluc increased (Fig. 3*A*). However, thrombin treatment induced a

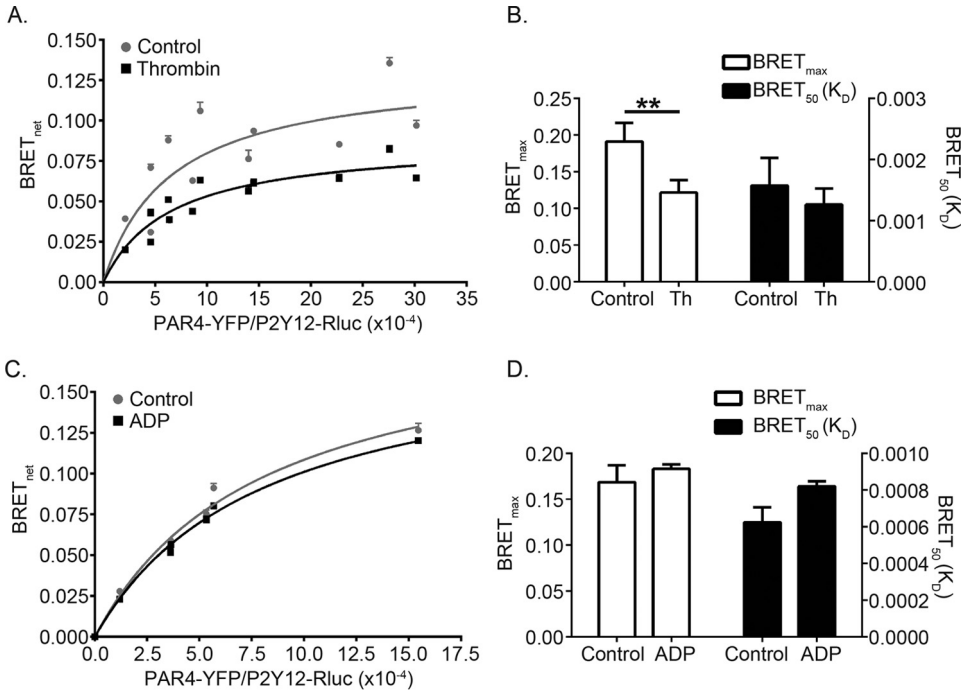

Figure 3. The PAR4-P2Y12 heterodimers display an agonist-induced change in BRET signal. *A* and *C*, COS-7 cells were transfected with a fixed amount of P2Y12-Rluc and increasing amounts of PAR4-YFP. $BRET_{net}$ was measured in triplicate at 37 °C after the addition of 30 nM thrombin (*A*) or 10 μM ADP (*C*). Buffer without agonist was added to control wells. $BRET_{net}$ is plotted as a function of the ratio of PAR4-YFP/P2Y12-Rluc expression. Data shown (mean ± S.E.) are representative of three independent experiments, which were used to calculate $BRET_{max}$ and $BRET_{50}$ values for cells treated with thrombin (*B*) and ADP (*D*) and were statistically significant as determined by paired *t* test (**, $p < 0.01$).

statistically significant decrease in the maximal BRET response elicited by the PAR4-P2Y12 heterodimer (Fig. 3*B*), with no accompanying change in $BRET_{50}$ (Fig. 3*B*). In contrast to thrombin, activation of P2Y12 with ADP failed to cause any significant change in PAR4-P2Y12-induced BRET signal compared with untreated control cells (Fig. 3, *C* and *D*). These data indicate that activation of PAR4, but not P2Y12, is sufficient to trigger a shift in the molecular arrangement of the GPCR protomers comprising the heterodimer complex, which suggests these changes may affect heterodimer function.

Activation of PAR4 drives P2Y12 co-internalization independent of β-arrestins

Previous studies indicate that P2Y12 internalization is dependent on β-arrestins, whereas β-arrestins are not required for PAR4 internalization (19, 20). As expected, in COS-7 cells, which are known to express low amounts of β-arrestins (24), ADP failed to induce internalization of the P2Y12 protomer (Fig. 4*A*), whereas agonist-induced PAR4 internalization remained intact in low β-arrestin–expressing COS7 cells (Fig. 4*B*). To confirm that the defect in P2Y12 trafficking was due to the absence of β-arrestins and not a cell-type specific effect, we co-expressed P2Y12 together with β-arrestin-2 (β-arr2) fused to GFP. As expected, ADP-stimulated internalization of P2Y12 was restored in COS-7 cells co-expressing β-arrestin-2 measured by immunofluorescence microscopy and cell-surface ELISA (Fig. 4, *C* and *D*). Whether the PAR4-P2Y12 heterodimer internalizes via a β-arrestin–dependent pathway is not known and was also examined using COS-7 cells. COS-7 cells co-expressing FLAG-PAR4 and HA-P2Y12 were labeled with receptor-specific antibodies and then stimulated with the PAR4-selective peptide agonist AYPGKF. In the absence of agonist, the PAR4-P2Y12 heterodimer was localized mainly at the cell surface (Fig. 4*E*, *top panels*), whereas stimulation with AYPGKF resulted in marked co-internalization of PAR4 and P2Y12 (Fig. 4*E*, *bottom panels*). Thus, activation of PAR4 drives β-arrestin–independent co-internalization of the PAR4-P2Y12 heterodimeric complex.

To determine if endogenous PAR4 and P2Y12 recapitulate PAR4-induced co-internalization of P2Y12, we performed immunofluorescence microscopy experiments in Dami megakaryocytic cells, which natively express PAR4 and P2Y12. In the absence of agonist, PAR4 and P2Y12 co-localized at the cell surface (Fig. 5*A*, *top panels*), whereas incubation with the PAR4-specific agonist peptide caused substantial co-internalization of PAR4 and P2Y12 from the cell surface to endocytic puncta (Fig. 5*A*, *bottom panels*). Next, we examined if activation of PAR4 triggers co-internalization of PAR1, a closely related family member shown previously to co-associate with PAR4 in platelets and other cell types (25, 26). In contrast to P2Y12, AYPGFK activation of PAR4 failed to drive PAR1 co-internalization (Fig. 5*B*). However, direct activation with the PAR1-specific agonist peptide TFLLRNPNDK resulted in robust receptor internalization (Fig. 5*C*), indicating that PAR1 is capable of internalizing in Dami cells. Thus, PAR4 specifically co-associates with P2Y12 and is the dominant driver of PAR4-P2Y12 co-internalization.

β-Arrestin recruitment to activated and internalized PAR4-P2Y12 heterodimers

β-Arrestins were shown to mediate PAR4-stimulated Akt activation, which also requires P2Y12 expression (16, 17). How-

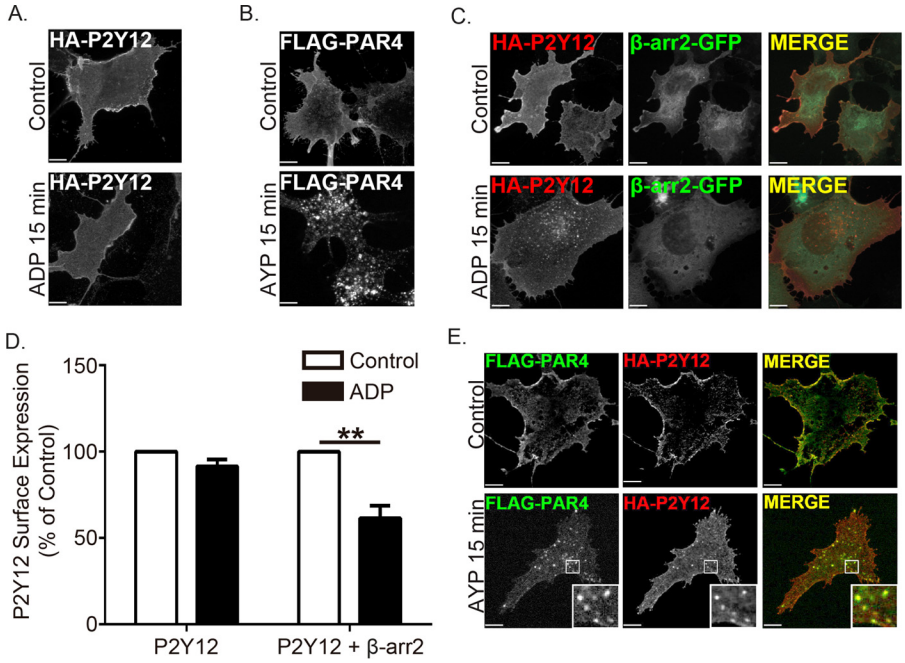

Figure 4. PAR4 and P2Y12 co-internalize and co-localize with β-arrestin2 on endosomes. COS-7 cells expressing either FLAG-PAR4 or HA-P2Y12 alone (*A* and *B*) or both receptors together with β-arrestin2-GFP (*C* and *E*) were pre-labeled with anti-FLAG and anti-HA antibodies, stimulated with 10 μM ADP (*A* and *C*) or 500 μM AYPGKF (*AYP*; *B* and *E*) or for 15 min at 37 °C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. The *yellow* puncta in the merged image of (*A*) are co-internalized PAR4 (*green*) and P2Y12 (*red*). Images shown are representative of three independent experiments (*Scale bars*, 10 μm). *D*, COS7 cells expressing P2Y12 alone or together with β-arr-2 were stimulated with 10 μM ADP for 15 min, fixed and the amount of receptor remaining on the cell surface determined by ELISA and were statistically significant as determined by paired *t* test (**, $p < 0.01$).

ever, it is not known how PAR4 and P2Y12 coordinate β-arrestin recruitment and were examined. In cells expressing PAR4 alone and β-arr2-GFP, incubation with AYPGKF failed to change subcellular localization of β-arr2 (Fig. 6*A*), which remained diffusely distributed throughout the cytoplasm. In contrast, AYPGKF treatment of cells co-expressing PAR4 wild type (WT) and P2Y12 receptor together with β-arr2-GFP caused a marked redistribution of β-arr2-GFP to PAR4-P2Y12–positive endocytic puncta (Fig. 6*B*, *middle panels*), indicated by the *white* color in the merged image. Although ADP treatment of these cells induced internalization of P2Y12, it failed to promote co-internalization of PAR4 and recruitment of β-arrestin-GFP to P2Y12-positive endocytic puncta (Fig. 6*B*, *bottom panels*). Next, we examined a PAR4 Y/A mutant in which tyrosine (*Y*) 264 and leucine (*L*) 268 were converted to alanine (*A*) and is defective in internalization (Fig. 6*C*) (20). Activation of PAR4 Y/A mutant with AYPGKF failed to promote P2Y12 co-internalization or recruitment of β-arr2-GFP (Fig. 6*C*). These data indicate that internalization of the activated PAR4-P2Y12 heterodimer complex is important for β-arrestin recruitment.

To confirm these findings, we examined agonist-induced PAR4-P2Y12 co-association with β-arr2 by co-IP. COS-7 cells expressing PAR4 WT, PAR4 WT, and P2Y12 or expressing PAR4 Y/A mutant and P2Y12 together with myc-tagged β-arr2 were treated with AYPGKF for various times at 37 °C. Cells were lysed, PAR4 was immunoprecipitated, and the presence of β-arrestins was detected using an anti-myc antibody. In cells expressing PAR4 WT alone, β-arrestin failed to associate with the receptor after agonist stimulation (Fig. 7*A*, *lanes 1–3*). However, we observed a significant ~1.75-fold increase in β-arr2 co-association with the PAR4 WT-P2Y12 heterodimeric complex after 15 min of agonist incubation (Fig. 7*A*, *lanes 4–6*), suggesting that β-arrestin recruitment to PAR4 requires co-expression of P2Y12. Remarkably, agonist activation of PAR4 Y/A mutant co-expressed with P2Y12 failed to promote β-arr2 co-association, providing additional evidence that internalization of PAR4 is necessary for β-arr2 recruitment (Fig. 7*A*, *lanes 7–9*). In contrast to PAR4, activation of P2Y12 with ADP for various times failed to promote co-association of β-arr2 when P2Y12 was expressed alone (Fig. 7*B*, *lanes 1–2*) or co-expressed with PAR4 (Fig. 7*B*, *lanes 3–5*), suggesting that P2Y12 facilitates β-arrestin association with activated PAR4.

Activation of the PAR4-P2Y12 heterodimer induces β-arr2 and Akt co-localization on intracellular vesicles

We next examined whether activated and internalized PAR4-P2Y12 heterodimer-induced localization of β-arr2 to endosomes results in recruitment of Akt. COS-7 cells co-expressing PAR4 and P2Y12 were co-transfected with β-arr2-GFP and myc-tagged Akt and stimulated with PAR4 agonist peptide AYPGKF, and then β-arr2 and Akt co-localization was assessed by immunofluorescence microscopy. In the absence of agonist, β-arr2 and Akt were diffusely distributed throughout the cytoplasm in cells co-expressing PAR4 and P2Y12 (Fig. 8*A*, *top panels*). However, after 15 min of AYPGKF stimulation, both β-arr2 and Akt showed a marked increase in co-localization at intracellular vesicles (Fig. 8*A*, *bottom panels*), indicated by the *yellow* color in the merged image and the overlapping line-scan intensity profiles. In contrast, AYPGKF stimulation of cells expressing PAR4 alone together with β-arr2-GFP and myc-Akt failed to induce β-arr2 endosomal localization and

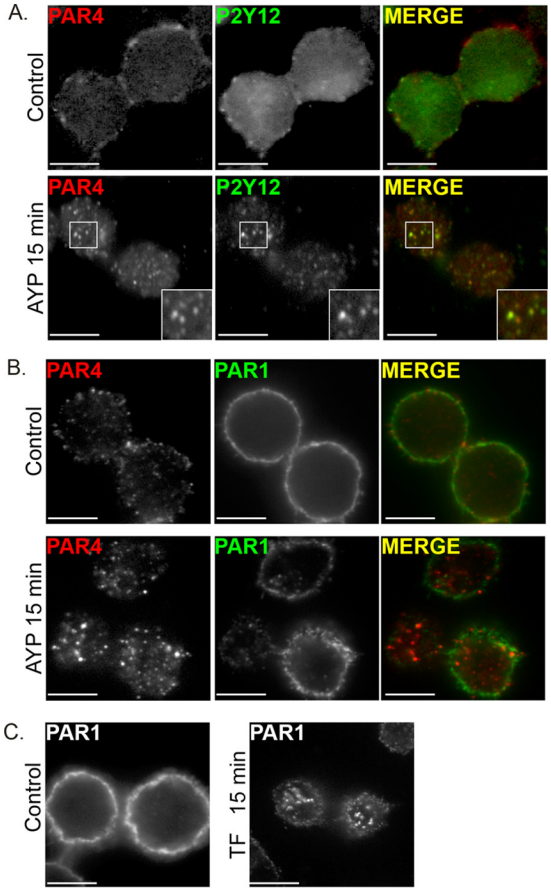

Figure 5. Endogenous P2Y12, but not PAR1, exhibits agonist-induced co-internalization with PAR4. *A–C*, Dami cells were plated on poly-lysine-coated glass coverslips and grown for 48 h. Cells were prelabeled with anti-PAR4 and anti-P2Y12 antibodies (*A*), anti-PAR4 and anti-PAR1 antibodies (*B*) antibodies, or anti-PAR1 antibody alone (*C*). Cells were stimulated with 500 μM AYPGKF (*AYP*; *A*) or 100 μM TFLLRNPNDK (*TF*) (*B* and *C*) for 15 min at 37 °C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. The *yellow* puncta in the merged image of *A* are co-internalized PAR4 (*red*) and P2Y12 (*green*). Images shown are representative of three independent experiments (*Scale bars*, 10 μm).

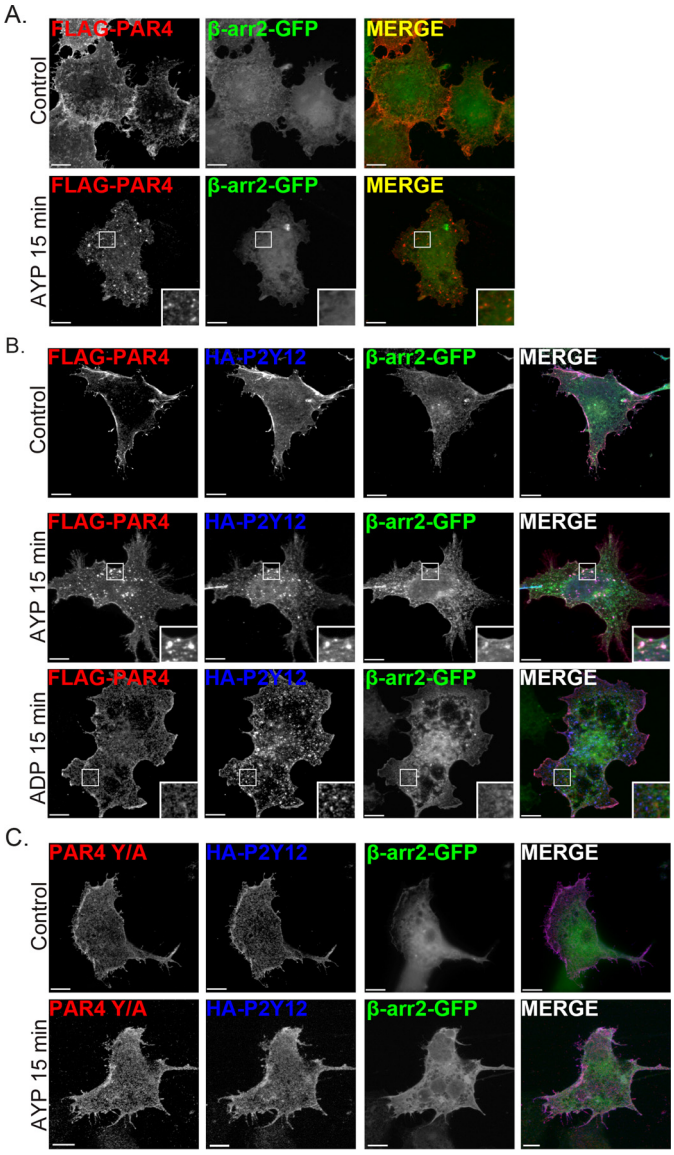

Figure 6. β-Arrestin2 is recruited to internalized PAR4-P2Y12 heterodimer complexes. *A–C*, COS-7 cells co-expressing β-arrestin2-GFP with either FLAG-PAR4 WT alone (*A*), FLAG-PAR4 WT and HA-P2Y12 (*B*), or FLAG-PAR4 Y/A mutant and HA-P2Y12 (*C*) were prelabeled with anti-FLAG antibody (*A*) or anti-FLAG and anti-HA antibodies (*B* and *C*), stimulated with 500 μM AYPGKF (*AYP*) for 15 min at 37 °C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. The *white* puncta in the merged image of *B* are colocalized PAR4 (*red*), P2Y12 (*blue*), and β-arrestin2-GFP (*green*). Images shown are representative of three independent experiments (*Scale bars*, 10 μm).

Akt recruitment (Fig. 8*B*, *bottom panels*). These results suggest that the activated and internalized PAR4-P2Y12 heterodimer coordinates β-arrestin2 and Akt recruitment at endosomes.

Agonist-induced internalization of the PAR4-P2Y12 heterodimer is required for Akt activation

We next examined if internalization of PAR4 is required for P2Y12-dependent activation of Akt. In HeLa cells expressing PAR4 alone, AYPGKF stimulation failed to induce Akt phosphorylation, whereas ERK1/2 activation was robust (Fig. 9*A*). This is likely due to either low or lost P2Y12 expression in HeLa cells, as these cells are not normally responsive to ADP stimulation (27). Thus, to examine if PAR4-P2Y12 heterodimer internalization mediates Akt signaling, HeLa cells were co-transfected with PAR4 WT or Y/A mutant together with P2Y12 and stimulated with AYPGKF for various times, and Akt activation was determined by immunoblotting using an anti-phospho-Akt antibody. In cells co-expressing P2Y12 and PAR4 WT, AYPGKF treatment elicited a significant and sustained increase in Akt phosphorylation compared with untreated control cells (Fig. 9*B*). In contrast, cells co-expressing P2Y12 and the internalization-deficient PAR4 Y/A mutant AYPGKF failed to induce Akt phosphorylation (Fig. 9*B*). Importantly, AYPGKF stimulation of cells expressing PAR4 WT or Y/A mutant together with P2Y12 caused a significant increase in ERK1/2 phosphorylation, indicating that the PAR4 mutant is not globally defective (Fig. 9*C*). To confirm a role for P2Y12 in endogenous PAR4-mediated Akt activation, we used Dami cells and PSB-0739, a potent and selective P2Y12 competitive antagonist (28). AYPGKF-induced Akt phosphorylation was significantly attenuated in Dami cells pretreated with PSB-0739 compared with control cells treated with DMSO (Fig. 9*D*), whereas ERK1/2 phosphorylation was unaffected (Fig. 9*E*). These data

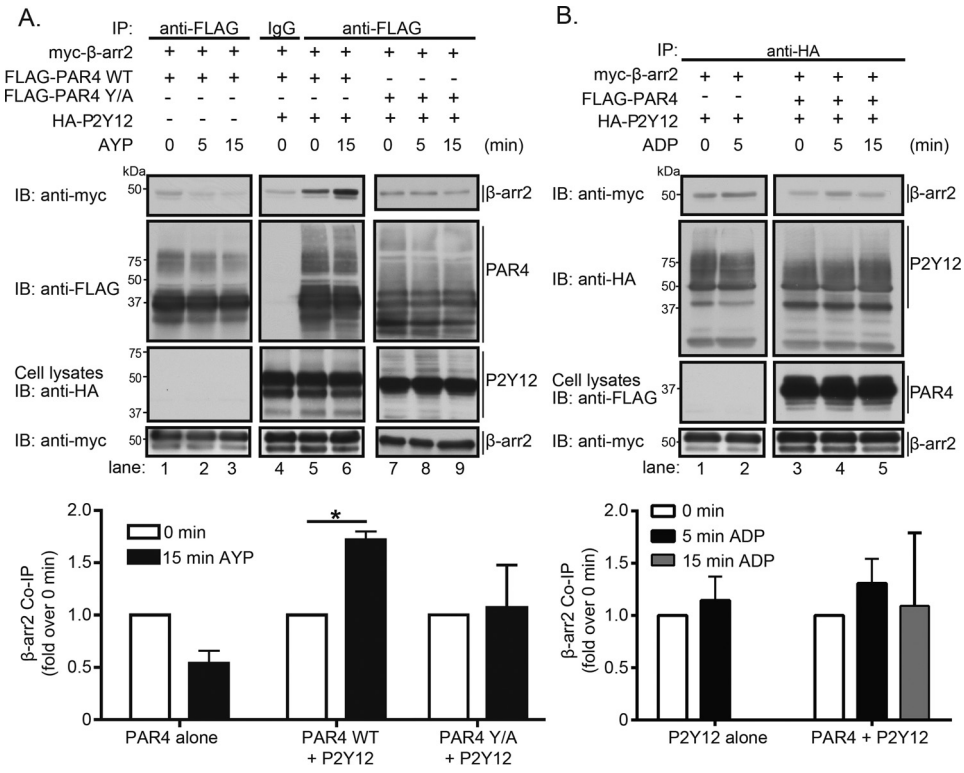

Figure 7. PAR4-P2Y12 association and internalization is required for agonist-induced β-arrestin2 recruitment. COS-7 cells co-expressing myc-β-arrestin2 with either FLAG-PAR4 WT, PAR4 Y/A mutant, or HA-P2Y12 alone, or PAR4 and P2Y12 together as indicated were stimulated with 500 μM AYPGKF (*A*) or 10 μM ADP (*B*) at 37 °C for the indicated times, then lysed, and immunoprecipitated. Cell lysates were resolved by SDS-PAGE on the same gel, immunoblotted (*IB*) with the indicated antibodies, and separated for labeling. Agonist-induced myc-β-arrestin2 co-IP was quantified by densitometry analysis of three independent experiments. The data (mean ± S.E.) are expressed as -fold change relative to untreated 0 min control and were statistically significant as determined by paired *t* test (*, $p < 0.05$).

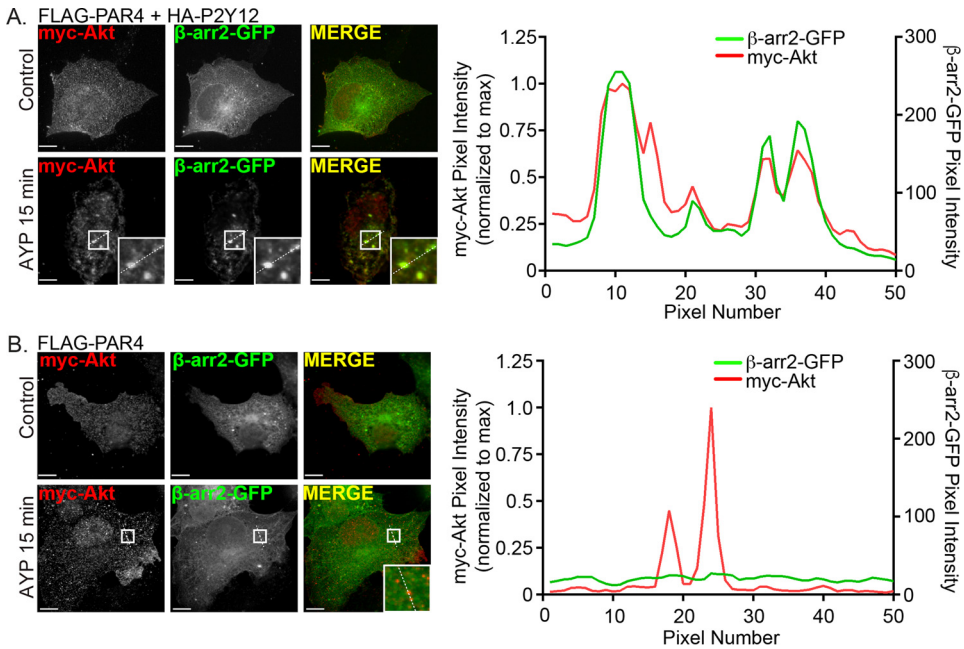

Figure 8. Coexpression with P2Y12 facilitates PAR4-induced recruitment of Akt to β-arrestin2 on endosomes. COS-7 cells co-expressing β-arrestin2-GFP, myc-Akt, and either FLAG-PAR4 and HA-P2Y12 (*A*) or FLAG-PAR4 alone (*B*) were stimulated with 500 μM AYPGKF for 15 min at 37 °C, fixed, permeabilized, immunostained, and imaged by confocal microscopy. *Yellow* puncta in the merged image of (*A*) are colocalized Akt (*red*) and β-arrestin2-GFP (*green*). The fluorescence intensity line scans were generated from the regions denoted by the *white dashed line* in the agonist-stimulated images (*A* and *B*, *lower panels*). Images shown are representative of three independent experiments (*scale bars*, 10 μm).

indicate that P2Y12 is a necessary component of the PAR4-mediated Akt signaling axis but not for activated PAR4 signaling in general. Together, these results suggest that co-internalization of the PAR4-P2Y12 heterodimer promotes the formation of an endosomal β-arrestin2-signaling complex that facilitates Akt activation.

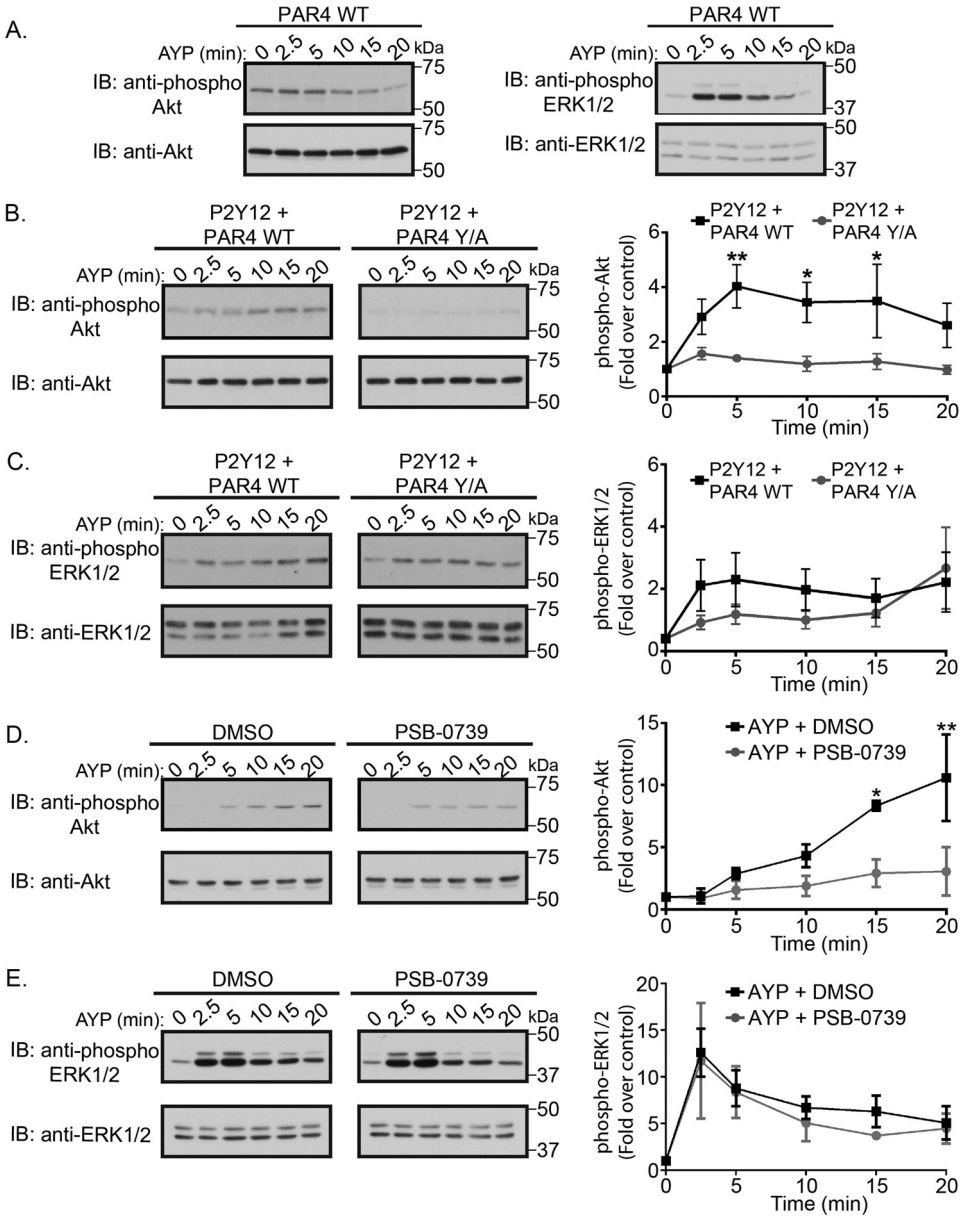

Figure 9. PAR4 internalization is required for PAR-P2Y12-mediated Akt activation. *A*, HeLa cells expressing FLAG-PAR4 WT only were treated with 500 μM AYPGKF for various times at 37 °C, lysed, and immunoblotted (*IB*) as shown. *B* and *C*, HeLa cells expressing HA-P2Y12 with either FLAG-PAR4 WT or FLAG-PAR4 Y/A mutant were treated with 500 μM AYPGKF (*AYP*) for various times at 37 °C. Cells were lysed and immunoblotted as indicated. Changes in phospho-Akt (*B*) and phospho-ERK1/2 (*C*) signals were quantified, normalized to total Akt (*B*) or total ERK1/2 (*C*), and expressed as a fraction of the untreated controls. *D* and *E*, Dami cells were pretreated with the P2Y12 antagonist 10 μM PSB-0739 or vehicle control for 1 h at 37 °C then treated with 500 μM AYPGKF for various times at 37 °C. Cell lysates were resolved by SDS-PAGE and immunoblotted as indicated. Changes in phospho-Akt (*D*) and phospho-ERK1/2 (*D*) signals were quantified, normalized to total Akt (*D*) or total ERK1/2 (*E*), and expressed as a fraction of the untreated controls. Cell lysates were resolved on the same gel and separated for labeling. Data shown (mean ± S.E.) are representative of three independent experiments, and statistical significance was calculated by two-way analysis of variance (*, $p < 0.05$; **, $p < 0.01$).

Discussion

GPCRs are known to form homodimers and heterodimers and can exist as larger oligomeric complexes. Although numerous studies have documented PAR-PAR homo-dimerization and hetero-dimerization in various model systems including native cells (29), the prerequisite of dimer or oligomer formation with function is not always clear. In the present study we sought to determine how the PAR4-P2Y12 heterodimer regulates β-arrestin–mediated Akt activation. We found that PAR4 and P2Y12 form heterodimers at the cell surface and intracellularly that may exist as dimers or higher-order oligomers. We further show that PAR4 and P2Y12 co-internalization is required for recruitment of β-arrestin on endosomes and Akt endosomal signaling. These studies are the first to demonstrate a role for PAR4-P2Y12 co-internalization in regulation of β-arrestin endosomal recruitment and Akt signaling.

Similar to other GPCR dimers, PAR4 and P2Y12 interaction is likely in a dynamic equilibrium between monomers and dimers of varying stability. Previous studies have shown agonist-dependent PAR4 co-association with P2Y12 in platelets (16) and in HEK293 cells ectopically expressing the receptor by co-IP and BRET analysis (17). Intriguingly, co-IP exper-

iments in the same studies clearly indicate that PAR4 and P2Y12 basally associate. However, we found that agonist stimulation of the PAR4-P2Y12 heterodimer at the cell surface fails to markedly enhance receptor-receptor interaction. These discrepancies could be due to cell-type differences, receptor construct variations, or high levels of receptor expression. However, our BRET studies further show that activation of PAR4 induces a molecular rearrangement of the PAR4-P2Y12 heterodimer similar to that previously reported (17). In contrast, however, activation of P2Y12 with ADP failed to induce any detectable change in PAR4-P2Y12 BRET signal; the underlying basis for this observation is not known. Regardless, these studies suggest that activation of PAR4 may be the critical driver for regulating PAR4-P2Y12 heterodimer function.

We also show that stimulation of the PAR4-P2Y12 heterodimer with AYPGKF results in recruitment of β-arrestin, whereas activation of the PAR4 protomer alone does not. This is not surprising given that we previously showed that β-arrestins are not essential for PAR4 internalization (20). We further report that AYPGKF-induced PAR4-P2Y12 co-internalization is required for recruitment of β-arrestins on endosomes. This is not observed with ADP activation of the PAR4-P2Y12 heterodimer or in cells expressing either the PAR4 or P2Y12 protomer alone. These findings are consistent with other reports showing that certain GPCR heterodimers acquire a unique capacity to recruit β-arrestins. Similar to PAR4, PAR1 does not require β-arrestins for internalization (30) and does not co-internalize with β-arrestins. However, activation of PAR1 in the PAR1-PAR2 heterodimer results in recruitment of β-arrestins and co-internalization to endosomes (31). Similarly, the μ-opioid receptor does not co-internalize with β-arrestins; however, activation of the μ-opioid receptor in the μ-opioid-nuerokinin-1 heterodimer results in recruitment and co-internalization of β-arrestins into the same endosomal compartment (32). Although it is clear that PAR4 internalization is required for PAR4-P2Y12 heterodimer recruitment of β-arrestins at endosomes, the molecular determinants in the dimer interface that facilitate β-arrestin recruitment are not known.

The PAR4-P2Y12 heterodimer appears to acquire an interface for β-arrestin recruitment that is distinct from the receptor protomers. Unlike PAR4, we show that agonist-induced internalization of P2Y12 requires β-arrestin expression, as previously reported (19). However, activation of P2Y12 alone is not sufficient to induce β-arrestin recruitment on endosomes. Interestingly, however, activation of PAR4-P2Y12 with the PAR4-specific agonist peptide drives internalization of the PAR4-P2Y12 heterodimer even in the absence of β-arrestins, suggesting that P2Y12 expressed in the context of the heterodimer bypasses the requirement for β-arrestins for internalization. Moreover, activation of P2Y12 with ADP is sufficient to induce its internalization but not co-internalization of exogenous or endogenous PAR4 when co-expressed in the same cells. Interestingly, although PAR4 and PAR1 have been reported to form heterodimers (25), activation of PAR4 failed to induce co-internalization of PAR1. These findings provide further evidence that the PAR4-P2Y12 heterodimer provides an interface for β-arrestin recruitment that is different compared with the P2Y12 protomer.

We further demonstrate that internalization of the PAR4-P2Y12 heterodimer is important for activation of Akt signaling. A PAR4 Y/A mutant that cannot internalize fails to recruit β-arrestins to endosomes even when P2Y12 is co-expressed. This suggests that internalization of PAR4 is necessary for association with β-arrestin, and raises the possibility that β-arrestin–mediated Akt signaling may occur on endosomes. Consistent with this idea, we show by immunofluorescence microscopy that activation of the PAR4-P2Y12 heterodimer induces β-arrestin co-localization with Akt on intracellular vesicles. In addition, activation of the PAR4 internalization-defective Y/A mutant in cells co-expressing P2Y12 fails to induce Akt activation although signaling to ERK1/2 remains intact. In addition, a previous study showed that PAR4 harboring mutations within the fourth transmembrane domain disrupts association with P2Y12 and prevents Akt activation (17), suggesting that PAR4-P2Y12 dimerization is also important. Given that the PAR4 Y/A mutation resides in the third intracellular loop, it is unlikely to affect PAR4-P2Y12 dimerization. Together, these findings suggest that activation and internalization of the PAR4-P2Y12 heterodimer is necessary for β-arrestin recruitment and Akt activation.

In summary, this work illustrates an important role for PAR4-P2Y12 heterodimer trafficking in β-arrestin endosomal recruitment and Akt activation. Arrestin-2 knock-out mice exhibit protection from ferric chloride-induced thrombosis compared with wild-type mice (16), indicating that β-arrestins play a critical role in Akt-mediated integrin activation. The work reported here now suggests that activation of this pathway requires internalization of the PAR4-P2Y12 heterodimer. Thus, in future studies it will be important to determine in platelets whether blockade of PAR4 internalization reduces integrin activation and platelet plug formation.

Experimental procedures

Reagents and antibodies

The PAR4 agonist peptide AYPGKF and PAR1 agonist peptide TFLLRNPNDK (TF) were synthesized as the carboxyl amides and purified by reverse-phase high-pressure liquid chromatography by the Tufts University Core Facility (Boston, MA). Human α-thrombin was obtained from Enzyme Research Technologies (South Bend, IN). Coelenterazine *h* (#10111) was purchased from Biotium (Fremont, CA). Protein A-Sepharose CL-4B beads were from GE Healthcare. Adenosine diphosphate (ADP) was purchased from Sigma. PSB-0739 (#3983) was purchased from Tocris Bioscience. Mouse monoclonal M2 anti-FLAG (#A2220) and rabbit anti-P2Y12 (#P4871) antibodies were purchased from Sigma. Polyclonal rabbit anti-FLAG (#600-401-383), mouse IgG, and mouse monoclonal anti-c-Myc (9E10) (sc-40) antibodies were purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse monoclonal anti-HA antibody (HA.11) (#MMS-101R) was purchased from Covance (Princeton, NJ). Rabbit anti-PAR1 polyclonal antibody was described previously (33). Mouse anti-PAR4 antibody was provided by Dr. Marvin Nieman (Case Western Reserve University, Cleveland, OH). Rabbit anti-p44/42 MAPK (#9102), mouse anti-phospho-p44/42 MAPK (Thr-202/Tyr-204)

(#9106), rabbit anti-Akt (#9272), and rabbit anti-phospho-Akt XP (Ser-473) (D9E) (#4060) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-mouse secondary antibodies conjugated to Alexa Fluor 488 (#A-11001), Alexa Fluor 594 (A-11005), and Alexa Fluor 647 (A-21235) and goat anti-rabbit secondary antibodies conjugated to Alex Fluor 488 (A-11008), Alex Fluor 594 (A-11012), and Alex Fluor 647 (A-21244) were purchased from Thermo Fisher Scientific (Waltham, MA). Goat anti-mouse (#170–6516) and goat anti-rabbit (#170–6515) secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Bio-Rad. HRP-conjugated mouse anti-HA (3F10) (#2013819) and HRP-conjugated mouse monoclonal anti-c-myc (9E10) (#1814150) antibodies were obtained from Roche Applied Science. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Thermo Fisher Scientific. FluorSave reagent was purchased from Calbiochem.

Cell culture and transfections

COS-7 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (v/v). The Dami human megakaryocytic cell line was purchased from ATCC and maintained in RPMI media supplemented with 10% (v/v) fetal bovine serum. HeLa and COS-7 cells were transiently transfected with cDNA plasmids using polyethyleneimine (PEI, Polysciences Inc., Warrington, PA) at a ratio of 6 μl of PEI (1 mg/ml solution) to 1 μg of plasmid.

Plasmid cDNAs

Human PAR4 wild type containing an N-terminal FLAG epitope was cloned into the pBJ mammalian vector as previously described (34). The PAR4 Y264A/L268A mutant designated as Y/A was generated as described previously (20). Full-length PAR4 containing a flexible linker region fused to YFP at the C terminus was cloned into the pRK6 vector, which was generously provided by Dr. Jean-Philippe Pin (Montpellier University, Montpellier, France). Full-length P2Y12 containing a flexible linker region fused to *Renilla* luciferase (Rluc) at the C terminus was cloned into the phRluc-N1 vector, which was a gift from Dr. Tracy Handel (University of California, San Diego, La Jolla, CA). The N-terminally HA-tagged P2Y12 construct was generated and cloned into the pcDNA3.1 vector. The β-arrestin-GFP cDNA plasmid was a gift from Dr. Marc Caron (Duke University Medical Center, Durham, NC). The myc-Akt cDNA plasmid was generously provided by Dr. Adriano Marchese (Medical College of Wisconsin, Milwaukee, WI). The myc-β-arrestin2 cDNA plasmid was a gift from Dr. Laura Bohn (The Scripps Research Institute, Jupiter, FL).

Immunoblotting

Cell lysates were collected in 2× Laemmli sample buffer containing 200 mM DTT. Samples were resolved by SDS-PAGE, transferred to PVDF membranes, immunoblotted with the appropriate antibodies, and then developed by chemiluminescence. Immunoblots were quantified by densitometry using ImageJ software (NIH, Bethesda, MD).

Immunoprecipitations

Cells were plated in 6-well dishes at a density of 2.5×10^5 cells per well and transfected the following day. After 48 h, cells were serum-starved for 1 h, then placed on ice, washed with PBS, and lysed with Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 1% Triton X-100) supplemented with a mixture of protease inhibitors. Cell lysates were cleared by centrifugation, and a BCA assay (Thermo Fisher Scientific) was performed to determine protein concentrations. Equal amounts of normalized lysates were immunoprecipitated with appropriate antibodies overnight at 4 °C. Immunoprecipitates were washed 3 times with lysis buffer, and proteins were eluted in 50 μl of 2× Laemmli sample buffer containing 200 mM DTT. Cell lysates and immunoprecipitates were analyzed my immunoblotting.

Sequential immunoprecipitations

Cells were transfected as above, starved for 1 h, and then incubated on ice for 1.5 h with primary antibody. Cells were lysed (using lysis buffer described above) and cleared by centrifugation, then protein concentration was determined by BCA assay. Cell lysates were normalized to obtain equal protein content, then each lysate was evenly divided into two tubes: whole cell and surface/intracellular. The whole-cell tube was incubated with primary anti-PAR4 antibody for 1.5 h at 4 °C followed by incubation with protein-A-Sepharose beads for 2 h at 4 °C to immunoprecipitate the whole-cell cohort of PAR4. The surface/intracellular tube was incubated with protein-A-Sepharose beads for 2 h at 4 °C to selectively immunoprecipitate the surface cohort of PAR4. The supernatant, which contained the unlabeled intracellular pool of PAR4, was collected and incubated with primary antibody for 1.5 h followed by protein-A-Sepharose beads for 2 h at 4 °C. Immunoprecipitates were washed and collected in sample buffer as above and analyzed by immunoblotting.

BRET assays

COS-7 cells were transfected for 48 h, lifted with Cellstripper (Mediatech), washed with PBS, and resuspended in PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose at a density of 5.0×10^5 cells/ml. Cells were added (80 μl per well) in triplicate to a 96-well microplate followed by 10 μl of coelenterazine *h* to obtain a final concentration of 5 mM. Basal BRET was obtained by measuring Rluc luminescence and YFP fluorescence signals after an 8-min delay using a TriStar LB 941 plate reader running MicroWIN 2000 software (Berthold Biotechnologies) using two filter settings (480 nm for Rluc and 530 nm for YFP). The BRET ratio was calculated as (emission at 530 nm)/(emission at 480 nm), and $BRET_{net}$ was calculated by subtracting the background BRET ratio (measured from cells expressing the Rluc-tagged protein alone). Total YFP signal was measured before the addition of coelenterazine *h* by excitation at 485 nm and detecting emission at 535 nm. Total luminescence was measured by integrating the signal for 1 s/well without filter selection. To measure agonist-induced BRET changes, cells were treated with agonist or buffer control immediately after obtain-

ing the basal BRET reading. All measurements were performed at 37 °C.

Immunofluorescence confocal microscopy

Cells were plated at a density of 0.4×10^5 cells per well on fibronectin-coated glass coverslips placed in a 12-well dish and grown overnight. Cells were transfected as described above, grown for 24 h, and then serum-starved in DMEM containing 1 mg/ml BSA and 10 mM HEPES overnight. Cells were then incubated at 4 °C with appropriate anti-PAR4 or -P2Y12 antibodies to label the surface population of receptors, then stimulated with agonist, fixed with 4% paraformaldehyde in PBS, permeabilized with methanol, immunostained with appropriate antibodies, and processed as described previously (35). In untransfected COS7 cells, no signal was detected with either anti-PAR4 or -P2Y12 antibodies (data not shown). Coverslips were mounted with FluorSave reagent. Confocal images of 0.28-μm x-y sections were collected sequentially using an Olympus IX81 DSU spinning confocal microscope fitted with a Plan Apo 60× oil objective and a Hamamatsu ORCA-ER digital camera using Metamorph 7.7.4.0 software (Molecular Devices). Fluorescence intensity line-scan analysis was performed using ImageJ software.

Cell-surface ELISA assays

P2Y12 receptor internalization assays were performed essentially as described previously (20). Briefly, transfected cells were serum-starved overnight then incubated on ice with anti-HA antibody to label surface HA-P2Y12. Cells were stimulated with agonist, fixed with 4% paraformaldehyde, and incubated with HRP-conjugated secondary antibody. The amount of antibody remaining at the cell surface was detected by incubation with ABTS for at least 20 min at room temperature, and HRP-ABTS reaction product was quantified by measuring absorbance at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

Signaling assays

Signaling assays were performed essentially as described previously (20). Briefly, HeLa or Dami cells were starved for 1 h at 37 °C. For experiments that included the P2Y12 antagonist 10 μM PSB-0739, cells were pretreated with the inhibitor or vehicle control during the 1-h starvation. Cells were stimulated with agonists for appropriate times at 37 °C, and cell lysates were collected by direct lysis in 2× Laemmli sample buffer containing 200 mM DTT. Samples were resolved by SDS-PAGE and immunoblotted.

Data analysis

Statistical significance was determined by paired t test or two-way analysis of variance using Prism 4.0 software (GraphPad).

Author contributions—T. H. S. designed and conducted most of the experiments; assistance was provided by J. G. L. (Figs. 6, 7, and 9) and M. R. D. (Fig. 8). T. H. S. analyzed the majority of the data and wrote most of the paper with assistance from J. T. T. H. S., J. T., and M. R. D. reviewed the paper.

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