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Clathrin-mediated integrin αIIbβ3 trafficking controls platelet spreading

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Abstract

Dynamic endocytic and exocytic trafficking of integrins is an important mechanism for cell migration, invasion, and cytokinesis. Endocytosis of integrin can be classified as clathrin dependent and clathrin independent manners. And rapid delivery of endocytic integrins back to the plasma membrane is key intracellular signals and is indispensable for cell movement. Integrin αIIbβ3 plays a critical role in thrombosis and hemostasis. Although previous studies have demonstrated that internalization of fibrinogen-bound αIIbβ3 may regulate platelet activation, the roles of endocytic and exocytic trafficking of integrin αIIbβ3 in platelet activation are unclear. In this study, we found that a selective inhibitor of clathrin-mediated endocytosis pitstop 2 inhibited human platelet spreading on immobilized fibrinogen (Fg). Mechanism studies revealed that pitstop 2 did not block the endocytosis of αIIbβ3 and Fg uptake, but inhibit the recycling of αIIbβ3 to plasma membrane during platelet or CHO cells bearing αIIbβ3 spreading on immobilized Fg. And pitstop 2 enhanced the association of αIIbβ3 with clathrin, and AP2 indicated that pitstop 2 inhibit platelet activation is probably due to disturbance of the dynamic dissociation of αIIbβ3 from clathrin and AP2. Further study demonstrated that Src/PLC/PKC was the key pathway to trigger the endocytosis of αIIbβ3 during platelet activation. Pitstop 2 also inhibited platelet aggregation and secretion. Our findings suggest integrin αIIbβ3 trafficking is clathrin dependent and plays a critical role in platelet spreading, and pitstop 2 may serve as an effective tool to address clathrin-mediated trafficking in platelets.

Keywords

Blood platelets, clathrin-coated vesicles, endocytosis, integrin αIIbβ3, pitstop 2

History

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Introduction

Integrins are a family of adhesive receptors [1]. Integrin interaction with extracellular ligand supplies a physical anchor for the cell and triggers the intracellular signaling events that manipulate cell fate [2]. Endocytic and exocytic trafficking of integrin is an important mechanism for rapid delivery of integrins back to the plasma membrane and facilitate adhesion turnover and provides the cell with a constant fresh pool of integrins to generate new adhesions [2], thereby regulating cell growth, proliferation, migration, invasion, and cytokinesis [3]. The role and mechanism of integrin endo/exocytic cycle are being increasingly recognized, and the basic idea is that the exocytic receptors are internalized by endocytosis and trafficked through the cells [4].

Integrin endocytosis can be triggered by intracellular signaling at the extracellular surface of the cell through clathrin-dependent and clathrin-independent manners [3]. In clathrin-mediated integrin endocytosis, the integrin budding-in process is initiated by a pit coated with clathrin assisted by a set of cytoplasmic proteins including integrin cytoplasmic domains [5], dynamin [6], and adaptors [7] such as adaptin [8]. The pit is further converted into a short-life clathrin-coated vesicle [9]. Then, the coat can be shed, and the remaining vesicle fuses with endosomes and proceeds down the endocytic pathway [10]. Tubular actin-dependent recycling endosomes [11] and ARF6 pathways [12] have been described in integrin recycling back to the plasma membrane, but whether clathrin-mediated trafficking functions in this process has not been determined.

In clathrin-mediated endocytosis, clathrin acts as a central organizing platform for coated pit assembly and dissociation via its terminal domain (TD) [13]. Recently, a selective inhibitor, pitstops, has been identified and shown to block endocytic ligand association with the clathrin TD [14]. Pitstops significantly interferes with receptor-mediated endocytosis, entry of HIV, and synaptic vesicle recycling, and therefore serves as a potential pharmacological tool to elucidate the functions and processes of clathrin-mediated endocytosis [15].

Integrin αIIbβ3, as a platelet-abundant specific adhesive receptor, plays a pivotal complex role in platelet physiology, such as adhesion, aggregation, clot formation, and retraction [16,17]. Despite the functions of αIIbβ3 internalization for uptake of Fg and downregulation of adhesiveness of αIIbβ3 were being partially clarified [12,18,19], the roles of clathrin-mediated αIIbβ3 trafficking in platelet activation remain unknown. Here, the role of integrin αIIbβ3 trafficking in human platelet was investigated

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using a novel clathrin terminal domain inhibitor pitstop 2. The data presented in this study have provided important insights into the αIIbβ3 trafficking in platelet activation.

Material and methods

Reagents

Apyrase, PGE1, Fg, indomethacin, and PF-573228 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagen was purchased from chrono-log (Havertown, PA, USA). α-Thrombin was from Enzyme Research Laboratories (South Bend, IN, USA). AP2-mu, tyrphostin A23, cytochalasin, taxol, clathrin antibodies and control IgG were purchased from Santa Cruz (Dallas, TX, USA). Monoclonal antibody SZ21 [20] against human platelet β3 for immunofluorescence was a gift from Xiaodong Xi (Shanghai Jiao Tong University School of Medicine). FITC-conjugated-CD41 antibody was purchased from Beckman Coulter (Brea, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse antibody and rhodamine affinipure goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Anti-LAMP1 rabbit antibody, anti-EEA1 rabbit antibody, pitstop 2 (ab120687), and its negative control (ab120688) were purchased from Abcam (Cambridge, MA, USA). Rhodamine-conjugated phalloidin was from Life Technologies (Gaithersburg, MD, USA). Super signal chemiluminescent substrate was from Pierce (Rockford, IL, USA). Rabbit anti-integrin β3 polyclonal antibody and mouse anti-GAPDH monoclonal antibody were from Cell Signaling Technology (Danvers, MA, USA). Anti-FAK rabbit polyclonal antibody was purchased from EMD Millipore (Billerica, MA, USA). Piceatannol was purchased from Cayman Chemical (Ann Arbor, MI, USA). U0126 was from Gene Operation (Ann Arbor, MI, USA). PP2 was purchased from Calbiochem (Darmstadt, Germany). Dynasore, U73122, apoptozole and Ro 31-8220 were purchased from Selleck (Houston, TX, USA).

CHO cells spreading on fibrinogen

CHO cells expressing αIIbβ3 were obtained from Xiaoping Du (University of Illinois) and incubated with pitstop 2 or control at the concentration of 5 μM in chamber slides coated with 50 μg/mL Fg. Intact cells were immobilized on poly-L-lysine after incubated with pitstop 2 or control. Adherent cells were rinsed with PBS and fixed with 4% paraformaldehyde. Then cells were washed and incubated with SZ21 and anti-EEA1 antibody or anti-LAMP1 antibody in labeling buffer (0.5% BSA and 0.5% Triton X-100 in PBS) overnight. The cells were washed 3 × 10 minutes with labeling buffer and incubated with secondary antibody and Rhodamine-conjugated Phalloidin in labeling buffer for 30 minutes. Then cells were washed and stained with DAPI. Cells were imaged with Zeiss LSM-710 confocal microscope.

Human platelet preparation, aggregation, and spreading on immobilized fibrinogen

After informed consent was obtained, blood was collected from healthy donors into empty syringes and then transferred to polypropylene centrifuge tubes containing 100 μl/mL whites anticoagulant (2.94% sodium citrate, 136 mM glucose, pH 6.4), 0.1 μg/mL PGE1, and 1 U/mL apyrase. Platelet-rich plasma (PRP) was prepared by differential centrifugation. Washed platelets were prepared as described [21,22]. Aggregation was measured in the Lumi-Aggregometer (Chrono-Log, Havertown, PA) using washed platelets (300 μL) adjusted to approximately 3×10^8/mL with stirring at 1000 rpm. Inhibitors were incubated with the platelets for 3 minutes prior to agonist stimulation.

Analysis of platelet spreading on immobilized Fg was done as described [23]. Briefly, washed human platelets (2×10^7 /mL) were incubated with pitstop 2 or control for 3 minutes prior to spreading on slides coated with Fg (50 μg/mL) for specific intervals. Then the platelets were stained with rhodamine-conjugated phalloidin and captured with Zeiss fluorescent microscope. The spreading area of platelets was quantified using the National Institutes of Health (NIH) Image J software.

Clot retraction

Clot retraction using human platelets was processed as described [24]. Clot size was quantified from photographs using NIH Image J software, and retraction was expressed as retraction ratio [1−(final clot size/initial clot size)].

Flow cytometry analysis

Analysis of FITC-Fg binding and p-selectin expression was done as described [24]. Briefly, human platelets were incubated with PE-CD62P or APC-Fg and thrombin for 20 minutes before measured by fluorescence-activated cell sorter (FACS).

Washed human platelets at a concentration of 3×10^7/mL incubated with FITC-CD41 or FITC-PAC1 were either kept resting or stimulated with α-thrombin (0.05 U/mL) to measure the surface levels of total and activated αIIbβ3. The mixture was analyzed by FACS.

Measurement of ATP secretion

Platelet secretion of adenosine trisphosphate (ATP) granule was measured in parallel with platelet aggregation as previously described [25, 26] using a Lumi-Aggregometer with stirring after the addition of the luciferin-luciferase reagent. To examine the effects of pitstop 2 on platelet function, platelets were incubated for 3 minutes with pitstop 2 or control before the addition of the platelet agonist. The results shown represented at least three independent experiments.

Integrin αIIbβ3 endocytosis

Washed platelets or CHO cells bearing αIIbβ3 were incubated with FITC-anti CD41 antibody (0.05mg/mL) for 1 hour at 37°C and then incubated with pitstop 2 or control. Intact cells were immobilized on poly-L-lysine. Platelets spreading on immobilized Fg were washed and fixed and then imaged with fluorescence microscopy survey. Cells spreading on immobilized Fg were washed and fixed. Then the cells were permeabilized with 0.25% Triton X-100 for 10 minutes followed by blocking with 5% BSA for 1 hour and incubated with anti-FAK rabbit polyclonal antibody overnight. The cells were washed with PBS and incubated with secondary antibody in 5%-BSA for 1 hour. Then cells were washed and stained with DAPI. Cells were imaged with Zeiss LSM-710 confocal microscope.

Platelets and cells spreading on immobilized Fg were washed and incubated with FITC-anti-CD41 antibody directly under non-permeabilizing conditions. Then they were fixed and imaged with fluorescence microscopy survey.

Immunoprecipitation and Western blot analysis

For the detection of AP2-mu and clathrin, spread platelet samples were added to the equal volume of 2× lysis buffer (100 mM Tris-HCl, pH 7.4; 2% NP-40; 300 mM NaCl; 2 mM EDTA; 2 mM PMSF; 2 μg/mL aprotinin, leupeptin, and pepstatin; 2 mM Na_3VO_4; 2 mM NaF; and a Complete Mini Protease Inhibitor

Cocktail Tablet). Next, the samples were incubated on ice for 30 minutes, and then 2 μg/mL anti-β3 was added, and the samples were incubated overnight at 4°C. Then, 30 μL Protein A/G PLUS-Agarose was added to each sample prior to incubation for 2 hours at 4°C. The beads were harvested by centrifugation at 3000 ×*g* for 2 minutes and washed three times with 500 μL lysis buffer and twice with PBS solution. Proteins were boiled in sample buffer and resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to PVDF membrane. Western blots were performed using anti-AP2-mu, anti-clathrin, or anti-β3 antibody at a 1:1000 dilution, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody at a 1:5000 dilution. Blots were developed using super signal chemiluminescent substrate.

Fitc-fg uptake assay

Analysis of FITC-Fg uptake was performed as previously described [12]. Briefly, washed human platelets were preincubated with pitstop 2, A23, dynasore or control, and then they were incubated with FITC-Fg (0.05 mg/mL) for 1 hour before being resuspended in Tyrode's buffer containing 0.35% BSA and 1 mM $CaCl_2$. Platelets then were incubated for 2 hours and fixed. Trypan blue (0.1%) was added prior to imaging. The number of FITC-positive punch per platelet was counted manually.

Results

Clathrin inhibitor pitstop 2 prevented platelet spreading

Integrin activation can be regulated by conformational change, clustering, and trafficking, including the delivery of newly synthesized integrins, integrin internalization, and recycling of internalized integrins [3]. Clathrin-mediated endocytosis is emerging as an important mechanism of integrin trafficking and has recently been widely investigated [19,27]. Integrin αIIbβ3, as the most abundant platelet surface receptor, plays an important role in platelet activation, and clustering of αIIbβ3 leads to platelet shape change and spreading [28]. To elucidate the roles of clathrin-mediated integrin αIIbβ3 trafficking in platelet activation, a novel selective clathrin terminal domain inhibitor pitstop 2 was used to examine its effects on platelet spreading on immobilized Fg and clot retraction.

We first evaluated if platelet size or morphology was changed by pitstop 2 treatment. As shown by supplemental Figure S1 and Table I pitstop 2 treatment had no significant effect on platelet size/morphology detected by flow cytometry and hemacytometer.

To focus on αIIbβ3-mediated outside-in signaling, additional agonists were not added. As shown in Figure 1A, pitstop 2 treatment caused a significant decrease in platelet ability to spread on Fg but had no obvious effect on platelet attachment ability. The statistical analysis showed that the average spreading area of the platelets treated with 5 μM pitstop 2 was significantly smaller than that in the presence of pitstop 2 specific control (ab120688) [29] (Figure 1B), demonstrating that pitstop 2 severely interferes with integrin αIIbβ3-mediated platelet spreading on immobilized Fg. Pitstop 2 treatment also caused a decrease of lamellipodia formation after platelets spreading for 30 minutes (Figure 1C), probably due to the inadequate membrane supplement for forming lamellipodia.

We also detected the effect of pitstop 2 on clot retraction. As shown in Figure 1D and 1E, pitstop 2 had no significant effect on clot retraction.

Pitstop 2 inhibited the recycling of αIIbβ3 in CHO cells

To further reveal the functions of clathrin on integrin αIIbβ3 trafficking, the dynamic location of integrin αIIbβ3 in CHO cells [30] spreading on immobilized Fg was traced by immunofluorescence staining as described in method. As shown in Figure 2, in resting CHO cells, F-actin and αIIbβ3 displayed a diffuse staining which was slightly brighter at the periphery of the cells. After spreading on Fg for 30 minutes, the majority of integrin αIIbβ3 in CHO cells treated with control was found to be into micro vesicles in the cytoplasm. To trace the internalized αIIbβ3, co-staining for early and late endosomes using an anti-EEA1 or anti-LAMP1 antibody with αIIbβ3 was carried out. The results presented in supplemental Figure S2 demonstrated that internalized αIIbβ3 fused partly with early and late endosomes. Over time, part of integrin αIIbβ3 was clustered and accompanied by the actin cytoskeletal reorganization. At 90 minutes, most of the integrin αIIbβ3 clusters located at focal adhesion sites that were interconnected by contractile actin fibers. There was no obvious difference of αIIbβ3 internalization in pitstop 2 treating CHO cells compared to that in control group at 30 minutes (Figure 2B). But the formation of integrin αIIbβ3 clusters and actin reorganization were severely inhibited at 60 minutes, and αIIbβ3 still existed in micro vesicles surrounded by actin in the cytoplasm after 90 minutes, leading to inadequate spreading of CHO cells in the pitstop 2 treatment group. These results demonstrated that pitstop 2 inhibited the recycling of αIIbβ3 to plasma membrane instead of blocking the endocytosis of αIIbβ3 during CHO cells spreading on immobilized Fg.

To directly observe the effects of pitstop 2 on internalized integrin αIIbβ3 instead of intracellular stored or newly synthesized αIIbβ3, CHO cells bearing αIIbβ3 were incubated with the FITC-conjugated-CD41 antibody in the presence of pitstop 2 or its control before spreading on immobilized Fg. Then the cells were fixed at specific intervals and stained with anti-FAK antibody. As shown in Figure 3A, the green fluorescence distributed evenly on the CHO cells membrane at resting state (control intact), whereas focal adhesion kinase (FAK) displayed a diffuse staining. After spreading for 30 minutes, the uniformity distribution of αIIbβ3 in CHO cells was disrupted and labeled αIIbβ3 were internalized as micro vesicles. At 60 minutes, the αIIbβ3 partially detached from vesicles and clustered along with the CHO cells spreading. At the time point of 90 minutes, the spreading process completed with the αIIbβ3 fully clustering at the focal adhesion sites and colocalized with FAK. Compared with the control group, endocytic αIIbβ3 stagnated at vascular stages in the pitstop 2 treated CHO cells and FAK failed to form bundles at 90 minutes (Figure 3B). The quantification assay demonstrated that pitstop 2 mainly functioned during the late stage of spreading (supplemental Figure S3). Spreading assay under nonpermeabilizing and permeabilizing was carried out to confirm the location of αIIbβ3. The results presented in supplemental Figure S4 convinced that the majority of αIIbβ3 was recycled to the membrane and formed clusters under nonpermeabilizing conditions. However, under permeabilizing conditions, αIIbβ3 displayed a more diffuse staining. The data also showed clearly that endocytic αIIbβ3 stayed inside the cells in the pitstop 2-treated group.

Pitstop 2 disturbed the dynamic dissociation of αIIbβ3 from clathrin and AP2

Platelets stained with FITC-conjugated-CD41 were carried out in the presence of pitstop 2 or control to detect its effects on αIIbβ3 trafficking in platelets. The fluorescence-labeled αIIbβ3 was present in a homogenous ring around the periphery of platelets treated with pitstop 2 or control at 0 minute (supplemental Figure S1). As shown in Figure 4A, after spreading for

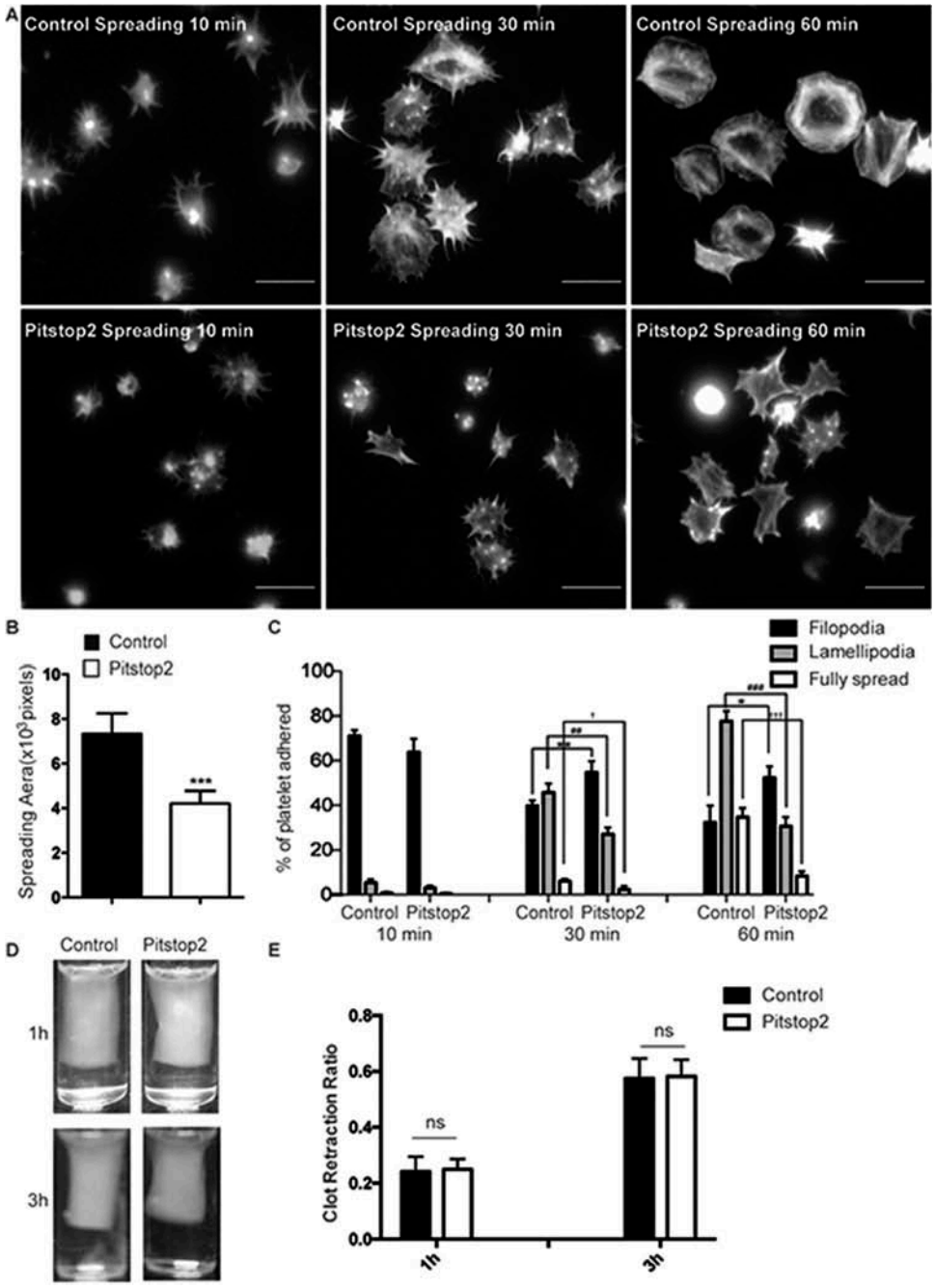

Figure 1. Clathrin inhibitor Pitstop 2 prevented platelet spreading. (A) Representative phalloidin-stained images of washed platelets preincubated with negative control or pitstop 2 spreading on immobilized Fg for 10, 30, and 60 minutes, respectively. Images were taken under oil immersion with magnification ×100. Scale bar, 5 μm. (B) Quantification of the platelet areas (pixel number) in 4 random fields per experiment and at least three independent experiments were performed. Statistical analyses were performed using the student t-test (mean ± standard deviation (SD); $^{***}P < 0.001$). (C) The numbers of platelets extending filopodia, lamellipodia, or fully spread per 100 platelets were counted manually at specific interval. The percentage platelets in each group relative to total were plotted as bar graph. Statistical significance was determined using the Student t-test (mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$). (D) Clot retraction of PRP containing platelets in the presence of pitstop 2 or negative control. (E) Two-dimensional retraction of clots was measured using NIH Image J software and data are retraction ratios. Statistical significance was determined using the Student t-test.

10 minutes, αIIbβ3 was internalized as vesicles, the empty vacuoles had a clear appearance on the surface of platelets. After 30 minutes, vesicular αIIbβ3 gradually turned into clustered αIIbβ3 appeared as large fluorescent particles. And the fluorescence labeled αIIbβ3 eventually distributed to the whole platelet at 60 minutes. On the contrary, in the pitstop 2 treated group, the fluorescence labeled αIIbβ3 was arrested at vesicular stages, and pitstop 2 treated human platelets finally formed the "ring" structure on immobilized Fg probably due to the inadequate cell membrane supplement for spreading. These results clearly indicated that pitstop 2 affected αIIbβ3 trafficking by interfering with the recycle process of internalized αIIbβ3.

Clathrin-coated pit initiation was thought to be triggered by the recruitment of the adaptor proteins and accessory proteins such as the most abundant endocytic clathrin adaptor, AP2 [6]. AP2-mediates cargo recruitment, maturation, and scission of the pit by binding cargo, clathrin, and accessory proteins. Recent study showed that AP2 interacted with integrin cytoplasmic conserved motif to selectively direct the internalization

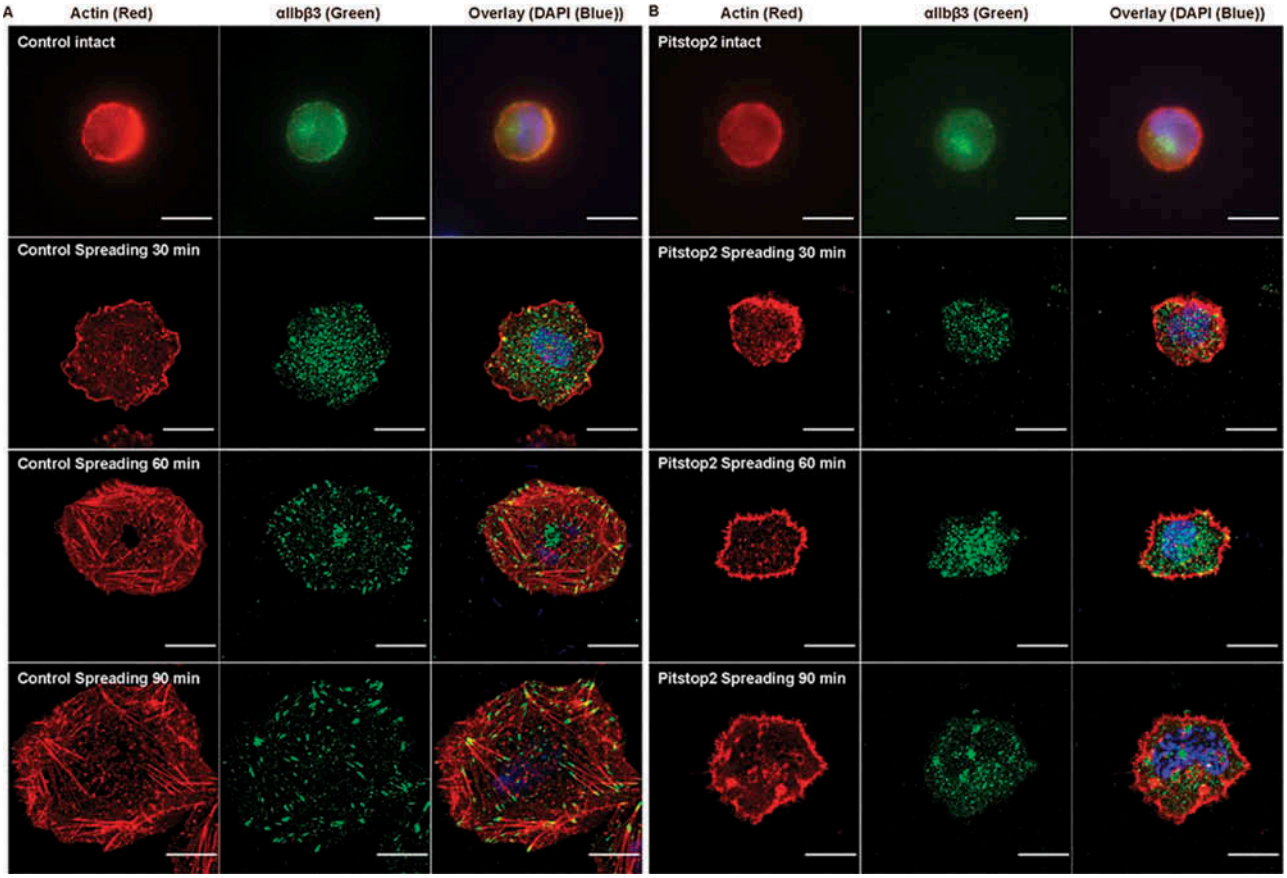

Figure 2. Pitstop 2 inhibited the recycling of αIIbβ3 to plasma membrane. (A, B) representative images of CHO cells bearing αIIbβ3 preincubated with negative control (A) or pitstop 2 (B) immunostained with SZ21 against human β3 antibody (green), rhodamine-conjugated phalloidin (red) and DAPI (blue) after immobilized in poly-L-lysine or spreading on immobilized Fg for 30, 60, and 90 minutes, respectively. Cells were imaged with Zeiss LSM-710 confocal microscope with magnification × 60. Scale bar, 20 μm.

of integrins [31]. Dynamin, as a mechanochemical enzyme, plays an important role in clathrin-coated vesicle formation and vesicle scission [32].

The functions of AP2 and dynamin in αIIbβ3-mediated platelet spreading were assessed using AP2-mu2 tyrosine-binding pocket inhibitor tyrphostin A23 [8] and dynamin selective inhibitor dynasore. The data presented in Figure 4A demonstrated that fluorescence labeled αIIbβ3 internalization was severely restrained in A23 and dynasore treated platelets, indicating that clathrin-coated pit, adaptor proteins and accessory proteins did play critical roles in platelet αIIbβ3 trafficking.

After detaching from the membrane, the clathrin coat is disassembled by the ATPase heat shock cognate 70 (HSC70), allowing the delivered protein to the next spot [6]. As pitstop 2 had little effect on αIIbβ3 internalization but inhibited it to recycle to the membrane, we speculated that pitstop 2 might play a role by influencing the uncoating reaction and clathrin component recycling. A selective inhibitor of HSC70, apoptozole [33], was used as a control to compare its effect on platelet trafficking with pitstop 2. As shown in Figure 4A, apoptozole stagnated the spreading at vesicular stage, similar to pitstop 2. This confirmed pitstop 2 may influence the uncoating reaction from a side.

Then we adopted immune precipitation assay to detect if pitstop 2 affected the dissociation of αIIbβ3 with clathrin. The data in Figure 4B demonstrated that integrin αIIbβ3 could co-immunoprecipitate with clathrin and AP2-mu subunit and pitstop 2 treatment caused higher levels of clathrin and AP2 association to αIIbβ3, indicating that pitstop 2 inhibited the recycling of αIIbβ3 probably by block the dissociation of αIIbβ3 with clathrin and AP2.

The functions of AP2 and dynamin in αIIbβ3-mediated platelet spreading were also assessed using phalloidin staining. The data presented in Figure 4C demonstrated that A23 and dynasore totally inhibited αIIbβ3-mediated platelet spreading on Fg.

Pitstop 2 had no effect on Fg uptake in vitro

As αIIbβ3 is the major receptor for Fg and mediates plasma Fg endocytosis, we examined the role of pitstop 2 in FITC-Fg uptake. Platelets preincubated with the inhibitors or control before incubated with FITC-Fg and then were fixed for imaging after trypan blue addition. In Figure 5A, pitstop 2-treated platelet had a comparable trypan blue-resistant internal pool of Fg compared with the control. The number of FITC-positive puncta per platelet counted manually was also similar between the two groups (Figure 5B). But Fg uptake was severely restrained in A23 or dynasore treated platelets, which had a higher percentage of platelets with fewer puncta (Figure 5A and B). These results are consistent with our observations in Figure 2–4, where pitstop 2 had no significant effect on endocytosis of αIIbβ3/Fg.

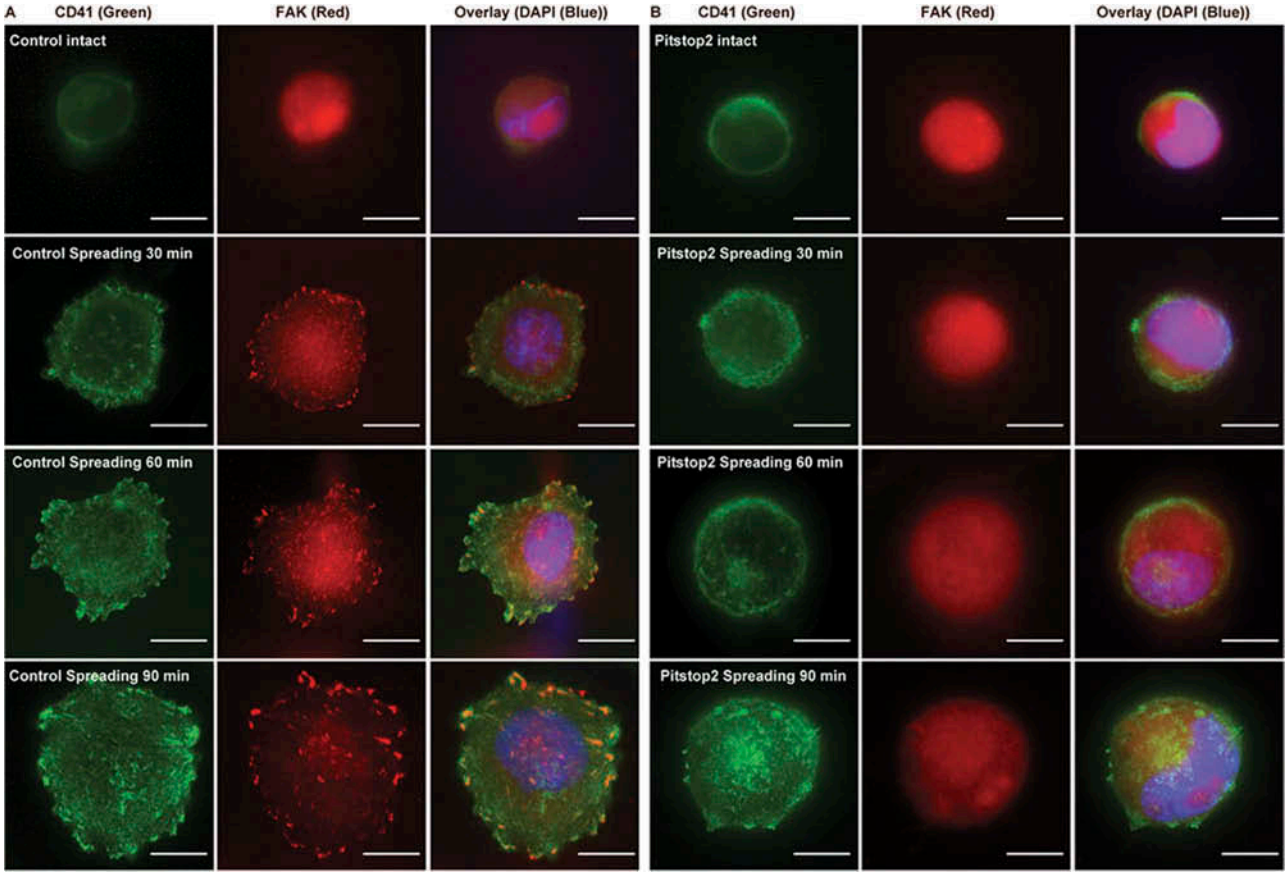

Figure 3. Pitstop 2 had no effect on endocytosis of αIIbβ3 but inhibited it trafficking back to membrane. Representative images of CHO cells incubated with FITC-conjucted CD41 (green) before treated with negative control (A) or pitstop 2 (B), then immobilized in poly-L-lysine or spreading on immobilized Fg for intervals as indicated and immunostained with FAK (red) and DAPI (blue). Cells were imaged with Zeiss LSM-710 confocal microscope with magnification × 60. Scale bar, 20 μm.

Src/plc/pkc signaling pathway may trigger integrin endocytosis

The data presented in this study indicated that endocytic and exocytic trafficking of integrin αIIbβ3 are critical steps for αIIbβ3-mediated platelet activation. However, the mechanism triggering the endocytosis of integrin αIIbβ3 is still poorly understood.

αIIbβ3 has the potential to interact its ligand and initiate a series of intracellular signaling events called "outside-in" signaling, leading to platelet spreading, granule secretion, and stable adhesion [34,35]. It has been reported that Src family kinases [36], FAK [37], Syk [37], and PLC/PKC [38], are involved in αIIbβ3-initiated outside-in signaling and play an important role in platelet activation. To explore the key signal molecules or signal pathway that initiates integrin αIIbβ3 endocytosis, specific inhibitors of Src, FAK, Syk, and PLC/PKC were used to resolve this issue.

The results presented in Figure 6 and supplemental Figure 4C showed that the Src inhibitor PP2, the PLC inhibitor U-73122, and the PKC inhibitor RO 31-8220 totally blocked αIIbβ3-bearing CHO cells spreading and αIIbβ3 endocytosis. However, the Syk inhibitor Picetannol and the FAK inhibitor PF-57322 partially inhibited CHO cells spreading, but had no effect on αIIbβ3 endocytosis. More interestingly, actin filaments blocker Cytochalasin D, but not microtubules stabilizer taxol, totally blocked αIIbβ3-mediated CHO cells spreading and αIIbβ3 endocytosis. These results suggested that the αIIbβ3-mediated Src/PLC/PKC pathway and actin filaments played critical roles in integrin αIIbβ3 endocytosis therefore αIIbβ3-mediated cell spreading on immobilized Fg.

Pitstop 2 prevented platelet aggregation and granule secretion

Further, the effects of pitstop 2 on platelet aggregation induced by collagen and thrombin were examined. As shown in Figure 7A and 7B, pitstop 2 inhibited collagen and thrombin induced platelet aggregation in a dose-dependent manner, with IC_{50} of 4.188 μM by 0.15 U/mL thrombin and 1.339 μM by 2 μg/mL collagen. The dense granule secretion monitored as ATP release was tested simultaneously and the results in Figure 7C and D showed that pitstop 2 inhibited dense granule secretion induced by collagen and thrombin in a concentration-dependent manner.

As pitstop 2 affected dense granule secretion, platelet spreading on immobilized Fg was repeated in the presence and absence of apyrase and indomethacin to exclude the effects of secondary signaling effects induced by secreted ADP or TXA_2. The results in Figure 7E and F showed that addition of pitstop 2 inhibited platelet spreading further on the basis of apyrase and indomethacin. These results demonstrated that the inhibition of spreading by pitstop 2 was not consequent directly to its interference with ADP or TXA_2 signaling.

When platelets exposed to agonists, various receptor-mediated intracellular signaling pathways are elicited and they converge to transform αIIbβ3 from a resting state to an

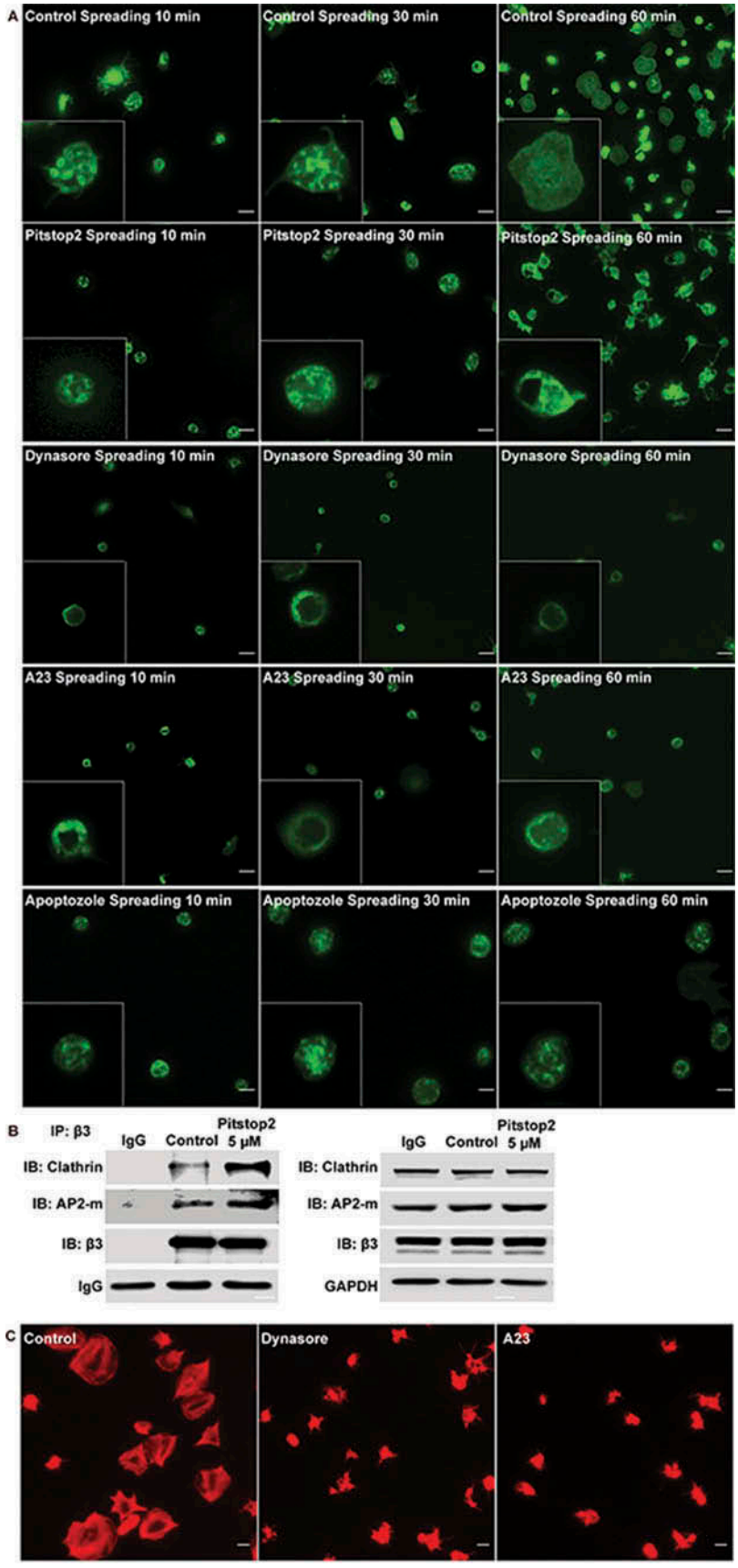

Figure 4. Pitstop 2 disturbed the dynamic dissociation of αIIbβ3 from clathrin and AP2. (A) representative images of platelets preincubated with FITC-conjugated CD41 before treated with negative control, pitstop 2, dynasore, A23, or apoptozole spreading on immobilized Fg for 10, 30, and 60 minutes, respectively. Images were taken under oil immersion with magnification × 100. Scale bar, 5 μm. (B) the rabbit-anti-β3 monoclonal antibody or rabbit IgG control was used to immunoprecipitate β3 from lysate of washed human platelets spreading on immobilized Fg for 90 minutes pretreated with negative control or pitstop 2. The immunoprecipitates were separated by SDS-PAGE and blotted with anti-clathrin, anti-AP2-mu, and anti-β3 antibodies for detection of the effect of pitstop 2 on clathrin and AP2 association with β3. IgG was used as a loading control. The expression levels of clathrin, AP2 and β3 in negative control and pitstop 2 treated platelets were examined by Western blotting. GAPDH was used to verify equal gel loading. (C) Representative phalloidin-stained images of washed platelets preincubated with control, dynasore, or Tyrphostin A23 spreading on immobilized Fg for 60 minutes. Scale bar, 1 μm.

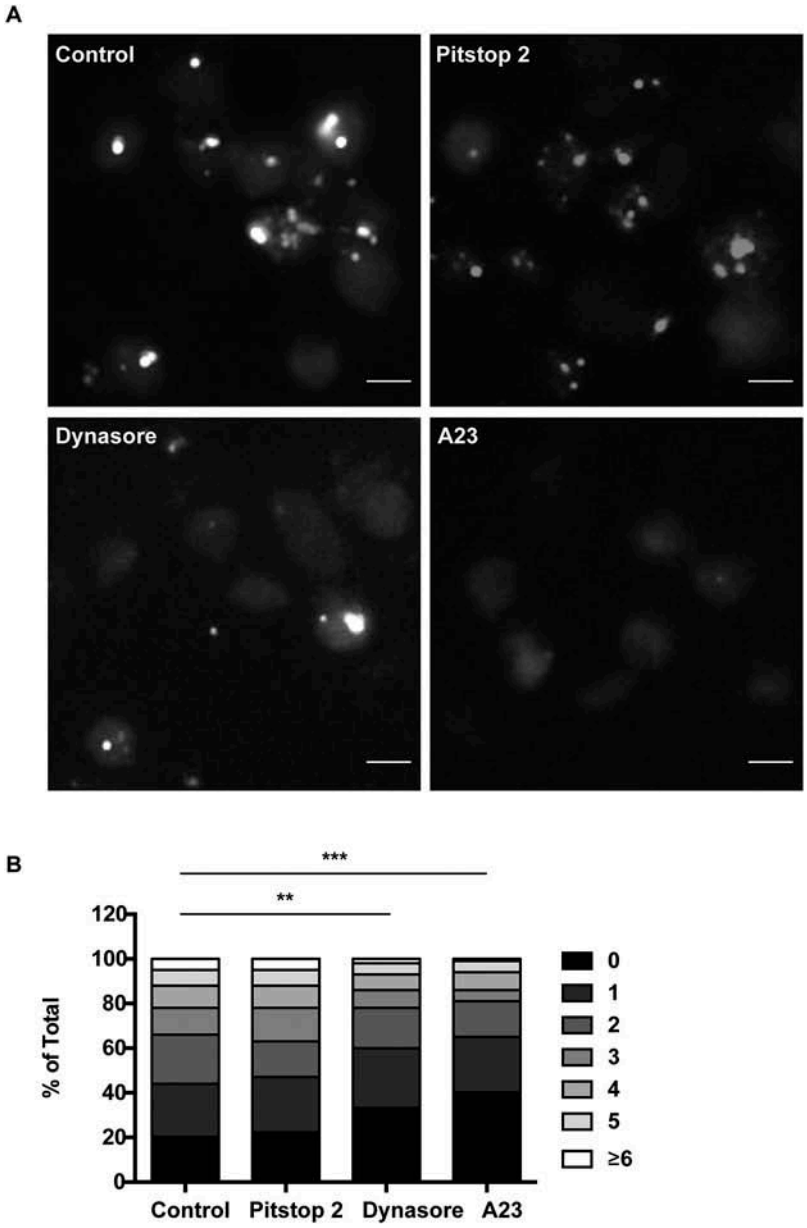

Figure 5. Pitstop 2 had no effect on Fg uptake in vitro. (A) Washed human platelets were incubated with pitstop 2, A23, dynasore, or negative control before incubated with FITC-Fg (0.05 mg/mL) for 1 hour at 37°C. Platelets were recovered and resuspended in Tyrode's buffer for another 2 hours. Platelets were fixed and subjected to epifluorescence microscopy. Representative images are presented after addition of trypan blue. Images were taken under oil immersion with magnification × 100. (B) The number of FITC-labeled puncta per platelet was counted manually. The percentage platelets in each group relative to total were plotted as a bar graph. Statistical significance was determined using the rank sum test (**$P < 0.01$, ***$P < 0.001$).

activated state with high affinity to ligands, which is called inside-out signaling [39]. We evaluated the effect of pitstop 2 on thrombin-induced αIIbβ3 activation using flow cytometric detection of FITC-conjugated-PAC1, an antibody specific for activated αIIbβ3, and APC-conjugated Fg binding to platelets. The results in Figure 8B and D demonstrated that αIIbβ3 activation and Fg binding induced by thrombin were restrained by pitstop 2 under non-stirring conditions. Also, expression of p-selectin on platelet surface was inhibited by incubation of pitstop 2 (Figure 8C). However, pitstop 2 had no significant influence on the total surface αIIbβ3 (Figure 8A). This may because FACS assays reflect steady state levels of surface αIIbβ3 and may not sensitive to small populations of rapid recycling αIIbβ3 [12].

Discussion

It has been proved that integrin endocytosis in nucleated cell can be achieved via two major approaches, clathrin-dependent manner and clathrin-independent manner such as caveolae and macropinocytosis. In this study, we found that pitstop 2, a selective inhibitor of clathrin, abolished integrin αIIbβ3-mediated human platelet aggregation and spreading on immobilized Fg in vitro, indicating that clathrin-mediated endocytosis plays a critical role in platelet activation. Clathrin-mediated integrin αIIbβ3 trafficking probably is an important way for platelet spreading. However, our study did not exclude the possibility of clathrin-independent αIIbβ3 trafficking involving in platelet activation, further investigation required to resolve this issue.

Clathrin is a three-legged protein complex with the heavy chain containing a globular N-terminal β-propeller domain (TD) [40]. During the endocytic process, clathrin functions as a central organizing platform for coated pit assembly and dissociation via its TD. Pitstop 2 was invented as a small molecule targeted on clathrin TD and has been confirmed as a useful tool to perturb clathrin-coated pits dynamics instead of inhibiting coated pit initiation [14]. However, a study recently reported that pitstop 2 prevent the massing of clathrin at internalization sites therefore attenuate the endocytosis of Dickkopf-1 receptors [41], which make the working mechanism of pitstop 2 controversy. In our study, we clearly showed that the membrane αIIbβ3 underwent internalizing, vesicular and clustering stages during the CHO cells spreading on

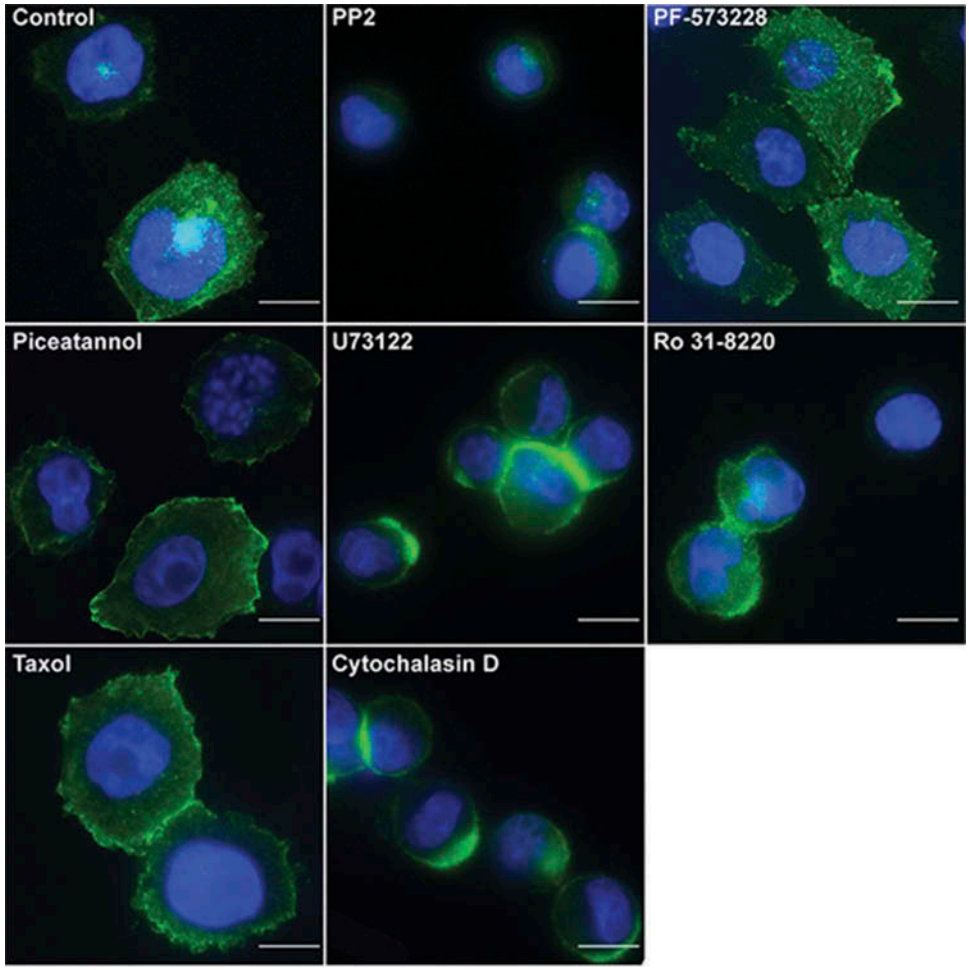

Figure 6. The Src/PLC/PKC signaling pathway may trigger integrin endocytosis. (A) Representative picture of CHO cells labeling with FITC-conjucted-CD41 spreading on immobilized Fg for 90 minutes after treated with the Src inhibitor PP2, FAK inhibitor PF-573228, Syk inhibitor Piceatannol, PLC inhibitor U73122, PKC inhibitor Ro 31-8220, microtubule inhibitor Taxol or actin polymerization inhibitor Cytochalasin D. Images were taken under oil immersion with magnification × 100. Scale bar, 20 μm.

immobilized Fg. And the pitstop 2 treatment did not affect the internalization αIIbβ3 in CHO cells and platelets, but caused the integrin αIIbβ3 arrested at vesicular stage in CHO cells and platelets spreading on immobilized Fg. The Fg uptake also showed that pitstop 2 treatment had no significant effect on internalization of αIIbβ3/Fg (Figure 5). These results indicated that pitstop 2 inhibited the recycling of αIIbβ3 to plasma membrane instead of initiation of the endocytosis of αIIbβ3. The immune precipitation assay showed that pitstop 2 treatment caused higher levels of clathrin and AP2 association with αIIbβ3 (Figure 4B), indicating that pitstop 2 inhibited αIIbβ3 recycling probably by block the dissociation of αIIbβ3 with clathrin and AP2. It has been reported that clathrin coat disassembly is implemented through the uncoating apparatus including ATPase heat shock cognate 70 (HSC70) and its co-chaperone auxilin [7,42] and auxilin binds physically to the clathrin TD [42]. Considering pitstop 2 targeted on clathrin TD and arrested recycling of integrin αIIbβ3, we proposed that pitstop 2 might work through regulating the uncoating apparatus. The role of uncoating apparatus in αIIbβ3 trafficking was also confirmed using HSC70 selective inhibitor apoptozole, which had a similar effect as pitstop 2 (Figure 4A). Although pitstop 2 had been reported to perturb clathrin-coated pits dynamics [14], we showed firstly that pitstop 2 inhibited αIIbβ3 recycling.

Another two critical components of clathrin-coated vesicles were also examined. AP2, as a main clathrin adaptor, plays a pivotal role in cargo selection and clathrin coat assembly. After the formation of clathrin-coated vesicles, the mechanochemical enzyme dynamin is recruited to the complex to help clathrin-coated vesicles detached from the neck. In our study, the inhibitors of AP2 and dynamin indeed impaired αIIbβ3 internalization (Figure 4C), confirming that αIIbβ3 traffic is mainly clathrin-mediated and pitstop 2 functioned through a different mechanism from the two inhibitors.

αIIbβ3 binding to its ligand can initiate outside-in signaling [34,35], and several kinases, including FAK, Src, and PI3K, have been shown as key molecules involving in this process [35]. In our study, Src, PLC, and PKC inhibitors totally blocked membrane integrin αIIbβ3 internalization, suggesting that αIIbβ3-mediated outside-in signaling initiates itself internalization. Src family kinases and PKC have been widely accepted for their effect on clathrin recruitment for clathrin coat pit formation through phosphorylation of the clathrin heavy chain [4], PKC can bind to β1 and regulate integrin internalization [43]. The function of PLC in integrin αIIbβ3 endocytosis had not been reported, but it is easy to speculate that PLC may function via the classical regulatory of PKC activity. As these kinases were well established in integrin signaling, we agreed with the opinion that integrin trafficking and signaling were probably tightly coupled [44].

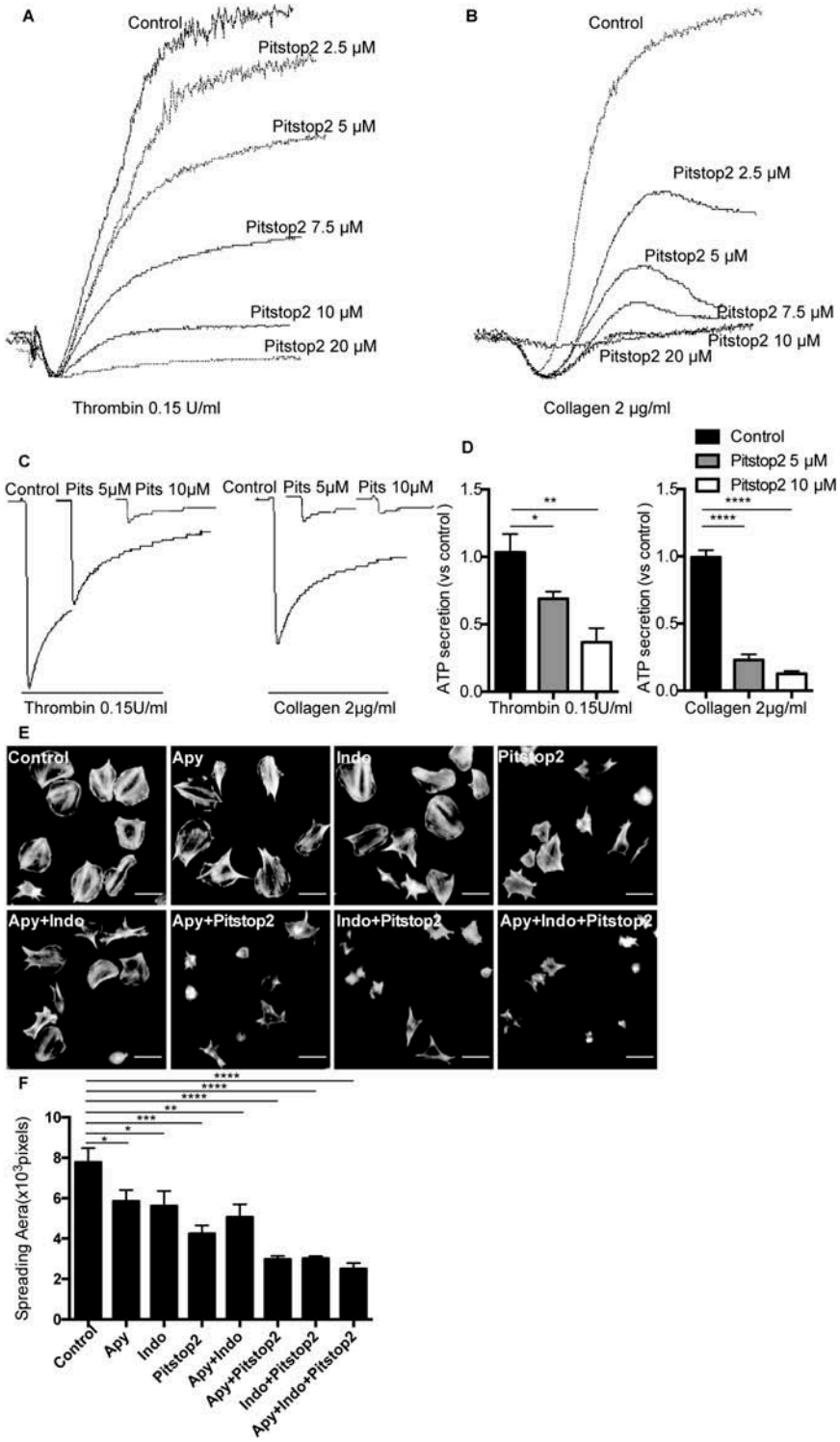

Figure 7. Pitstop 2 prevented platelet aggregation and dense granule secretion. (A, B) aggregations of washed platelets preincubated with negative control or pitstop 2 were studied by aggregometery in response to agonists as indicated. (C, D) Platelet secretion of ATP was recorded in the presence of luciferin-luciferase agent. At least three independent experiments were performed. Statistical analyses were performed using Student t-test, mean ± SD, $*P < 0.05$, $** P < 0.01$, $****P < 0.0001$. Pit, pitstop 2. (E) Representative phalloidin-stained images of washed platelets preincubated with negative control, apyrase (10 U/mL), indomethacin (75 μM), pitstop 2, apyrase + indomethacin, apyrase + pitstop 2, indomethacin + pitstop 2 or apyrase + indomethacin + pitstop 2 spreading on immobilized Fg for 60 minutes, respectively. Images were taken under oil immersion with magnification × 100. Scale bar, 5 μm. (F) Quantification of the platelet areas (pixel number) in 5 random fields per experiment and at least three independent experiments were performed. Statistical analyses were performed using the Student t-test (mean ± SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$). Apy, apyrase. Indo, indomethacin.

However, the precise mechanisms of these kinases involving in initiation of αIIbβ3 internalization still need further declaration. In addition, cytochalasin D, but not taxol, severely inhibited the endocytosis of αIIbβ3, suggesting that actin filaments, but not microtubules played a more pivotal role in regulating membrane αIIbβ3 trafficking.

Except for integrin αIIbβ3, platelets express many other receptors including adhesion receptors and G-protein-coupled receptors. As clathrin-mediated endocytosis may also be involved in the trafficking process of the receptors [45,46] and granule secretion [47], it is understandable that pitstop 2 interfered with platelet aggregation and secretion. But the precise component responsible for the phenomenon is worthy further investigation. Pitstop 2 also affected Fg binding and αIIbβ3 activation, demonstrating an effect on αIIbβ3-mediated inside-out signaling. However, the extent of αIIbβ3 internalization triggered by immobilized Fg was not influenced by pitstop 2. These results indicated that αIIbβ3 activation may not be necessary for αIIbβ3 endocytosis. Further study is required to clarify the mechanism.

In summary, our study presented here suggested integrin αIIbβ3 trafficking was mainly clathrin-dependent and played a

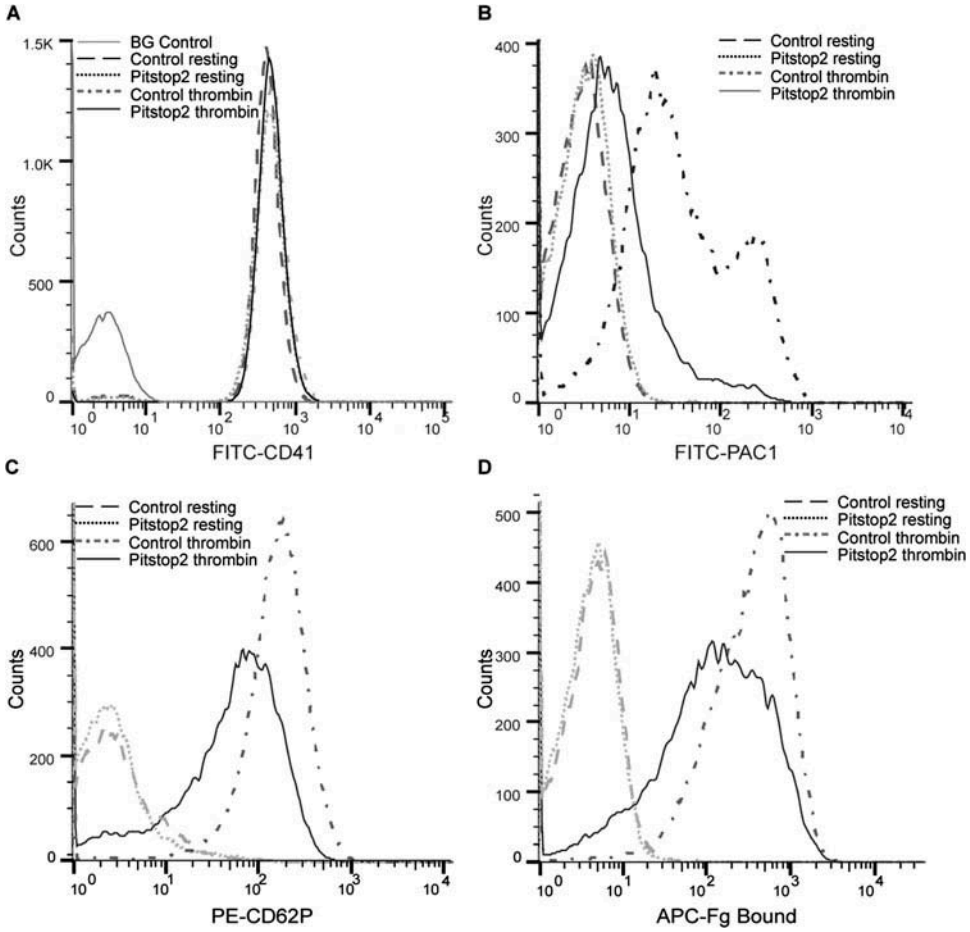

Figure 8. Pitstop 2 restrained platelet inside-out signaling. (A, B) Washed human platelets at a concentration of 3×10^7/mL were incubated with FITC-anti-CD41 antibody to measure levels of total integrin αIIbβ3 (A) or FITC-PAC1 antibody to measure levels of activated integrin αIIbβ3 (B) in the presence of 0.05 U/mL thrombin. Unlabeled platelets were used as the background control. (C) Washed human platelets were incubated with PE-CD62P monoclonal antibody at 25°C for 20 minutes in the presence of 0.05 U/mL thrombin. The expression of CD62P was analyzed using flow cytometer. (D) Binding of Alexa 647-Fg to washed human platelets stimulated with 0.05 U/mL thrombin.

critical role on platelet spreading. Pitstop 2 may serve as an effective tool to address clathrin-mediated trafficking in platelets.

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Supplemental data

Supplemental data for this article can be accessed on the publisher's website.

References

1. Gironcel D, Racaud-Sultan C, Payrastre B, Haricot M, Borchert G, Kieffer N, Breton M, Chap H. alphaIIb beta 3-integrin mediated adhesion of human platelets to a fibrinogen matrix triggers phospholipase C activation and phosphatidylinositol 3′,4′-biphosphate accumulation. FEBS Lett 1996;389(3):253–256. doi:10.1016/0014-5793(96)00595-9
2. De Franceschi N, Hamidi H, Alanko J, Sahgal P, Ivaska J. Integrin traffic: The update. J Cell Sci 2015;128(5):839–852. doi:10.1242/jcs.161653
3. Margadant C, Monsuur HN, Norman JC, Sonnenberg A. Mechanisms of integrin activation and trafficking. Curr Opin Cell Biol 2011;23(5):607–614. doi:10.1016/j.ceb.2011.08.005
4. Pellinen T, Ivaska J. Integrin traffic. J Cell Sci 2006;119(Pt 18):3723–3731. doi:10.1242/jcs.03216
5. Israeli-Rosenberg S, Chen C, Li R, Deussen DN, Niesman IR, Okada H, Patel HH, Roth DM, Ross RS. Caveolin modulates integrin function and mechanical activation in the cardiomyocyte. Faseb J 2015;29(2):374–384. doi:10.1096/fj.13-243139
6. Kosaka T, Ikeda K. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of Drosophila melanogaster, shibirets1. J Cell Biol 1983;97(2):499–507. doi:10.1083/jcb.97.2.499
7. Ht M, Boucrot E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 2011;12(8):517–533. doi:10.1038/nrm3151
8. Dn B, Oakley JD, Sessions RB, Banting G. Tyrphostin A23 inhibits internalization of the transferrin receptor by perturbing the interaction between tyrosine motifs and the medium chain subunit of the AP-2 adaptor complex. J Biol Chem 2003;278(14):12022–12028. doi:10.1074/jbc.M211966200
9. Schmid SL. Clathrin-coated vesicle formation and protein sorting: An integrated process. Annu Rev Biochem 1997;66:511–548. doi:10.1146/annurev.biochem.66.1.511
10. Irannejad R, Tsvetanova NG, Lobingier BT, Von Zastrow M. Effects of endocytosis on receptor-mediated signaling. Curr Opin Cell Biol 2015;35:137–143. doi:10.1016/j.ceb.2015.05.005
11. Puthenveedu MA, Lauffer B, Temkin P, Vistein R, Carlton P, Thorn K, Taunton J, Weiner OD, Parton RG, Von Zastrow M. Sequence-dependent sorting of recycling proteins by actin-stabilized

endosomal microdomains. Cell 2010;143(5):761–773. doi:10.1016/j.cell.2010.10.003
12. Huang Y, Joshi S, Xiang B, Kanaho Y, Li Z, Bouchard BA, Moncman CL, Whiteheart SW. Arf6 controls platelet spreading and clot retraction via integrin alphaIIbbeta3 trafficking. Blood 2016;127(11):1459–1467. doi:10.1182/blood-2015-05-648550
13. Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. J Cell Biol 2009;187(5):733–747. doi:10.1083/jcb.200904054
14. Von Kleist L, Stahlschmidt W, Bulut H, Gromova K, Puchkov D, Robertson MJ, MacGregor KA, Tomilin N, Pechstein A, Chau N, Chircop M, Sakoff J, Von Kries JP, Saenger W, Krausslich HG, Shupliakov O, Robinson PJ, McCluskey A, Haucke V. Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. Cell 2011;146(3):471–484. doi:10.1016/j.cell.2011.06.025
15. Guo S, Zhang X, Zheng M, Zhang X, Min C, Wang Z, Cheon SH, Oak MH, Nah SY, Kim KM. Selectivity of commonly used inhibitors of clathrin-mediated and caveolae-dependent endocytosis of G protein-coupled receptors. Biochim Biophys Acta 2015;1848(10 Pt A):2101–2110. doi:10.1016/j.bbamem.2015.05.024
16. Gruner S, Prostredna M, Schulte V, Krieg T, Eckes B, Brakebusch C, Nieswandt B. Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo. Blood 2003;102(12):4021–4027. doi:10.1182/blood-2003-05-1391
17. Sm S, Ono A, Nesbitt WS, Lim J, Jarman K, Jackson SP. Phosphoinositide 3-kinase p110 beta regulates integrin alpha IIb beta 3 avidity and the cellular transmission of contractile forces. J Biol Chem 2010;285(4):2886–2896. doi:10.1074/jbc.M109.029132
18. Re B, Norman JC, Caswell PT. Integrin trafficking at a glance. J Cell Sci 2012;125(Pt 16):3695–3701. doi:10.1242/jcs.095810
19. Ch Y, Rafiq NB, Cao F, Zhou Y, Krishnasamy A, Biswas KH, Ravasio A, Chen Z, Wang YH, Kawauchi K, Jones GE, Sheetz MP. Integrin-beta3 clusters recruit clathrin-mediated endocytic machinery in the absence of traction force. Nat Commun 2015;6:8672. doi:10.1038/ncomms9672
20. Yx Z, Zhu MQ, Jiang M, Zuo B, Wu QY, Ruan CG, He Y. An improved flow cytometric immunobead array to detect autoantibodies in plasma from patients with immune thrombocytopenic purpura. Clin Chim Acta 2015;438:396–400. doi:10.1016/j.cca.2014.09.018
21. Liu J, Jackson CW, Gruppo RA, Jennings LK, Gartner TK. The beta3 subunit of the integrin alphaIIbbeta3 regulates alphaIIb-mediated outside-in signaling. Blood 2005;105(11):4345–4352. doi:10.1182/blood-2004-07-2718
22. Weng Z, Li D, Zhang L, Chen J, Ruan C, Chen G, Gartner TK, Liu J. PTEN regulates collagen-induced platelet activation. Blood 2010;116(14):2579–2581. doi:10.1182/blood-2010-03-277236
23. Yin H, Liu J, Li Z, Berndt MC, Lowell CA, Du X. Src family tyrosine kinase Lyn mediates VWF GPIb-IX induced platelet activation via the cGMP signaling pathway. Blood 2008;112(4):1139–1146. doi:10.1182/blood-2008-02-140970
24. Fan X, Shi P, Dai J, Lu Y, Chen X, Liu X, Zhang K, Wu X, Sun Y, Wang K, Zhu L, Zhang CC, Zhang J, Chen GQ, Zheng J, Liu J. Paired immunoglobulin-like receptor B regulates platelet activation. Blood 2014;124(15):2421–2430. doi:10.1182/blood-2014-03-557645
25. Liu J, Fitzgerald ME, Berndt MC, Jackson CW, Gartner TK. Bruton tyrosine kinase is essential for botrocetin/VWF-induced signaling and GPIb-dependent thrombus formation in vivo. Blood 2006;108(8):2596–2603. doi:10.1182/blood-2006-01-011817
26. Mj C, Liu J, Pestina TI, Steward SA, Thomas DW, Coffman TM, Wang D, Jackson CW, Gartner TK. The roles of alpha IIb beta 3-mediated outside-in signal transduction, thromboxane A2, and adenosine diphosphate in collagen-induced platelet aggregation. Blood 2003;101(7):2646–2651. doi:10.1182/blood-2002-05-1363
27. Dejonghe W, Kuenen S, Mylle E, Vasileva M, Keech O, Viotti C, Swerts J, Fendrych M, Ortiz-Morea FA, Mishev K, et al. Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification. Nat Commun 2016;7:11710. doi:10.1038/ncomms11710
28. Unsworth AJ, Kriek N, Bye AP, Naran K, Sage T, Flora GD, Gibbins JM. PPARgamma agonists negatively regulate alphaIIbbeta3 integrin outside-in signaling and platelet function through up-regulation of protein kinase A activity. J Thromb Haemost 2017;15(2):356–369. doi: 10.1111/jth.13578
29. Hf C, Bzeih H, Schirra C, Chitirala P, Halimani M, Cordat E, E K, Rettig J, Pattu V. Endocytosis of Cytotoxic Granules Is Essential for Multiple Killing of Target Cells by T Lymphocytes. J Immunol 2016;197(6):2473–2484. doi:10.4049/jimmunol.1600828
30. Li Z, Zhang G, Feil R, Han J, Du X. Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. Blood 2006;107(3):965–972. doi:10.1182/blood-2005-03-1308
31. De Franceschi N, Arjonen A, Elkhatib N, Denessiouk K, Wrobel AG, Wilson TA, Pouwels J, Montagnac G, Owen DJ, Ivaska J. Selective integrin endocytosis is driven by interactions between the integrin alpha-chain and AP2. Nat Struct Mol Biol 2016;23(2):172–179. doi:10.1038/nsmb.3161
32. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 2006;10(6):839–850. doi:10.1016/j.devcel.2006.04.002
33. Hj C, Kim GH, Park SH, Hyun JY, Kim NK, Shin I. Probing the effect of an inhibitor of an ATPase domain of Hsc70 on clathrin-mediated endocytosis. Mol Biosyst 2015;11(10):2763–2769. doi:10.1039/C4MB00695J
34. Ma YQ, Qin J, Plow EF. Platelet integrin alpha(IIb)beta(3): Activation mechanisms. J Thromb Haemost 2007;5(7):1345–1352. doi:10.1111/j.1538-7836.2007.02537.x
35. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol 2010;30(12):2341–2349. doi:10.1161/ATVBAHA.110.207522
36. Gong H, Shen B, Flevaris P, Chow C, Lam SC, Voyno-Yasenetskaya TA, Kozasa T, Du X. G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling. Science 2010;327(5963):340–343. doi:10.1126/science.1174779
37. Ya S, Mazharian A, Mori J. Src family kinases: At the forefront of platelet activation. Blood 2014;124(13):2013–2024. doi:10.1182/blood-2014-01-453134
38. Wonerow P, Pearce AC, Vaux DJ, Watson SP. A critical role for phospholipase Cgamma2 in alphaIIbbeta3-mediated platelet spreading. J Biol Chem 2003;278(39):37520–37529. doi:10.1074/jbc.M305077200
39. Estevez B, Shen B, Du X. Targeting integrin and integrin signaling in treating thrombosis. Arterioscler Thromb Vasc Biol 2015;35(1):24–29. doi:10.1161/ATVBAHA.114.303411
40. Fm B. Diversity of clathrin function: new tricks for an old protein. Annu Rev Cell Dev Biol 2012;28:309–336. doi:10.1146/annurev-cellbio-101011-155716
41. Hishinuma S, Nozawa H, Akatsu C, Shoji M. C-terminal of human histamine H1 receptors regulates their agonist-induced clathrin-mediated internalization and G-protein signaling. J Neurochem 2016. doi:10.1111/jnc.13834
42. Lemmon SK, Traub LM. Getting in touch with the clathrin terminal domain. Traffic 2012;13(4):511–519. doi:10.1111/j.1600-0854.2011.01321.x
43. Ng T, Shima D, Squire A, Bastiaens PI, Gschmeissner S, Humphries MJ, Parker PJ. PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic. Embo J 1999;18(14):3909–3923. doi:10.1093/emboj/18.14.3909
44. Korolchuk V, Banting G. Kinases in clathrin-mediated endocytosis. Biochem Soc Trans 2003;31(Pt 4):857–860. doi:10.1042/bst0310857
45. Baurand A, Eckly A, Hechler B, Kauffenstein G, Galzi JL, Cazenave JP, Leon C, Gachet C. Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? Mol Pharmacol 2005;67(3):721–733. doi:10.1124/mol.104.004846
46. Sw B, Nooh MM. Barcoding of GPCR trafficking and signaling through the various trafficking roadmaps by compartmentalized signaling networks. Cell Signal 2017;36:42–55. doi:10.1016/j.cellsig.2017.04.015
47. Michaux G, Dyer CE, Nightingale TD, Gallaud E, Nurrish S, Cutler DF. A role for Rab10 in von Willebrand factor release discovered by an AP-1 interactor screen in C. Elegans J Thromb Haemost 2011;9(2):392–401. doi:10.1111/j.1538-7836.2010.04138.x