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β -arrestin-1 participates in thrombosis and regulates integrin $\alpha_{IIb}\beta_3$ signalling without affecting P2Y receptors desensitisation and function

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Summary

β -arrestin-1 (β -arr1) and β -arrestin-2 (β -arr2) are cytosolic proteins well-known to participate in G protein-coupled receptor desensitisation and signalling. We used genetically-inactivated mice to evaluate the role of β -arr1 or β -arr2 in platelet function, P2Y receptor desensitisation, haemostasis and thrombosis. Platelet aggregation, soluble fibrinogen binding and P-selectin exposure induced by various agonists were near normal in β -arr1^{-/-} and β -arr2^{-/-} platelets. In addition, deficiency in β -arr1 or β -arr2 was not critical for P2Y receptors desensitisation. A functional redundancy between β -arr1 and β -arr2 may explain these unchanged platelet responses. Interestingly, β -arr1^{-/-} but not β -arr2^{-/-} mice were protected against laser- and FeCl₃-induced thrombosis. The tail bleeding times, number of rebleeds and volume of

blood loss were unchanged in β -arr1^{-/-} and β -arr2^{-/-} mice, suggesting no defect in haemostasis. β -arr1^{-/-} platelet activation upon adhesion to immobilised fibrinogen was inhibited, as attested by a $37 \pm 5\%$ ($n = 3$, $p < 0.0001$) decrease in filopodia extension, suggesting defective signalling through integrin $\alpha_{IIb}\beta_3$. β -arr1 appeared to be located downstream of Src family kinases and to regulate $\alpha_{IIb}\beta_3$ signalling by increasing Akt phosphorylation. Overall, this study supports a role for β -arr1 in promoting thrombus formation, in part through its participation in $\alpha_{IIb}\beta_3$ signalling, and no role of β -arr1 and β -arr2 in agonist-induced platelet activation and P2Y receptors desensitisation.

Keywords

β -arrestins, platelets, thrombosis, $\alpha_{IIb}\beta_3$, Akt

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Introduction

β -arrestin-1 (β -arr1) and β -arrestin-2 (β -arr2), also called arrestin-2 and arrestin-3, respectively, are ubiquitously-expressed cytosolic proteins found in mammals, and form the non-visual arrestin family (1). They bind to activated G protein-coupled receptors (GPCRs) and were originally described as serving to desensitise activation of G proteins and to target the receptors to clathrin-coated pits for endocytosis, thereby terminating the agonist-induced signalling. More recently, it has been established that both β -arr1 and β -arr2 also transduce signals of agonist-occupied GPCRs by acting as adaptors, recruiting a variety of proteins including GTPases and diverse kinases such as phosphoinositide 3-kinase (PI3K), Akt, Src kinases and mitogen-activated protein kinases (1). The resulting multiprotein scaffolding promotes G protein-independent alternative signalling which regulate many cellular responses such as adhesion, proliferation, differentiation, migration, and apoptosis.

P2Y₁ and P2Y₁₂ are G protein-coupled adenosine 5'-diphosphate (ADP) receptors playing a key role in platelet activation and thrombosis (2). Platelets activated by ADP become refractory to restimulation, a phenomenon which has been attributed to P2Y₁ desensitisation and internalisation (3–5). Although a substantial fraction of P2Y₁₂ is rapidly internalised upon stimulation (4), whether this process is accompanied by a functional desensitisation remains unclear. We previously published that ADP-pre-

treated platelets retained P2Y₁₂ activity (3, 4). For unidentified reasons, this was not the case in another study by Hardy et al. (5), suggesting that P2Y₁₂ underwent desensitisation. Both β -arr1 and β -arr2 were shown to associate with stimulated P2Y₁ and P2Y₁₂ in transfected cell systems (6–8). However, whether β -arrestins mediate P2Y receptor desensitisation in platelets remains to be established.

Integrin $\alpha_{IIb}\beta_3$ plays a pivotal role in haemostasis and arterial thrombosis by supporting platelet adhesion and aggregation at sites of vascular injury (9). A key attribute of $\alpha_{IIb}\beta_3$ is its capacity to shift from a resting low to an activated high affinity conformation for its main ligand, fibrinogen. This transformation is mediated by platelet-activating inside-out signals derived from tyrosine kinase-linked and G protein-coupled receptors. Upon fibrinogen binding, $\alpha_{IIb}\beta_3$ initiates its own signalling cascade involving several proteins notably Src kinases, phospholipase C γ 2, PI3K and Akt (9–11). The so-called outside-in $\alpha_{IIb}\beta_3$ signalling promotes intracellular Ca²⁺ mobilisation and cytoskeletal reorganisation leading to filopodia emission, and is important to platelet function in haemostasis and arterial thrombosis (12–15). While β -arrestins also regulate a variety of non GPCRs, their importance in $\alpha_{IIb}\beta_3$ function is unknown (1).

Recently, it has been described that β -arr1 deficiency resulted in a thrombosis defect in a FeCl₃ carotid injury model (16). *In vitro* studies showed reduced soluble fibrinogen binding after stimu-

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lation of the platelet protease-activated receptor (PAR) 4. Whether the decreased thrombosis observed in β -arr1^{-/-} mice is due to the defective response through PAR4 or arises from additional signalling pathways remains to be established.

The objectives of this study were to evaluate the role of β -arr1 and β -arr2 in (i) agonist-induced platelet activation, (ii) P2Y₁ and P2Y₁₂ desensitisation, and (iii) haemostasis and experimental thrombosis. Mouse platelets with deficiency in β -arr1 or β -arr2 responded normally to a series of agonists, and displayed similar desensitisation to ADP as wild-type (WT) platelets, suggesting that these proteins do not play a key role in these processes. We observed a contribution of β -arr1, but not β -arr2, to arterial thrombosis after laser or FeCl₃ injury. The tail bleeding times, rebleeding occurrences and volume of blood loss were unchanged in β -arr1^{-/-} and β -arr2^{-/-} mice, suggesting no defect in haemostasis. Upon adhesion to immobilised fibrinogen, β -arr1-knock-out platelets exhibited defective integrin $\alpha_{IIb}\beta_3$ -mediated shape change. The role of β -arr1 in $\alpha_{IIb}\beta_3$ outside-in signals appeared to be dependent on Src family kinases and linked to the upregulation of Akt phosphorylation.

Materials and methods

Materials

Alexa Fluor 488-labelled fibrinogen, DIOC₆ (3,3'-dihexyloxacarbocyanine iodide) and the indicators Oregon Green 488 BAPTA/AM-1 and Calcein red-orange/AM were from Molecular Probes (Paisley, UK). FITC-coupled anti-P-selectin antibody (RB40.34) was purchased from BD Pharmingen (Le Pont-De-Claix, France) and FeCl₃ solution from VWR Prolabo (Fontenay-sous-Bois, France). Fatty acid-free human serum albumin (HSA), fura-2/AM, TRITC (tetramethylrhodamine isothiocyanate)-phalloidin, bovine thrombin, U46619, ADP, ADP β S, indomethacin, PP1 and PP2 were provided by Sigma-Aldrich (Lyon, France). AR-C69931MX (Cangrelor), a P2Y₁₂ antagonist, was from the Medicines Company (Parsippany, NJ, USA) and MRS2179, a P2Y₁ blocker, from Tocris Bioscience (Bristol, UK). Cross-linked collagen-related peptide (CRP-XL) was obtained from Dr. R.W. Farndale (University of Cambridge, Cambridge, UK), PAR4-selective agonist AYPGKF peptide was synthesised by PolyPeptide Group (Strasbourg, France) and Horm fibrillar type I collagen from equine tendon was from Nycomed (Zurich, Switzerland). Human fibrinogen was provided by Kabi (Bad Homburg, Germany) and 1-paraformaldehyde (PFA) by Electron Microscopy Sciences (Euromedex, Souffelweyersheim, France). Recombinant hirudin was from Transgene (Illkirch-Grattenstaden, France) and acid citrate dextrose (ACD) solution from Bioluz (St-Jean-de-Luz, France). Apyrase was purified from potatoes as previously described (17). Monoclonal antibodies to total Akt and phosphorylated Akt at Ser473 (D9E) or Thr308 (244F9) were from Cell Signaling Technology (Ozyme, St-Quentin-en-Yvelines, France).

Mice

β -arr1- and β -arr2-deficient mice (18, 19) were provided by Pr. R.J. Lefkowitz (Duke University, Durham, NC, USA) and backcrossed for six generations on C57BL/6 background. WT mice were purchased from Charles River (L'Arbresle, France). Mice were maintained in the animal facilities of the Etablissement Français du Sang-Alsace. We used 8- to 10-week-old mice, anaesthetised intraperitoneally with a mixture of xylazine (20 mg per kg body weight, Rompun[®], Bayer, Leverkusen, Germany) and ketamine (100 mg per kg body weight, Imalgene 1,000[®], Merial, Lyon, France). All experiments conformed to the French legislation for animal experimentation and followed the recommendations of the Guide for the Care and Use of Laboratory Animals.

Washed platelet preparation

Blood was drawn from the abdominal aorta into ACD anticoagulant, pooled (4-6 animals), and platelets were washed by sequential centrifugations and adjusted to 3×10^8 platelets/ml in Tyrode's albumin buffer containing 0.02 U/ml apyrase (17, 20)

Aggregation studies

Washed platelets aggregation was measured turbidimetrically using a four-channel CARAT TX4 aggregometer (Entec, Ilmenau, Germany) as previously described (17).

Measurement of fibrinogen binding and P-selectin exposure

Washed platelets (5×10^7 /ml) were stimulated with the indicated agonists in the presence of Alexa Fluor 488-fibrinogen (20 μ g/ml) or FITC-anti-P-selectin antibody (25 μ g/ml), mixed and at predetermined time points fixed with PBS-20 mg/ml PFA for 20 minutes (min). Platelets were pelleted by centrifugation at $1,000 \times g$ for 2 min and resuspended in 500 μ l phosphate-buffered saline (PBS). The fluorescence intensity of each platelet sample was measured in the range 525-50 nm after excitation with a 488-nm argon-ion laser using a Gallios flow cytometer (Beckman Coulter, Villepinte, France). The extents of integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure were represented by the levels of Alexa Fluor 488-fibrinogen and FITC-anti-P-selectin binding, respectively, expressed as the geometric mean of the relative fluorescence intensity of 10,000 platelets (in arbitrary units) (Kaluza software, Beckman Coulter). Basal fluorescence, as measured in resting platelets, was subtracted from all data points.

ELISA-based VASP whole blood assay

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation state was determined using a standardised ELISA (CY-QUANT VASP/P2Y12[®], Biocytex, Marseille, France) (21, 22). Briefly, hirudinized (100 U/ml) whole blood drawn from the abdominal aorta was pretreated for 20 min with Tyrode's albumin buffer or ADP (10 μ M). Each sample was then incubated for 10 min at room temperature (RT) with either prostaglandin E₁ (PGE₁) or PGE₁ plus ADP in two separate wells of a 96-well plate. After cell lysis, VASP was captured by a selective anti-VASP antibody coated on the wells. A peroxidase-conjugated anti-phosphorylated VASP antibody was then added, and the plates were incubated with a chromogenic substrate solution and read at 450 nm. The platelet reactivity index (PRI), which reflects the ability of P2Y₁₂ to inhibit PGE₁-induced VASP phosphorylation, was calculated from blank-corrected optical densities (OD) as follows: PRI (%) = [(OD_{PGE1} - OD_{PGE1 + ADP}) / OD_{PGE1}] x 100.

Static adhesion assay

Glass coverslips were coated with 100 μ g/ml fibrinogen for 2 hours (h) at RT and blocked with PBS-10 mg/ml HSA for 1 h. Washed platelets (3 x 10⁷/ml) were pretreated for 15 min with AR-C69931MX (10 μ M), MRS2179 (100 μ M) and indomethacin (100 μ M) alone or in the presence of either vehicle (1:1,000 DMSO), PP1 or PP2 (10 μ M), and allowed to adhere to the coated surfaces at 37°C. After 20 min, non-adherent platelets were washed away and adherent cells were fixed with PBS-40 mg/ml PFA, stained with TRITC-phalloidin (2 μ g/ml) and imaged using a Leica DMI 4000 B epifluorescence microscope with a 63x, 1.4 numerical aperture oil objective (Leica Microsystems, Wetzlar, Germany) (23).

Scanning electron microscopy (SEM)

Adherent platelets were fixed with 25 mg/ml glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 20 mg/ml sucrose. SEM was performed as described elsewhere (23).

Analysis of cytosolic Ca²⁺ fluxes

ADP-induced Ca²⁺ elevations were measured as previously reported (24). Briefly, washed platelets resuspended in Ca²⁺-free Tyrode's buffer (7.5 x 10⁸ cells/ml) were loaded with fura-2/AM (15 μ M) for 45 min at 37°C. The dyed platelets were pelleted at 1,900 x g for 3 min, resuspended to 2 x 10⁸/ml in Tyrode's buffer containing apyrase and 1 mg/ml HSA, and challenged with P2Y receptor agonists using a PTI Deltascan spectrofluorimeter (Photon Technology International, Ford, UK). Fluorescence measurements were

performed at 510 nm after exciting alternatively at 340 and 380 nm, and converted to intracellular Ca²⁺ levels. Cytosolic Ca²⁺ changes upon platelet adhesion to fibrinogen were measured using a previously published method (20). Briefly, washed platelets (5 x 10⁸/ml) were simultaneously loaded with Oregon Green 488 BAPTA/AM-1 (5 μ M), Calcein red-orange/AM (10 μ M) and the membrane transporter inhibitor probenecid (2.5 μ M) for 45 min at 37°C. The dyed platelets were pelleted at 1,900 x g for 3 min, re-suspended at 1 x 10⁸/ml in Tyrode's albumin buffer, and allowed to adhere to fibrinogen-coated (100 μ g/ml) coverslips for 20 min. The increases in platelet and Ca²⁺-dependent fluorescence intensity upon adhesion were measured in the ranges 572–700 nm and 495–535 nm, respectively, by confocal laser scanning microscopy, and converted to intracellular Ca²⁺ levels (Leica TCS SP5).

Clot retraction

Blood drawn into sodium citrate (3.15 mg/ml) from the abdominal aorta of 2–3 mice was pooled, centrifuged at 250 x g for 1 min and platelet-rich plasma (PRP) was adjusted to 8 x 10⁸ platelets/ml with autologous platelet-poor plasma. PRP (500 μ l) was added to quartz cuvettes maintained at 37°C in the presence of 20 mM CaCl₂. The clots were allowed to retract for up to 80 min and photographed every 5 min. The extent of retraction was then quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Measurement of Akt phosphorylation

Platelets adhering to fibrinogen were incubated in lysis buffer (1:50 Triton X-100, 40 mM Tris (tris(hydroxymethyl)aminomethane), 10 mM Na₂VO₃, 100 mM NaF, 2 mM EDTA, 2 mM EGTA, 300 mM NaCl, pH 7.4) for 10 min at 4°C and centrifuged at 7,000 x g for 7 min. The supernatants were resolved by SDS-PAGE and transferred to PVDF membranes. The levels of total and phosphorylated Akt were determined by Western blotting using monoclonal antibodies as previously described (25).

Laser-induced mesenteric artery thrombosis model

Thrombosis was performed as previously described (26). Briefly, platelets were labelled by injection of DIOC₆ (5 μ l of a 100 μ M solution per g body weight), a membrane fluorescent dye, and a localised deep injury of a mesenteric arteriole was induced with a high intensity 440-nm-pulsed nitrogen dye laser applied for 30–40 seconds (s) (300–400 hits) with a Micropoint system (Photonic Instruments, Andor Technology, Belfast, UK). Thrombus formation was monitored (1 image/s) by fluorescence microscopy using a Sensicam charge-coupled device (CCD) camera (The Cooke Cor-

Table 1: Platelet count and surface expression of different glycoproteins in WT, β -arr1^{-/-} and β -arr2^{-/-} mice. Platelet counts are the mean \pm SEM for groups of at least 12 mice. Surface expression of the indicated glycoproteins was determined in whole blood by staining with selective antibodies followed by flow cytometry analysis. Platelets were gated by FSC/SSC characteristics (10,000 platelets per sample). Results are expressed as the geometric mean \pm SEM of the relative fluorescence intensity, in arbitrary units (three mice per group).

	WT	β -arr1 ^{-/-}	β -arr2 ^{-/-}
GPIIb/IIIa ($\alpha_{IIb}\beta_3$)	3.9 \pm 0.1	4.2 \pm 0.04	4.5 \pm 0.1
GPIIa (β_1)	0.43 \pm 0.02	0.56 \pm 0.09	0.46 \pm 0.06
GPIa (α_2)	0.73 \pm 0.03	0.68 \pm 0.07	0.69 \pm 0.01
GPVI	0.20 \pm 0.01	0.19 \pm 0.01	0.21 \pm 0.01
GPV	0.65 \pm 0.01	0.63 \pm 0.03	0.60 \pm 0.02
GPIX	1.47 \pm 0.06	1.44 \pm 0.01	1.49 \pm 0.03
GPIb α	1.74 \pm 0.07	1.65 \pm 0.06	1.64 \pm 0.01
Platelet count (x10 ⁶ /ml)	1,038 \pm 47	969 \pm 53	1,151 \pm 35

poration, Romulus, MI, USA), and images were analysed with the SlideBook software (Intelligent Imaging Innovations, Göttingen, Germany).

FeCl₃-induced carotid artery thrombosis model

DIOC₆ was injected into the jugular vein (5 μ l of a 100 μ M solution per g body weight) to label platelets and assist thrombus visualisation. The left common carotid artery was exposed and vascular injury was induced by topically applying over the adventitia for 2 min a Whatman #1 filter paper (1 x 0.5 mm) saturated with 0.2 μ l of a 75 mg/ml FeCl₃ saline solution (27). The artery was then rinsed with saline and thrombus formation was monitored in real time under a fluorescent microscope (Macrofluor, Leica Microsystems) using a 5x, 0.5 numerical aperture objective. Images (1 image/s) were acquired using a CCD camera (CoolSNAP HQ2, Photometrics, Roper Scientific, Evry, France) and analysed with Metamorph software version 7.6 (Molecular Devices, Roper Scientific).

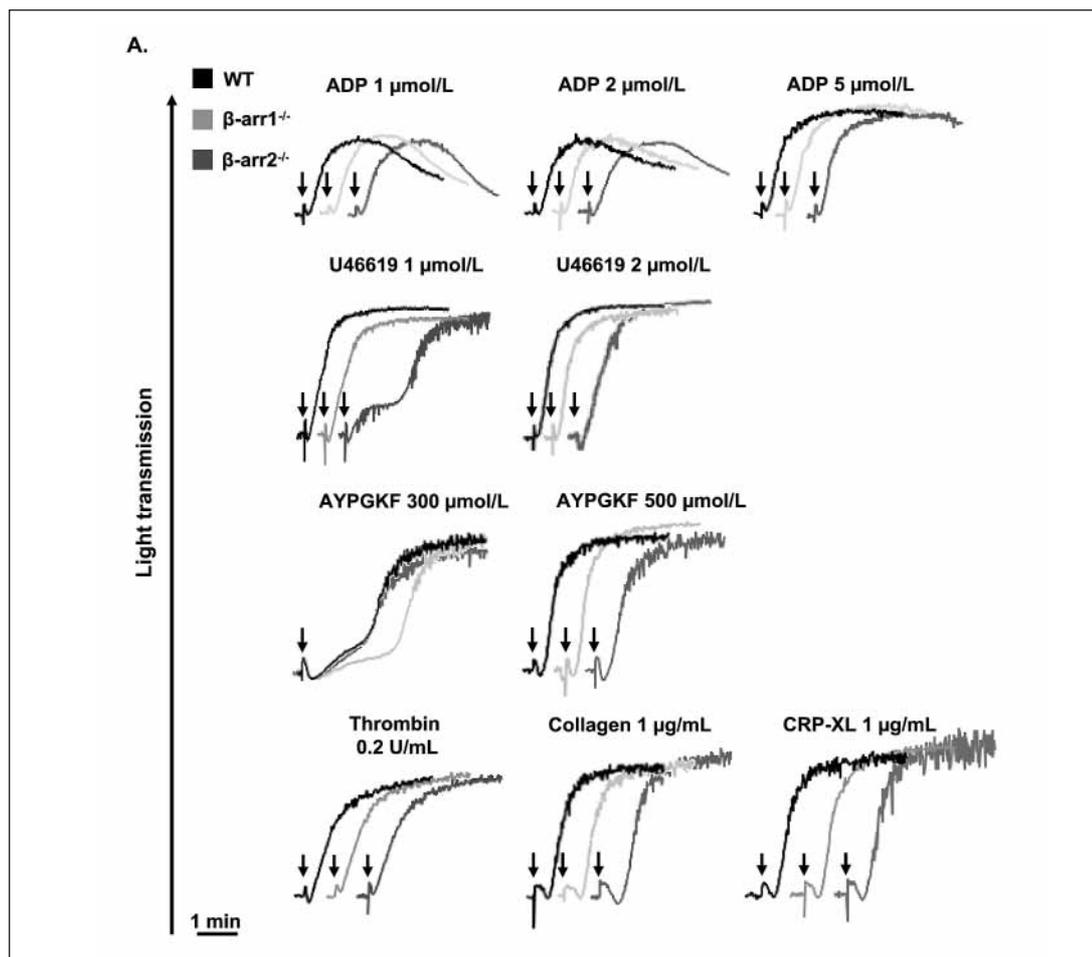
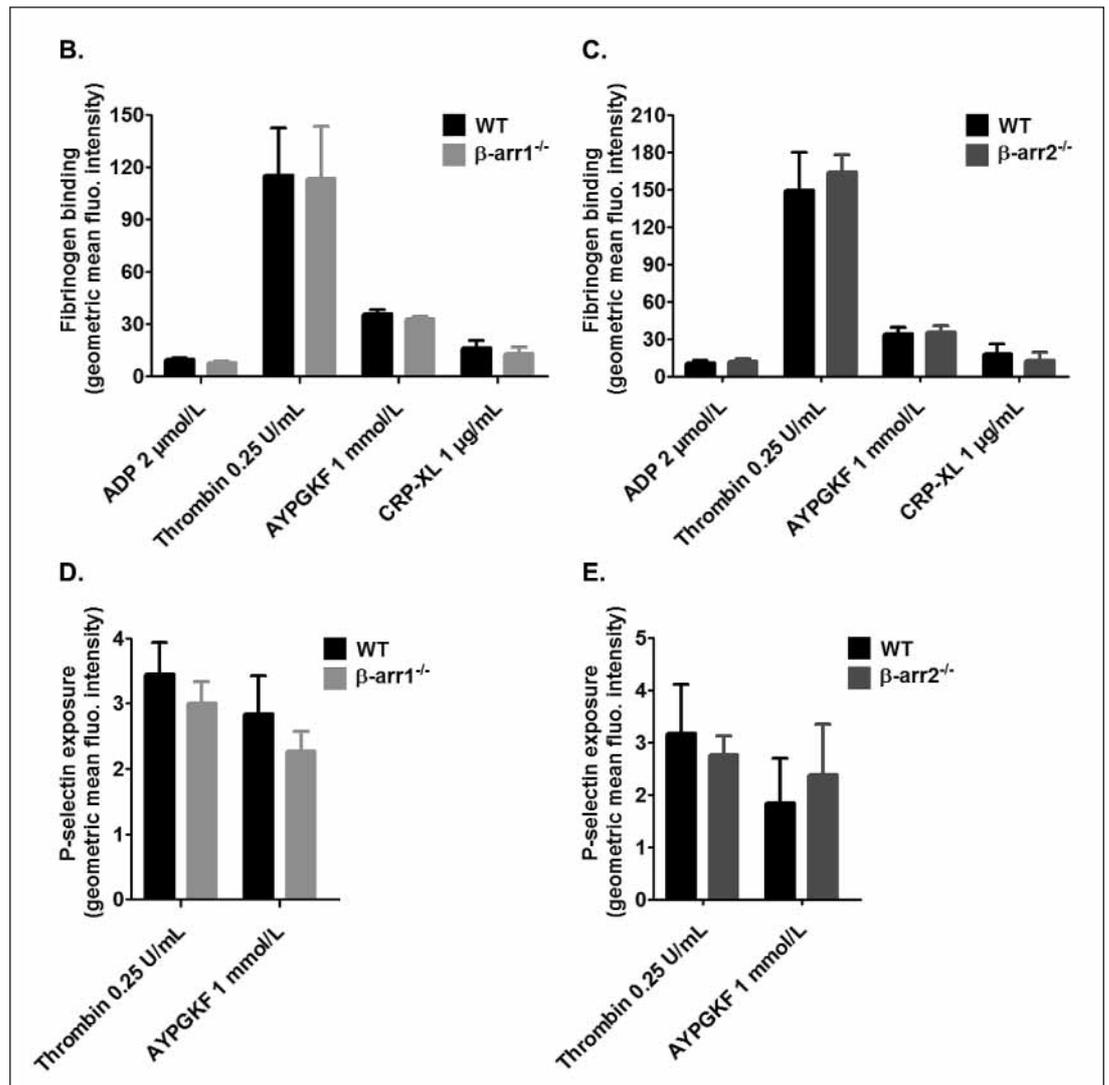


Figure 1: Role of β -arr1 and β -arr2 in platelet aggregation, soluble fibrinogen binding and P-selectin exposure. A) Washed platelets (2×10^8 /ml) from WT, β -arr1^{-/-} and β -arr2^{-/-} mice were aggregated by a range of agonists in the presence (ADP, U46619, AYPGKF, type I collagen and CRP-XL) or absence (thrombin) of fibrinogen (64 μ g/ml). Arrows indicate the point of agonist addition. Aggregation profiles are representative of four separate experiments.

Figure 1: B-E) WT, β -arr1^{-/-} (B, D) and β -arr2^{-/-} (C, E) washed platelets (5×10^7 /ml) were stimulated with various agonists in the presence of Alexa Fluor 488-fibrinogen (B, C) or a FITC-anti-P-selectin antibody (D, E). After 20 or 10 min, the samples were fixed and the extent of fibrinogen binding and P-selectin exposure was measured by flow cytometry. Results represent the geometric mean \pm SEM of the relative fluorescence intensity in arbitrary units, obtained in four separate experiments performed in duplicate (10,000 platelets analysed per experiment).



Bleeding time

The bleeding time was measured by transversally severing a 3-mm segment from the distal tail of 8- to 10-week-old isoflurane-anesthetised mice. The amputated tail was immediately immersed in 9 mg/ml isotonic saline at 37°C during 30 min and the time required for arrest of bleeding was recorded. The tube containing saline with blood was then homogenised and centrifuged at 550 \times g during 5 min. The supernatant was removed and 2 ml of lysis buffer (NH₄Cl 150 mM, KHCO₃ 1 mM, EDTA 0.1 mM, pH 7.2) was added to the pellet. After homogenisation, the optical density was read at 540 nm and compared to a standard curve to determine the volume of blood loss.

Statistical analyses

All values are reported as mean \pm standard error of the mean

(SEM) unless otherwise indicated. Data were compared by two-tailed Student's t-tests using Prism software (GraphPad, La Jolla, CA, USA) and differences were considered significant at $p < 0.05$.

Results

Platelet aggregation, soluble fibrinogen binding and P-selectin exposure in β -arr1- and β -arr2-deficient platelets

Deletion of β -arr1 or β -arr2 did not affect the platelet count and expression of the major surface glycoproteins (► Table 1). We examined the ability of β -arr1^{-/-} and β -arr2^{-/-} platelets to become activated after stimulation with a range of agonists. As shown in ► Figure 1A and ► Suppl. Figure 1 (available online at www.thrombosis-online.com), WT and knock-out platelets displayed equivalent aggregation profiles in response to various concentrations of

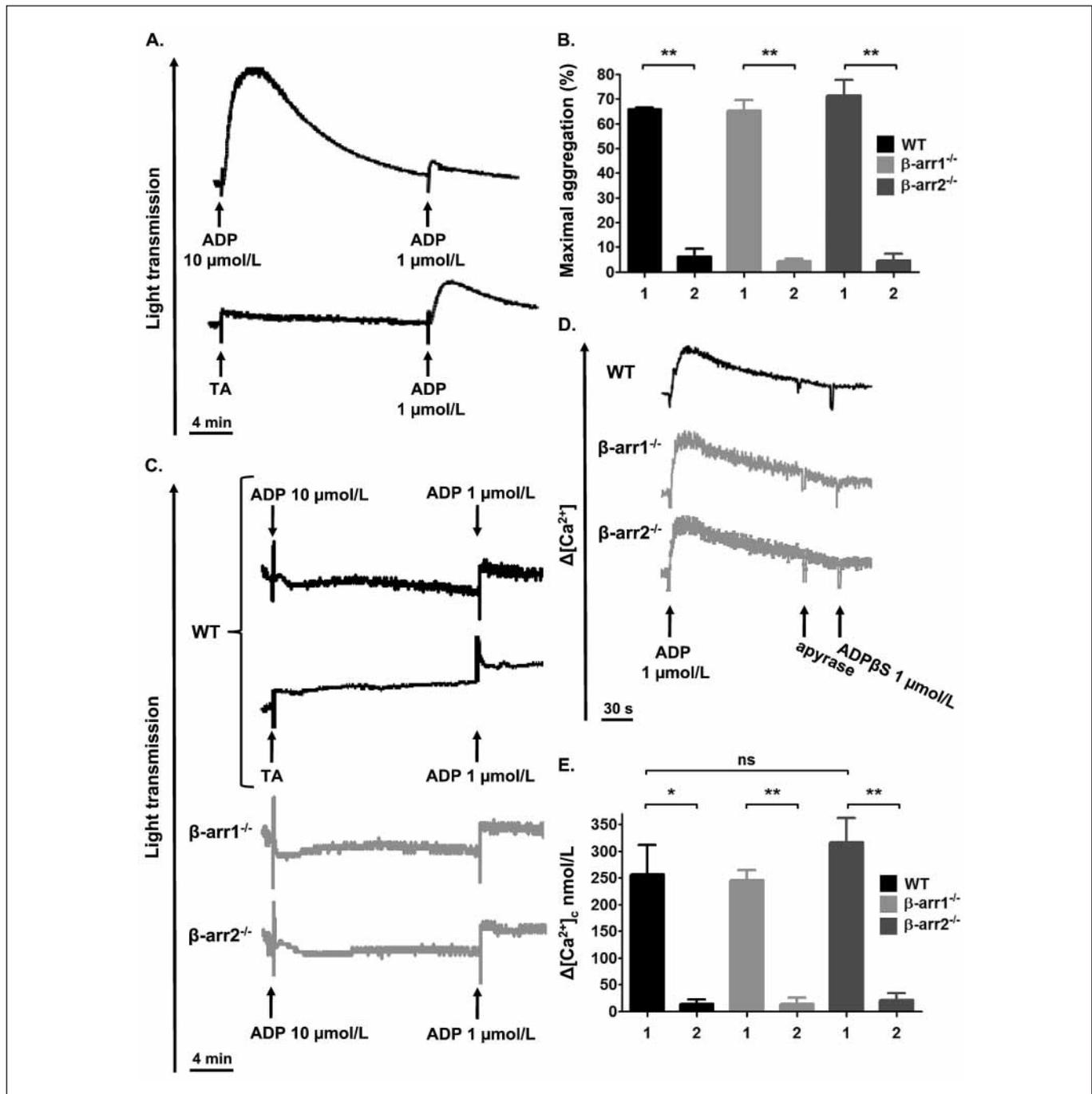
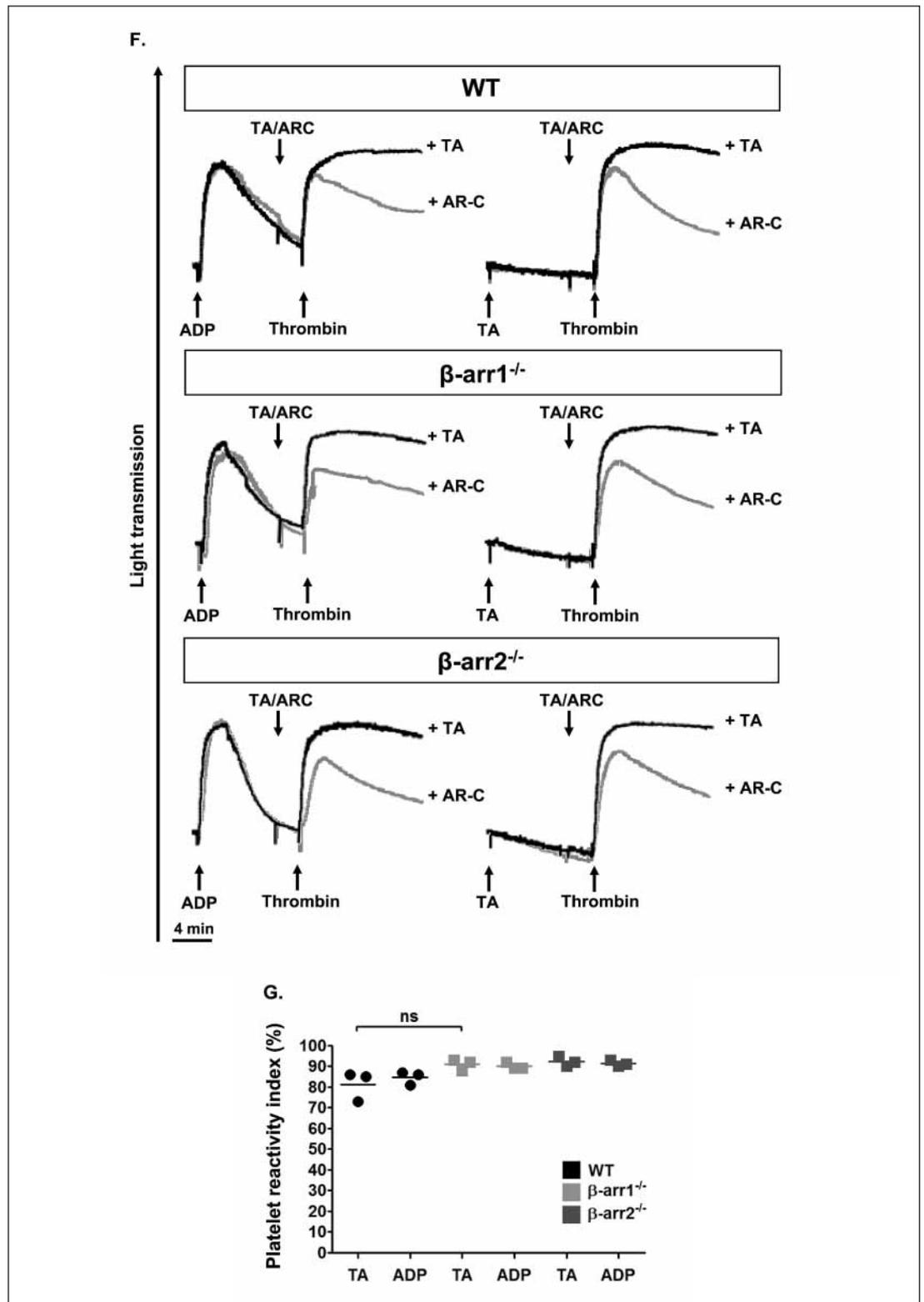


Figure 2: Role of β -arr1 and β -arr2 in P2Y receptors desensitisation. A) Top: WT platelets (2×10^8 /ml) were aggregated by $10 \mu\text{M}$ ADP in the presence of $64 \mu\text{g/ml}$ fibrinogen, followed by a second challenge with ADP ($1 \mu\text{M}$) after 20 min. Bottom: WT platelets were incubated with vehicle (Tyrode's albumin buffer, [TA]) and stimulated with $1 \mu\text{M}$ ADP after 20 min. B) Washed platelets (2×10^8 /ml) from WT, β -arr1 $^{-/-}$ and β -arr2 $^{-/-}$ mice were aggregated as in A. Bars represent the maximal amplitude of aggregation after addition of $10 \mu\text{M}$ ADP (1) and restimulation with $1 \mu\text{M}$ ADP (2). Data are the mean \pm SEM from three separate experiments. $^{**}p < 0.01$ C) Aggregation: WT, β -arr1 $^{-/-}$ and β -arr2 $^{-/-}$ platelets (2×10^8 /ml) pretreated with AR-C69931MX ($10 \mu\text{M}$) for 15 min were incubated with $10 \mu\text{M}$ ADP or TA in the presence of $64 \mu\text{g/ml}$ fibrinogen, followed by a second challenge with ADP ($1 \mu\text{M}$) after 20 min. D, E) Variations in Ca^{2+} levels were measured by spectrofluorimetry in fura-2/AM-loaded ($15 \mu\text{M}$) WT, β -arr1 $^{-/-}$ and β -arr2 $^{-/-}$ platelets (2×10^8 /ml) stimulated with $1 \mu\text{M}$ ADP. After 2 min, apyrase (0.2 U/ml) was added for 30 s and the cells were restimulated with $1 \mu\text{M}$ ADP β S. Representative profiles are shown (D). Bars represent the maximal increase in cytosolic Ca^{2+} after addition of ADP (1) and restimulation with ADP β S (2). Data are the mean \pm SEM from three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ (E).

Figure 2: F) Black curves: aggregation of WT, β -arr1^{-/-} and β -arr2^{-/-} platelets (2×10^8 /ml) in response to 0.05 U/ml thrombin, 13 min after a first addition of either 10 μ M ADP or TA in the presence of 64 μ g/ml fibrinogen. Grey curves: WT, β -arr1^{-/-} and β -arr2^{-/-} platelets were aggregated or not by ADP as above, followed by addition of AR-C69931MX (10 μ M), 3 min before stimulation with thrombin. The profiles are representative of at least three independent experiments. G) Hirudinized (100 U/ml) whole blood from WT, β -arr1^{-/-} and β -arr2^{-/-} mice was pretreated or not with 10 μ M ADP for 20 min, and the ability of P2Y₁₂ to inhibit PGE₁-induced VASP phosphorylation was determined by ELISA. Distribution of the platelet reactivity index in three different mice is shown. The mean value is indicated with horizontal bars.



ADP, thrombin, type I fibrillar collagen and CRP-XL, a glycoprotein VI-selective ligand. In addition, serotonin-induced shape change and the ability of either adrenaline or serotonin to potentiate aggregation in response to 1 μ M ADP were unchanged (data not shown). By contrast, β -arr2^{-/-} but not β -arr1^{-/-} platelet aggregation was delayed and exhibited a biphasic appearance restricted to the lowest concentration (1 μ M) of the thromboxane A₂ (TxA₂) analog U46619, indicating minor participation of β -arr2 in TP receptor signalling (► Fig. 1A). A similar defect was observed in β -arr1^{-/-} platelets using subthreshold concentrations (300 μ M) of AYPGKF, a PAR4 agonist peptide (► Fig. 1A). In parallel, we observed that soluble fibrinogen binding induced by ADP (2 μ M), thrombin (0.25 U/ml), AYPGKF (1 mM) and CRP-XL (1 μ g/ml) was normal in β -arr1- and β -arr2-null compared to control platelets (► Fig. 1B, C). Finally, no defective P-selectin exposure, a marker of granule secretion, was found following thrombin (0.25 U/ml) and AYPGKF (1 mM) stimulation (► Fig. 1D, E). Altogether, these results suggested that β -arr1 and β -arr2 play no major role in the signalling machinery leading to activation of platelets by a wide range of agonists.

β -arr1 and β -arr2 are not critical for P2Y₁ and P2Y₁₂ receptors desensitisation

WT platelets activated by ADP become refractory to restimulation, a phenomenon attributed to P2Y receptor desensitisation (3–5), and illustrated by absence of shape change and reduced aggregation in response to a second ADP challenge (► Fig. 2A and ► Suppl. Fig. 2A and C, available online at www.thrombosis-online.com). A similar refractoriness was observed in the knock-out platelets, suggesting that neither β -arr1 nor β -arr2 play a critical role in P2Y receptor desensitisation (► Fig. 2B and ► Suppl. Fig. 2B and D, available online at www.thrombosis-online.com). To investigate this in more details, we studied P2Y₁ desensitisation in platelets pretreated with 10 μ M of the P2Y₁₂ blocker AR-C69931MX. As expected, addition of ADP (10 μ M) to WT platelets caused a shape change without aggregation (► Fig. 2C) (2, 28). Upon restimulation with 1 μ M ADP, no response was observed, reflecting desensitisation of P2Y₁ (► Fig. 2C). Platelets lacking either β -arr1 or β -arr2 exhibited similar responses, indicating that these proteins do not contribute to P2Y₁ desensitisation (► Fig. 2C). These results were confirmed by looking at ADP-induced Ca²⁺ mobilisation, which is mediated by P2Y₁ (3, 24). Similarly as in the WT, β -arr1^{-/-} and β -arr2^{-/-} platelets stimulated with ADP followed by its removal with apyrase, were unable to exhibit Ca²⁺ rises in response to a second challenge with the metabolically stable P2Y receptor agonist ADP β S (► Fig. 2D, E). Although a substantial fraction of P2Y₁₂ is rapidly internalised upon stimulation (4), whether this phenomenon is accompanied by a functional desensitisation is still a matter of debate (3–5). Therefore, we re-examined P2Y₁₂ desensitisation and the potential role of β -arrestins in this process. We used the capacity of P2Y₁₂ to potentiate thrombin-induced platelet aggregation (► Fig. 2F, grey curves) as a marker of its sensi-

sation state (2, 4). As shown in ► Figure 2F, the rate and amplitude of aggregation in response to 0.05 U/ml thrombin were similar in WT platelets pre-stimulated or not with 10 μ M ADP, suggesting no functional desensitisation of P2Y₁₂. Equivalent aggregation profiles were obtained when these experiments were performed in β -arr1^{-/-} and β -arr2^{-/-} platelets (► Fig. 2F). To get a more direct measurement of P2Y₁₂ signalling, we analysed intraplatelet VASP phosphorylation, a method used to monitor the efficacy of anti-P2Y₁₂ therapy (21, 22). ELISA showed that in WT platelets pretreated with 10 μ M ADP, P2Y₁₂ was still able to profoundly inhibit PGE₁-induced VASP phosphorylation, reflecting once again absence of desensitisation (► Fig. 2G). Similar results were found in β -arr1^{-/-} and β -arr2^{-/-} platelets, confirming that these proteins are unlikely to contribute to P2Y₁₂ desensitisation (► Fig. 2G).

β -arr1 participates in integrin $\alpha_{IIb}\beta_3$ -mediated filopodia extension

We next evaluated the impact of a β -arrestin deficiency on platelet activation mediated by adhesion receptors. As compared to the WT, the ability of β -arr1^{-/-} and β -arr2^{-/-} platelets to adhere to von Willebrand factor or collagen and undergo shape change, which reflects activation of the cells, was unchanged (data not shown). In contrast, following adhesion to fibrinogen, the proportion of β -arr1^{-/-} but not β -arr2^{-/-} platelets extending filopodia was reduced by 37 \pm 5% (n = 3, p < 0.0001) (► Fig. 3A, B), while the number of adherent cells remained unaltered (data not shown). As these experiments were performed in the presence of ADP and TxA₂ blockers, this would suggest abnormal cytoskeletal reorganisation through integrin $\alpha_{IIb}\beta_3$, the main platelet receptor for fibrinogen.

β -arr1 functions downstream of Src family kinases and regulates Akt phosphorylation mediated by $\alpha_{IIb}\beta_3$

Integrin $\alpha_{IIb}\beta_3$ signalling has been reported to be initiated by Src family kinases such as c-Src and Fyn (9). To explore whether β -arr1 is a target of Src kinases, washed platelets were preincubated with PP1 and PP2, two pan-inhibitors of this family of proteins, prior to adhesion to fibrinogen. As shown in ► Figure 3B, PP1 reduced the proportion of WT (31 \pm 6%) and β -arr1^{-/-} (25 \pm 5%) platelets extending filopodia to a similar extent (n = 3, p = 0.44). Similar results were obtained with PP2 (30 \pm 4% of WT platelets with filopodia versus 33 \pm 3% in mutants, n = 4, p = 0.50), suggesting that β -arr1 is located downstream of Src kinases in $\alpha_{IIb}\beta_3$ signalling. Src kinases have been proposed to modulate various signalling events including Ca²⁺ mobilisation (9). Upon adhesion to fibrinogen, equivalent Ca²⁺ increases were observed in WT and mutant platelets, indicating that β -arr1 does not participate in this process (► Fig. 3C). In addition, β -arr1^{-/-} platelets exhibited normal fibrin clot retraction, another event in which $\alpha_{IIb}\beta_3$ and Src kinases play a

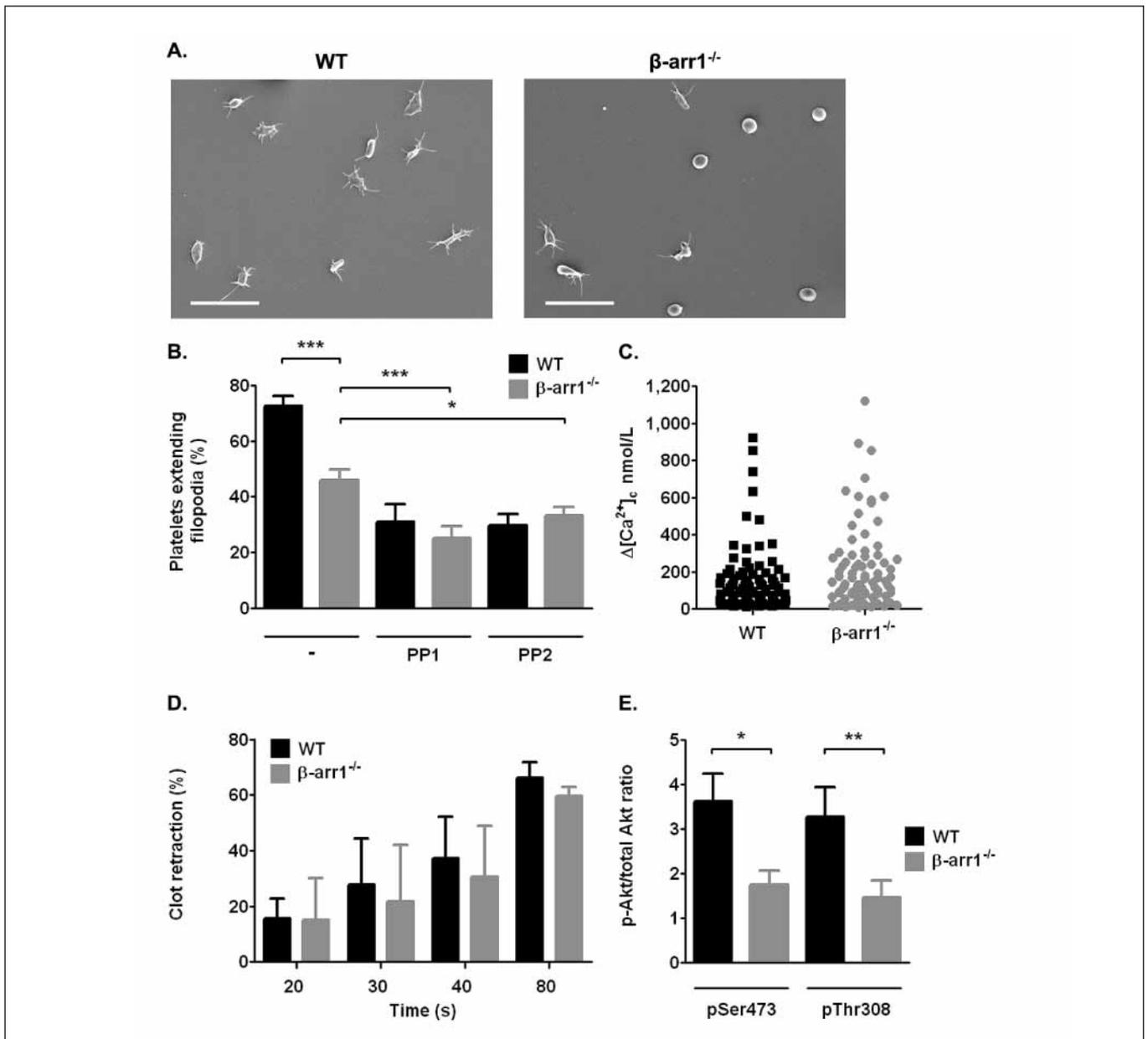


Figure 3: Role of β -arr1 in $\alpha_{IIb}\beta_3$ signalling. A, B) WT and β -arr1^{-/-} washed platelets were pretreated for 15 min with AR-C69931MX (10 μ M), MRS2179 (100 μ M) and indomethacin (100 μ M) alone or in the presence of PP1 or PP2 (10 μ M), and applied for 20 min to fibrinogen-coated (100 μ g/ml) coverslips. Adherent platelets were fixed and examined by SEM. Representative images of WT and β -arr1^{-/-} platelets are shown. Scale bars, 10 μ m (A). The adherent platelets were also stained with TRITC-phalloidin and the mean percentage of cells with filopodial extensions was determined by epifluorescence microscopy in eight random fields in at least three independent experiments (B). C) WT and β -arr1^{-/-} washed platelets loaded with Ca^{2+} and morphological dyes were allowed to adhere to fibrinogen-coated (100 μ g/ml) coverslips as in A, B. Changes in fluorescence were monitored for 20 min by confocal microscopy, and cytosolic Ca^{2+} concentrations were determined. The dot plot dis-

tribution of the relative maximal increase in individual adherent platelets is shown. D) Citrated platelet-rich plasma from WT and β -arr1^{-/-} mice was added to quartz cuvettes maintained at 37°C in the presence of 20 mM CaCl_2 and clot retraction, visible as a consolidated morphology and transparent clot liquor, was photographed every 5 min for 80 min. Bars represent the mean percentage reduction of the initial clot surface area at different time points, determined by two-dimensional image analysis. E, WT and β -arr1^{-/-} washed platelets were allowed to adhere to fibrinogen as in A, B, lysed, centrifuged and the supernatants were resolved by SDS-PAGE followed by Western blotting. Bars represent the relative ratio of phosphorylated to total Akt, calculated by densitometry analysis. Data represent the mean \pm SEM of three or four independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

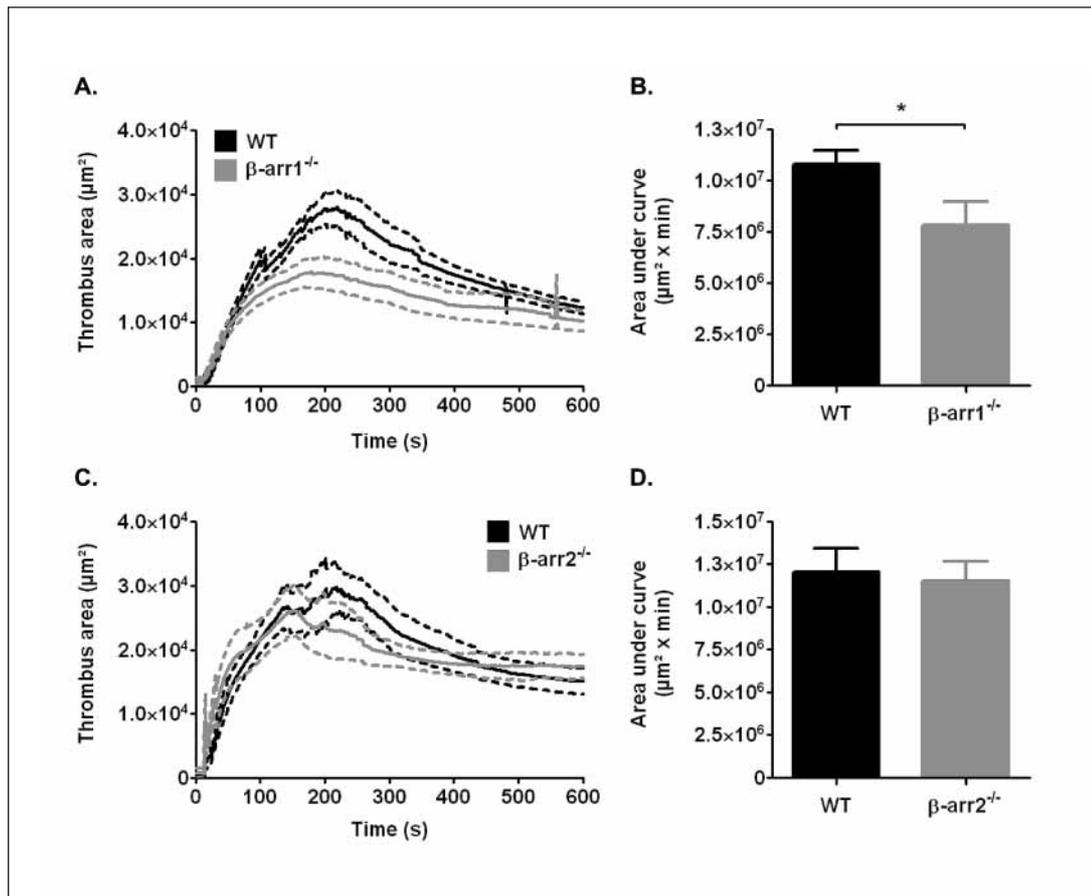


Figure 4: β -arr1^{-/-} but not β -arr2^{-/-} mice exhibit a decreased thrombotic response after laser injury of the mesenteric artery. A localised deep injury of an exposed mesenteric arteriole of WT, β -arr1^{-/-} (A, B) and β -arr2^{-/-} (C, D) mice was generated with a high intensity 440-nm-pulsed nitrogen dye laser applied for 30 s, and thrombus formation was monitored by fluorescence microscopy (26). A, C) Time-course of the thrombus growth, represented by its mean surface area. Dotted curves represent the SEM. B, D) Bar graphs represent the area under the curves shown in A and C, respectively. Data are from 12 vessels in five mice (A, B), three vessels in three β -arr2^{-/-} mice or five vessels in three WT mice (C, D). * p <0.05.

critical role (► Fig. 3D). The downstream effectors of Src kinases also include Akt (10, 11), which has been proposed to be targeted by β -arr1 in various cellular systems (1, 16, 29). To determine whether β -arr1 participates in $\alpha_{IIb}\beta_3$ signalling by regulating Akt, WT and β -arr1^{-/-} washed platelets were allowed to adhere to fibrinogen for 20 min and then lysed, and the level of Akt phosphorylation was measured by Western blotting. Interestingly, β -arr1^{-/-} platelets presented a decrease of $52 \pm 9\%$ ($n = 4$, $p < 0.05$) and $56 \pm 12\%$ ($n = 4$, $p < 0.01$) in the phosphorylation of Ser473 and Thr308, respectively (► Fig. 3E). Overall, these results suggested that upon binding of fibrinogen to $\alpha_{IIb}\beta_3$, β -arr1 regulates filopodia emission through a process involving Src kinases and increased Akt phosphorylation.

β -arr1^{-/-} but not β -arr2^{-/-} mice are protected in two models of arterial thrombosis

The role of β -arr1 and β -arr2 in arterial thrombosis was investigated using two *in vivo* models. When mesenteric arterioles were injured with a laser beam, a deficiency in β -arr1 led to a $28 \pm 10\%$ ($n = 12$ vessels in five mice, $p < 0.05$) reduction in total thrombus surface area as compared to the WT (► Fig. 4A, B). This result is in agreement with a recent study revealing that β -arr1^{-/-} mice are less

sensitive to FeCl₃-induced carotid thrombosis (16). Using a similar model, we observed a delay in thrombus growth with a thrombus area which was significantly reduced at early (350 s: $32 \pm 9\%$ reduction, $n = 6$, $p < 0.05$) but not later times (>480 s) (► Fig. 5A-C). In contrast, thrombus formation was comparable between WT and β -arr2 knock-outs (► Figs. 4C and D, 5D and E), pointing to a modest role of β -arr1 and no role of β -arr2 in this process.

β -arr1-deficient mice exhibit normal tail bleeding

To establish whether the decreased thrombosis observed in β -arr1-deficient mice is mirrored by defective haemostasis, standardised tail-bleeding experiments were performed. The bleeding time was equivalent in β -arr1-null (149 ± 33 s, $n = 12$) and WT (137 ± 26 s, $n = 12$) mice (► Fig. 6A), as were the number of re-bleeds (► Fig. 6B) and total bleeding time (► Fig. 6C) over 30 min. Despite an increased blood loss in a few mutant mice, the mean volume was not significantly different in WT (► Fig. 6D). The bleeding phenotype was also unaltered in β -arr2^{-/-} mice (► Fig. 6A-D). Overall, these results suggested a minor contribution of β -arr1 and β -arr2 to primary haemostasis.

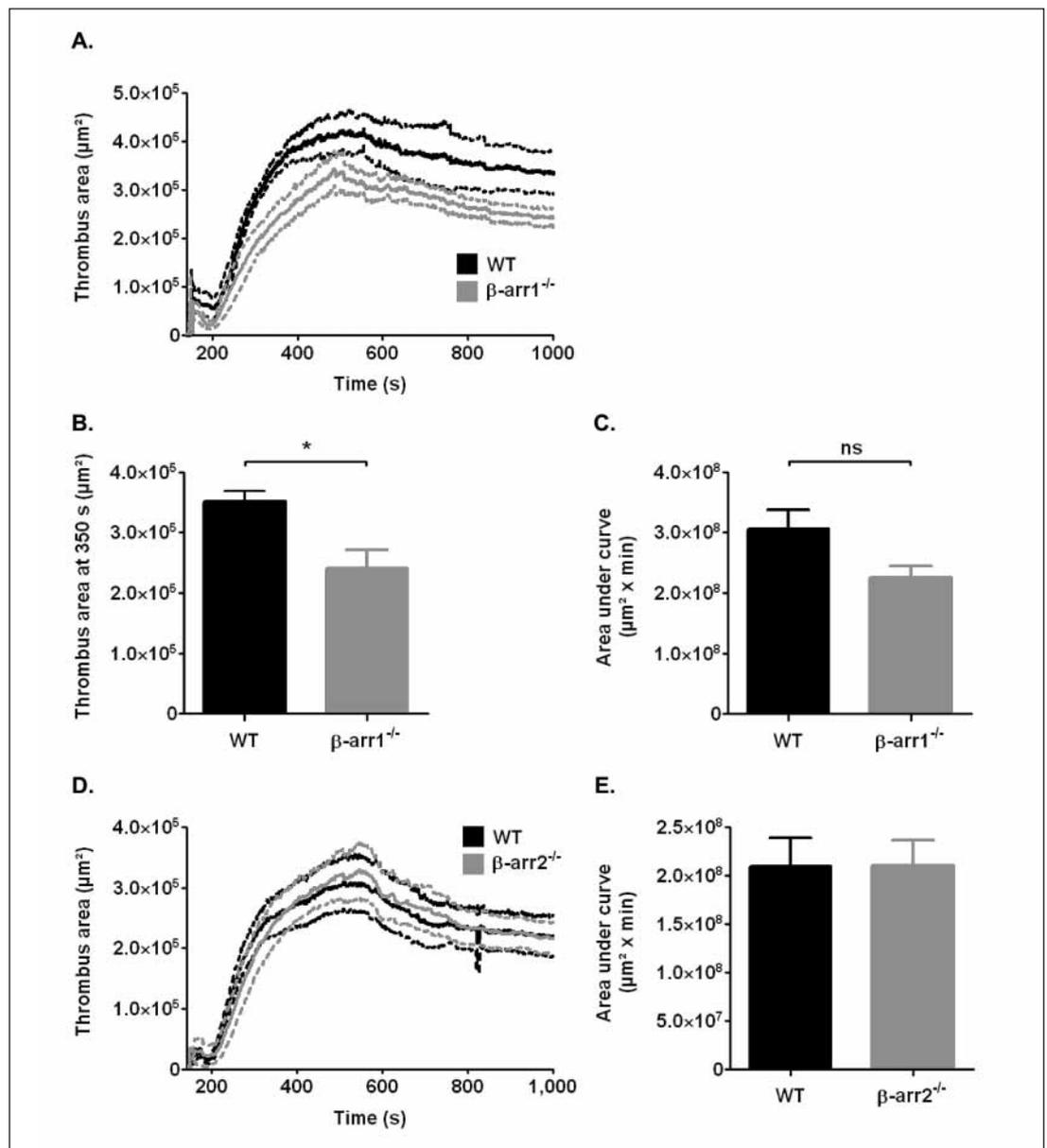


Figure 5: β -arr1^{-/-} but not β -arr2^{-/-} mice exhibit a delayed thrombotic response after FeCl₃ injury of the carotid artery. A 75 mg/ml FeCl₃ solution was applied for 2 min to the exposed carotid artery of WT, β -arr1^{-/-} (A-C) and β -arr2^{-/-} (D, E) mice, and thrombus growth was recorded by video microscopy (27). A, D) Time-course of the thrombus growth, represented by its mean surface area. Dotted curves represent the SEM. B) Mean thrombus area after 350 s in WT and β -arr1^{-/-} mice. C, E) Bar graphs represent the area under the curves shown in A and D, respectively. Data are from six (A-C) or seven (D, E) mice. * $p < 0.05$.

Discussion

In the present study, we addressed the question of the role of β -arrestins in platelet functions. Neither β -arr1 nor β -arr2 appeared to critically regulate inside-out signals induced by a wide range of agonists. We also provide evidence that P2Y₁ receptor desensitisation and platelet refractoriness to ADP still occur in β -arr1^{-/-} and β -arr2^{-/-} platelets. Two *in vivo* models supported a modest role of β -arr1 and no role of β -arr2 in arterial thrombosis. The lack of β -arr1 or β -arr2 expression was not critical for haemostasis since the tail bleeding phenotype was unchanged. Our results identified a novel function for β -arr1 through the regulation of integrin $\alpha_{\text{IIb}}\beta_3$ signalling. Upon fibrinogen binding to $\alpha_{\text{IIb}}\beta_3$, β -arr1 participates in cytoskeletal reorganisation leading to filopodia extension by a process involving Src kinases and probably upregulation of

Akt activity.

In agreement with our previous studies (3, 4), we observed that mouse platelets activated by ADP were unable to fully re-aggregate in response to a second challenge, as a result of P2Y₁ but not P2Y₁₂ receptor desensitisation. Most of GPCRs desensitise through phosphorylation by GPCR kinases (GRKs), which promotes β -arrestin recruitment thereby precluding G protein coupling and ultimately leading to receptor internalisation (1). Although β -arr1 and β -arr2 were reported to interact with activated P2Y₁ in transfected HEK-293 cells (6, 7), our study shows that the deficiency in either of these proteins did not prevent platelet P2Y₁ desensitisation, as judged by the absence of re-aggregation and Ca²⁺ elevation in response to a second ADP challenge. However, one cannot exclude a compensatory mechanism between the two isoforms. This hypothesis is supported by the fact that in mouse embryonic fibrob-

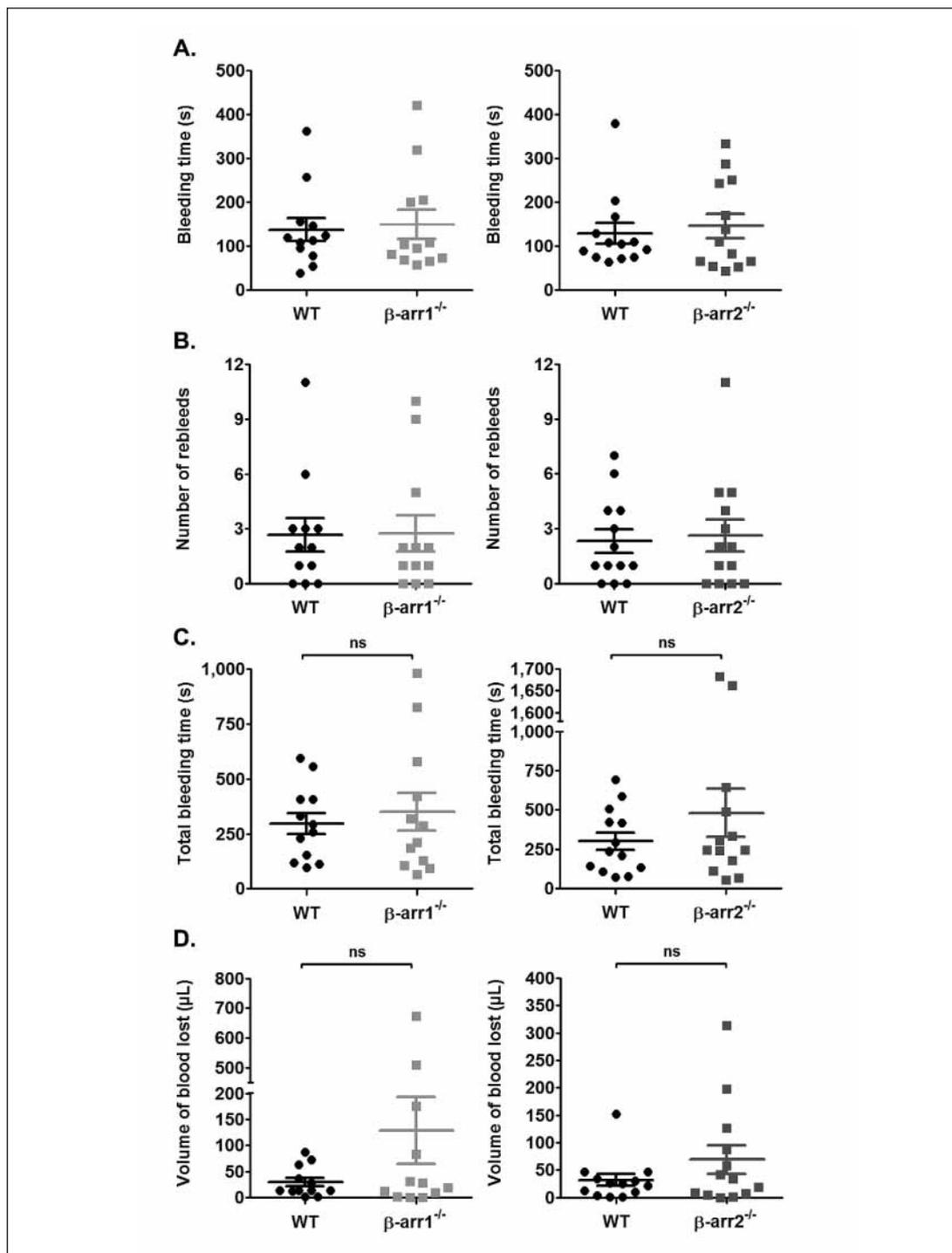


Figure 6: β -arr1^{-/-} and β -arr2^{-/-} mice present normal tail bleeding. A 3-mm segment from the distal tail of 8- to 10-week-old WT, β -arr1^{-/-} and β -arr2^{-/-} mice was amputated and the time required for initial arrest of bleeding was measured (A). The rebleeding occurrences (B), total bleeding time (C) and volume of blood lost (D) were then recorded over a 30-min period. Symbols represent individual mice (n = 12 or 13) and the mean \pm SEM is indicated with horizontal bars.

lasts ablation of both β -arr1 and β -arr2 was required to inhibit desensitisation of the angiotensin II type 1A receptor (30). Since double β -arr1/ β -arr2 deficiency is embryonically lethal, tissue-specific knock-outs would be needed to definitely resolve this question. Alternatively, P2Y₁ phosphorylation on its own may be sufficient to induce desensitisation without any need for a β -arrestin. Such a mechanism has been proposed in transfected 1321N1 cells in which P2Y₁ phosphorylation and internalisation did not

require GRKs but protein kinase C (5, 8). Concerning P2Y₁₂, we previously demonstrated that even though it was rapidly and transiently internalised upon stimulation, no functional desensitisation was objectivated (3, 4). In agreement with these observations, the present study did not highlight a functional desensitisation of P2Y₁₂ in mouse platelets. These results suggested that β -arrestins are not key players of P2Y₁₂ desensitisation. However, recent identification of a patient carrying a mutation in P2Y₁₂ gene

suggested that β -arrestins may bind a 4-amino acid motif at the extreme C-terminus of P2Y₁₂, thereby promoting its internalisation and subsequent recycling (31).

Arrestins also act as adaptors, allowing for the assembly of multi-protein complexes which transduce the signals of a variety of receptors in many cell types (1). We provided evidence that β -arr1 and β -arr2 were not critical for the activation of platelets by a large series of agonists including ADP. This is in accordance with a recent report by Li et al. (16) showing normal ADP-induced Akt phosphorylation in β -arr1^{-/-} platelets. These results appear surprising since β -arr1 and β -arr2 were proposed to bind and potentially regulate both P2Y₁ and P2Y₁₂ (6–8), which are indispensable for normal platelet response to ADP (2). As suggested above, a functional redundancy between β -arr1 and β -arr2 may help maintain normal P2Y receptor function in the knock-out platelets. According to Li et al. (16), β -arr1 modulates PAR4 signalling, as evidenced by a decrease in Alexa Fluor 488-labelled fibrinogen binding upon stimulation of β -arr1^{-/-} platelets with 50 to 100 μ M of the selective agonist AYPGKF. Using three different assays, we observed no defect in β -arr1^{-/-} platelet activation in response to the lowest concentrations of AYPGKF sustaining maximal effect. However, when stimulated with a subthreshold concentration (300 μ M), β -arr1^{-/-} platelets exhibited a slight delay in aggregation relative to control. Lower doses, as those used by Li et al. (16), could not be tested since they induced no functional response in any of the assays we utilised. The reason for this is unclear, but is probably related to differences in the platelet washing procedure causing various levels of sensitivity to agonists. Overall, our results are in agreement with those of Li et al. (16), suggesting that β -arr1 participates in PAR4 signalling, but its role could be limited since the defect was restricted to stimulations with low concentrations of the agonist.

Besides GPCRs, β -arrestins bind to other classes of surface receptors specifically single-membrane-spanning receptors for transforming growth factor- β , insulin-like growth factor 1 and low density lipoprotein (1). We propose a novel function of β -arr1 as a positive regulator of integrin $\alpha_{IIb}\beta_3$ outside-in signalling, as indicated by decreased filopodia emission and Akt phosphorylation in β -arr1^{-/-} platelets adhering to fibrinogen. This is in agreement with the role of Akt in regulating cytoskeletal reorganisation which has been revealed in Akt-1-deficient mice (32). Whether β -arr1 and integrin $\alpha_{IIb}\beta_3$ physically interact is unknown. β -arr1 directly binds to specific phosphorylated serine and threonine residues of agonist-occupied GPCRs, thereby competing with G proteins (1). Since the β_3 integrin subunit has been shown to bind the G protein α_{13} subtype (33), a similar mechanism might occur for $\alpha_{IIb}\beta_3$. However, our studies on platelet lysates using various types of detergents failed to co-precipitate β -arr1 and β_3 (data not shown). Alternatively, since we provided evidence that β -arr1 is located downstream of Src family kinases, which are known to constitutively bind the cytoplasmic tail of β_3 and initiate signal transduction (9), one may speculate that Src kinases recruit β -arr1 to the vicinity of $\alpha_{IIb}\beta_3$. β -arr1 would in turn facilitate the formation of a signalling complex leading to Akt phosphorylation and its subsequent activation. This is supported by the fact that Src kinases and β -arr1

What is known about this topic?

- β -arrestin-1 (β -arr1) and β -arrestin-2 (β -arr2) are important regulators of G protein-coupled receptors desensitisation and signalling.
- P2Y₁, a G protein-coupled ADP receptor known to desensitise, can interact with both β -arr1 and β -arr2.
- A contribution of β -arr1 in PAR4-mediated platelet activation and in arterial thrombosis was recently revealed.

What does this paper add?

- β -arr1 and β -arr2 do not regulate agonist-induced platelet activation or P2Y receptors desensitisation.
- β -arr1^{-/-} but not β -arr2^{-/-} mice are less sensitive to FeCl₃- and laser-induced thrombosis but exhibit normal tail bleeding.
- β -arr1 plays a role in integrin $\alpha_{IIb}\beta_3$ -mediated filopodia extension by a process involving Src family kinases and probably Akt.

have been reported to associate downstream of PAR1 and PAR4, resulting in increased PI3K activity and Akt phosphorylation (16, 29). Although $\alpha_{IIb}\beta_3$ -mediated activation of Akt is known to depend upon phosphatidylinositol 3,4-bisphosphate generated by PI3K (11), whether β -arr1 also regulates PI3K function downstream of $\alpha_{IIb}\beta_3$ remains to be established. Recently, the small GTPase Cdc42 was proposed to participate in Akt phosphorylation and $\alpha_{IIb}\beta_3$ -dependent filopodia formation in mouse platelets, suggesting that it could be involved in β -arr1-mediated responses (34).

Whether the modest decrease in thrombus formation in β -arr1^{-/-} mice could arise from reduced signalling through $\alpha_{IIb}\beta_3$ is still speculative at this point. However, there are precedents for diminished thrombosis in relation with abnormal $\alpha_{IIb}\beta_3$ outside-in signalling, notably DiYF mice, which have a mutation on Tyr747/Tyr759 of β_3 (12, 13). In addition, two mouse strains lacking either tetraspanin TSSC6 or the adaptor Lnk and exhibiting defective signalling through $\alpha_{IIb}\beta_3$ have also been described to be protected against experimental thrombosis (14, 15). Interestingly, while β -arr1 deficiency had no impact on the bleeding phenotype, DiYF, TSSC6^{-/-} as well as Lnk^{-/-} mice displayed a pronounced tendency to rebleed. One notable difference which could explain these differences is that clot retraction, which is important for thrombus stabilisation, occurred normally in β -arr1^{-/-} mice while it was delayed and less effective in DiYF, TSSC6^{-/-} and Lnk^{-/-} mice.

In conclusion, despite their well-recognised role in GPCR regulation, β -arr1 and β -arr2 were not critical in agonist-induced platelet activation and P2Y receptors desensitisation. Studies of mice genetically ablated for β -arr1 support a modest role of this protein in promoting thrombosis, at least in part through its participation in platelet integrin $\alpha_{IIb}\beta_3$ signalling, while not affecting haemostasis. Given the ubiquitous expression of β -arrestins and integrins, it would be interesting to determine whether β -arrestins regulate integrin function in other cell types.

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Conflict of interest

None declared.

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