Arrestin Scaffolds NHERF1 to the P2Y\textsubscript{12} Receptor to Regulate Receptor Internalization*

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Shaista P. Nisar\textsuperscript{1}, Margaret Cunningham\textsuperscript{1}, Kunal Saxena, Robert J. Pope, Eamonn Kelly, and Stuart J. Mundell\textsuperscript{2}

From the School of Physiology and Pharmacology, Medical Sciences Building, University of Bristol, Briston BS8 1TD, United Kingdom

Background: The PDZ-binding motif of the P2Y\textsubscript{12} receptor regulates correct receptor traffic in human platelets.

Results: The PDZ-binding protein NHERF1 binds to the P2Y\textsubscript{12} receptor to promote agonist-dependent internalization.

Conclusion: Arrestin scaffolds NHERF1 to the P2Y\textsubscript{12} receptor to facilitate effective NHERF1-dependent receptor internalization.

Significance: A novel model of arrestin-dependent GPCR internalization.

We have recently shown in a patient with mild bleeding that the PDZ-binding motif of the platelet G protein-coupled P2Y\textsubscript{12} receptor (P2Y\textsubscript{12}R) is required for effective receptor traffic in human platelets. In this study we show for the first time that the PDZ motif-binding protein NHERF1 exerts a major role in potentiating G protein-coupled receptor (GPCR) internalization. NHERF1 interacts with the C-tail of the P2Y\textsubscript{12}R and unlike many other GPCRs, NHERF1 interaction is required for effective P2Y\textsubscript{12}R internalization. In vitro and prior to agonist stimulation P2Y\textsubscript{12}R/NHERF1 interaction requires the intact PDZ binding motif of this receptor. Interestingly on receptor stimulation NHERF1 no longer interacts directly with the receptor but instead binds to the receptor via the endocytic scaffolding protein arrestin. These findings suggest a novel model by which arrestin can serve as an adaptor to promote NHERF1 interaction with a GPCR to facilitate effective NHERF1-dependent receptor internalization.

Agonist-induced internalization and subsequent intracellular traffic of many G protein-coupled receptors (GPCRs)\textsuperscript{3} mediates important functions for the cell, tuning cell responsiveness to ligands over both short term and long term periods and regulating receptor coupling to signal transduction pathways (1, 2). Many GPCRs possess specific cytoplasmic sequences required for the efficient trafficking of the receptor into either lysosomal or recycling pathways (3). Among these motifs, postsynaptic density 95/disc large/zonula occludens-1 (PDZ) binding sequences that interact with PDZ domain-containing proteins are frequently used to regulate protein traffic (4). PDZ ligands are short amino acid sequences usually found at the very extreme C terminus of various transmembrane proteins including ion channels, transporters, and GPCRs. Based on sequence analysis, more than 50 GPCRs contain a PDZ ligand consensus sequence in their cytoplasmic C termini; however, for the majority of these GPCRs the functional significance of this motif remains unexplored.

For those GPCRs that have been investigated, in particular the \(\beta\textsubscript{2}\)-adrenoreceptor (\(\beta\textsubscript{2}\)AR) and the parathyroid hormone receptor type 1 (PTH1R), studies have revealed the importance of the PDZ ligand for regulation of GPCR signaling and trafficking. PDZ ligands interact with PDZ domain containing proteins, the most widely studied of which have been the Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor (NHERF) family proteins. NHERF1 and -2 have been shown to bind to the PDZ ligands of a number of GPCRs, including the P2Y\textsubscript{1}, \(\beta\textsubscript{2}\)AR, mGLuR5, and PTH1R (5–7) and regulate their ability to signal by scaffolding GPCRs, G proteins, and protein kinases in close proximity at the cell membrane. With regards to the regulation of GPCR traffic, recent studies looking at the role of endogenous PDZ proteins have begun to explain why PDZ ligands in different GPCRs appear to have contrasting roles. For example, NHERF1 stabilizes PTH1R at the cell membrane but does not affect receptor recycling, whereas PDZ-regulated recycling of the \(\beta\textsubscript{2}\)AR appears to be mediated via another PDZ domain-containing protein, SNX27 (8).

We have recently identified a patient with a mild bleeding disorder and dysregulated P2Y\textsubscript{12} receptor (P2Y\textsubscript{12}R) function (9). The P2Y\textsubscript{12}R plays a critical role in the regulation of primary hemostasis with P2Y\textsubscript{12}R antagonists a mainstay in antithrombotic drug therapy. We have previously shown that upon agonist exposure P2Y\textsubscript{12}Rs on human platelets rapidly desensitize, internalize, recycle, and resensitize, allowing platelets to maintain responsiveness (10). The P2Y\textsubscript{12}R possesses a type 1 PDZ ligand (ETPM) at its C terminus and our recent study showed that a point mutation within this motif (P341A) led to a reduced efficiency in platelet P2Y\textsubscript{12}R surface receptor expression and function (9). In addition the P341A-P2Y\textsubscript{12}R variant failed to recycle following agonist-induced internalization, highlighting the critical importance of this motif in the regulation of normal receptor function (9). In this study we sought to identify PDZ-binding domain containing proteins that may interact with and regulate P2Y\textsubscript{12}R function. We report a novel role for endoge-
NHERF1/Arrestin Regulate Receptor Internalization

ous NHERF1 in the regulation of P2Y$_{12}$R internalization, showing for the first time that NHERF1 is required for effective internalization of this receptor via the formation of a tertiary complex between receptor, arrestin, and NHERF1.

**EXPERIMENTAL PROCEDURES**

**Materials**

Mouse monoclonal anti-NHERF1 antibody was from BD Biosciences. The donkey anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody was purchased from Abcam. The P2Y$_{12}$-R-specific antibody, directed to the extracellular surface N terminus of the receptor was from Imgenex. Mouse anti-GFP antibody and complete protease inhibitor tablets were from Roche Diagnostics Ltd. Goat anti-mouse rhodamine-conjugated secondary antibody, Lipofectamine 2000, and DMEM were purchased from Invitrogen. siRNAs were from Thermo Scientific Dharmacon. The alkaline phosphatase substrate kit was from Bio-Rad. The Myc antibody was a kind gift from Dr. Harry Mellor (University of Bristol). The Duolink system was from Olink Biosciences. All other reagents were from Sigma.

**cDNA and Cell Culture**

HA-tagged human P2Y$_1$ and P2Y$_{12}$ wild-type receptor constructs and 1321N1 cell stable transfectants of HA-P2Y$_1$ and HA-P2Y$_{12}$ using pCMVneo were generated as previously described (11). The PD2Δ mutant of the HA-tagged P2Y12R was engineered using standard PCR techniques by introducing a stop codon following E339 and 1321N1 cell stable cell lines expressing HA-P2Y$_{12}$ PD2Δ were generated. 1321N1 human astrocytoma cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 400 μg/ml of geneticin at 37 °C in a humidified atmosphere of 95% air and 5% CO$_2$. HEK cells were cultured under the same conditions, in the absence of geneticin. 1321N1 cells were lysed in ice-cold PBS lysis buffer (20 mM NaCl, 10 mM EDTA, 20 mM Hepes, 1% Triton X-100, pH 7.4) containing protease inhibitors (Roche Applied Science). Human platelets were lysed in 2× PBS lysis buffer. Samples were incubated with ~20 μg of GST fusion protein bound to glutathione-Sepharose-agarose beads overnight at 4 °C. Beads were washed three times in 1 ml of wash buffer (20 mM NaCl, 10 mM EDTA, 20 mM Hepes, 0.2% Triton X-100, pH 7.4) containing protease inhibitors and proteins were eluted in 5× SDS sample buffer (62.5 mM Tris, 350 mM DTT, 25% glycerol, 2% SDS). Proteins were resolved by SDS-PAGE and immunoblotted for NHERF1 or NHERF2. Inputs were immunoblotted for α-tubulin to show an equal amount of protein across samples. Immunoblots were developed with ECL (see below).

**siRNA Transfection**

1321N1 cells were seeded at a density of ~2.5 × 10$^5$ cells per 10-cm dish and grown overnight. Cells were transfected with 33 nm (600 pmol) siRNA with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Experiments were carried out 72 h post-transfection. Specific siRNA (arrestin 2/3) and a nonspecific siRNA (negative control) were from Thermo Scientific Dharmacon and were designed to target the following mRNA sequences: Arrestin2/3 siRNA (5’-ACC UGC GCC UUC CGC UAU G-3’), and nonspecific sequence (5’-GAC AAG AAC CAG AAC GCC A-3’). The NHERF1 siRNA was from Qiagen (Crawley, West Sussex, UK) and targeted the following mRNA sequence: NHERF1 (5’-CAG AAG GAG AAC AGT CGT GAA-3’).

**Preparation of Human Platelets**

Human blood was drawn from healthy, drug-free volunteers, and washed platelets were isolated from platelet-rich plasma as previously described (12). Platelets were allowed to rest for a further 30 min in the presence of 0.02 units/ml of apyrase and 10 μM indomethacin, prior to stimulation or lysis.

**GST Pulldown Assay**

Three glutathione S-transferase (GST) fusion proteins were used in this study: GST receptor COOH-terminal tails (CT) of the human P2Y$_1$, P2Y$_{12}$, and μ-opioid receptor 1 (MOR1). GST fusion proteins of the P2Y$_1$ receptor C termini were made by polymerase chain reaction (PCR) using cDNA encoding the full-length HA-tagged receptors as a template. GST fusion proteins of P2Y$_1$ and P2Y$_{12}$ receptor C termini (GST-P2YR CT) were cloned into the bacterial expression vector pGEX-4T-1 and the recombinant pGEX-4T-1 vectors were transformed into *Escherichia coli* strain of BL21 (DE3, Promega) competent cells for protein expression.

1321N1 cells were lysed in ice-cold PBS lysis buffer (200 mM NaCl, 10 mM EDTA, 20 mM Hepes, 1% Triton X-100, pH 7.4) containing protease inhibitors (Roche Applied Science). Human platelets were lysed in 2× PBS lysis buffer. Samples were incubated with ~20 μg of GST fusion protein bound to glutathione-Sepharose-agarose beads overnight at 4 °C. Beads were washed three times in 1 ml of wash buffer (20 mM NaCl, 10 mM EDTA, 20 mM Hepes, 0.2% Triton X-100, pH 7.4) containing protease inhibitors and proteins were eluted in 5× SDS sample buffer (62.5 mM Tris, 350 mM DTT, 25% glycerol, 2% SDS). Proteins were resolved by SDS-PAGE and immunoblotted for NHERF1 or NHERF2. Inputs were immunoblotted for α-tubulin to show an equal amount of protein across samples. Immunoblots were developed with ECL (see below).

**Co-immunoprecipitation and Western Blotting**

Following drug treatment, cells were washed twice with ice-cold PBS and lysed in co-immunoprecipitation lysis buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 10 mM glycerol phosphate, 200 μM sodium orthovanadate, 2.5 mM sodium pyrophosphate plus protease inhibitors). HA-tagged Rs were immunoprecipitated overnight at 4 °C with anti-HA-agarose. Arrestin-2-GFP was immunoprecipitated with a monoclonal GFP antibody (3 μg/sample) and precipitates were collected with protein A/G-agarose beads. Protein samples were boiled in SDS sample buffer and subjected to Western analysis. Endogenous NHERF1 and Arrestin-2-GFP were detected by immunoblotting with a monoclonal anti-NHERF1 and anti-GFP antibody, respectively. Myc-tagged NHERF1 was detected using a polyclonal anti-Myc sheep antibody. HRP-conjugated secondary antibodies were detected with chemiluminescence reagent (GE Healthcare). Where relevant, densitometry of blots was performed using ImageJ software (National Institutes of Health).

**Immunofluorescence in Human Platelets and Cell Lines**

Human Platelets—Immunofluorescence confocal microscopy of human platelets was adapted from protocols previously described by Pula and co-workers (13). Human platelets were prepared as described above and incubated with anti-P2Y$_{12}$ antibody targeted to the receptor N terminus (9) at 15 °C to label cell surface receptors and stimulated with ADP (10 μM) for 5 min at 37 °C. Platelet suspensions (2 × 10$^7$ platelets/ml) were allowed to settle onto the fibrinogen (0.1 mg/ml)-coated glass coverslips for 30 min. Platelets were fixed in 4% paraformaldehyde (15 min) permeabilized with 0.05% Triton X-100/
PBS (10 min) blocked with 1% BSA in 0.05% Triton X-100/PBS (30 min) and subsequently incubated with primary antibodies for 1 h at room temperature and then incubated with fluorophore-conjugated species-specific antibodies and then mounted onto slides with DABCO/Mowiol solution. Slides were subsequently imaged as described below.

**1321N1 Cells**—Cellular distribution of HA-tagged receptor or FLAG-tagged receptor in 1321N1 cells was assessed by immunofluorescence microscopy (11). Briefly, cells were grown on poly-L-lysine-coated coverslips in 6-well plates. Twenty-four hours later, receptor distribution was assessed using a primary anti-HA monoclonal antibody (HA-11; 1:200) and goat anti-mouse fluorescein-conjugated secondary antibody (1:200).

For the proximal-ligation assay (PLA) to detect the interaction between Myc-NHERF1 and GFP-arrestin-2 in HEK293 cells rabbit anti-Myc and mouse anti-GFP were used with a DuoLink PLA assay kit (Olink Bioscience). In brief, HEK293 cells were transfected with GFP-arrestin-2 and/or Myc-tagged NHERF1 fixed in 3.7% paraformaldehyde and antigenically labeled for Myc or GFP. The PLA detection was then performed according to the manufacturer’s specifications. In all cases slides were imaged using an upright Leica TCS-NT confocal laser scanning microscope attached to a Leica DM IRBE epifluorescence microscope with a Plan-Apo ×63 oil immersion objective. Images were analyzed using Photoshop 6.0 (Adobe). In receptor colocalization experiments individual spots (intracellular or membrane) were identified and subsequently quantified (contribution from red and green channels, each assigned a densitometric measurement on a 0–255 scale) using Volocity software (Improvision, Coventry, UK).

**Assay of Receptor Internalization**

Receptor internalization of HA-tagged surface receptors were assessed by ELISA as described previously (11). Briefly, 1321N1 cells were split into 24-well tissue culture dishes coated with 0.1 mg/ml of poly-L-lysine. Twenty-four hours later, cells were incubated with DMEM containing apyrase (0.2 units/ml) for 1 h at 37 °C, washed, and then challenged with DMEM containing ADP (10 μM) for 0–60 min at 37 °C. Changes in surface receptor expression were subsequently determined by an immunosorbent assay (ELISA) taking advantage of the HA epitope tag and expressed as either % surface receptor or % surface receptor loss with the background signal from pcDNA3-transfected controls subtracted from all receptor-transfected values.

**Measurement of cAMP Accumulation in 1321N1 Cells**

Desensitization and signaling of P2Y<sub>12</sub>R responses in 1321N1 cells were measured as previously described (14). In desensitization/resensitization experiments, cells were exposed to a desensitizing dose of ADP (10 nM; 15 min) in the presence of the phosphodiesterase inhibitor Ro201724 (250 μM). Apyrase (0.2 units/ml) was then added directly to each well to promote receptor resensitization and incubated at 37 °C (30 min) to remove the desensitizing ADP. P2Y<sub>12</sub>R activity was assessed by adding forskolin (1 μM) to cells in the absence or presence of ADP (0.01 μM to 10 μM), and plates were incubated at 37 °C for 10 min. Cyclic AMP levels were subsequently assessed as previously described (14). Data are expressed as the percentage of inhibition of forskolin-stimulated adenylyl cyclase.

**RESULTS**

**The P2Y<sub>12</sub>R Receptor C-tail Binds Endogenous NHERF1 and NHERF2 in Vitro**—Following our recent identification of a patient with a heterozygous mutation in the PDZ binding sequence of their P2Y<sub>12</sub>R (P341A) associated with reduced receptor expression and aberrant receptor traffic in human platelets (9) we aimed to discover PDZ domain binding partners that may regulate receptor function. Previous studies have described an interaction between the P2Y<sub>1</sub> receptor (P2Y,R), also expressed in platelets, and the first PDZ domain of NHERF2 (6, 15). Importantly the interactions between P2Y<sub>12</sub> receptors, which like the P2Y,R possess a type I PDZ ligand, and NHERF proteins have not been explored.

Initially, we generated GST fusion proteins of the P2Y<sub>1</sub> and P2Y<sub>12</sub> C termini and investigated interactions with endogenous NHERF1 and NHERF2 proteins from human platelet cell lysates and from 1321N1 cells in which we have extensively characterized the molecular mechanisms regulating P2Y<sub>12</sub> receptor. The C termini of both the P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors bound NHERF1 from platelet and cell lysates (Fig. 1A). Importantly GST beads alone did not bind NHERF1 from either platelets or 1321N1 cells, whereas a GST fusion protein of the μ-opioid receptor 1 (MOR1) C terminus, which lacks a distal PDZ ligand, also failed to bind NHERF1 from these cells (Fig. 1A). Quantification of band intensities revealed an apparent difference in the ability of P2Y<sub>1</sub> and P2Y<sub>12</sub> C termini to bind NHERF1 showing that the P2Y<sub>12</sub> receptor C-terminal tail consistently bound significantly more NHERF1 than the P2Y<sub>1</sub> C-tail from both platelet (Fig. 1B) and 1321N1 (Fig. 1C) cell lysates.

We next investigated interactions between NHERF2 and P2Y<sub>12</sub>R receptor C termini. In accordance with published data using overexpressed NHERF (6, 15), we showed that the P2Y<sub>1</sub> C terminus was able to strongly bind endogenous NHERF2 from 1321N1 cells, whereas MOR1 C-tail and GST alone did not (Fig. 1D). The C terminus of P2Y<sub>12</sub> also bound NHERF2, although this interaction appeared to be considerably weaker than that between NHERF2 and the P2Y<sub>1</sub> C terminus (Fig. 1E). In these studies we were unable to detect binding of any receptor tails and endogenous NHERF2 from platelet lysates, likely due to the lower levels of NHERF2 expression in platelets. Taken together, these in vitro data show that P2Y<sub>1</sub> and P2Y<sub>12</sub> C termini are able to interact with both NHERF1 and NHERF2 with differing affinity.

**Full-length P2Y<sub>12</sub>R Interacts with NHERF1 in an Agonist-dependent Manner**—Given the high expression of NHERF1 in platelets and an apparent strong interaction with the P2Y<sub>12</sub>R C terminus detected in the pulldown assays, co-immunoprecipitation studies were carried out to further investigate whether full-length P2Y<sub>12</sub> receptors are likely to interact with NHERF proteins in intact cells. Previous studies have shown that GPCRs can associate with NHERF proteins upon agonist stimulation or dissociate from NHERF upon activation. For example, κ opioid receptors undergo an agonist-dependent associa-
tion with NHERF1 (16), whereas the PTH1R, which constitutively binds NHERF1, is thought to uncouple from NHERF1 upon activation by a full agonist (17). In light of these observations the NHERF1/P2Y12R interaction was investigated in the absence and presence of agonist stimulation. HA-tagged P2Y12Rs were immunoprecipitated from stably transfected 1321N1 cells and associations with endogenous NHERF1 were examined using an anti-NHERF1 antibody. Under basal conditions NHERF1 associated with P2Y12R (Fig. 2A). Importantly ADP treatment (10 μM; 5 min) caused a clear increase in the amount of co-immunoprecipitated NHERF1 (Fig. 2, A and B).

Interestingly, NHERF1 also underwent a small molecular mass increase of ∼3–4 kDa upon agonist treatment. This molecular weight shift may be a consequence of NHERF1 phosphorylation following receptor activation that is postulated to increase receptor affinity for NHERF1 (18, 19).

We subsequently attempted to co-immunoprecipitate endogenous P2Y12R with NHERF1 from human platelets. Unfortunately these studies failed to show any definitive interaction between these proteins most likely due to low levels of receptor expression in these cells (copy number around 200 receptors per platelet (12)). We therefore assessed by confocal microscopy whether NHERF1 and P2Y12R co-localize in human platelets (Fig. 2C). Isolated platelets were incubated with an N-terminal anti-P2Y12 antibody (9) to label cell surface receptors and then treated with ADP for 5 min. Following stimulation, platelets were fixed, permeabilized, and then stained for endogenous NHERF1. Under basal conditions, P2Y12Rs were localized primarily to the platelet membrane, whereas NHERF1 was found distributed throughout the cell in a punctuate localization. Following ADP treatment, P2Y12R was inter-

FIGURE 1. In vitro association of NHERF proteins isoforms with the C-tail of the P2Y1 and P2Y12Rs. A and C, human platelet (first to fourth lanes) or 1321N1 (fifth to eighth lanes) cell lysates were incubated with GST fusion proteins containing the P2Y1, P2Y12, or MOR1 receptor cytoplasmic tails (CT). Samples were resolved by SDS-PAGE and immunoblotted (IB) for NHERF1 (A) or NHERF2 (C). Inputs were immunoblotted for α-tubulin to show equal protein across samples. Blots shown are representative of five independent experiments. Multiple experiments were quantified by densitometry (B) shows quantified NHERF1 binding from platelets and 1321N1 cell lysates, respectively, **, p < 0.05 comparing level of P2Y1/NHERF1 to level of P2Y12/NHERF1 interaction (Mann-Whitney U test) (D) shows quantified NHERF2 binding from 1321N1 cell lysates. **, p < 0.05 comparing level of P2Y1/NHERF2 to level of P2Y12/NHERF2 interaction (Mann Whitney U test) (data are represented as mean ± S.E. (n = 5) in B).

FIGURE 2. Agonist-dependent association of NHERF1 with full-length P2Y12R. A, 1321N1 cells stably expressing HA-tagged P2Y12R were incubated in the absence or presence ADP (10 μM, 5 min). Cells were lysed and receptors were immunoprecipitated using an HA antibody. Samples were resolved by SDS-PAGE and immunoblotted for associated NHERF1 (top panel) and reprobed with an anti-HA antibody to show total receptor immunoprecipitated (middle panel). Inputs were immunoblotted for NHERF1 (bottom panel) to show equal protein concentrations across samples. Blots shown are representative of three independent experiments. B, quantification of co-immunoprecipitated NHERF1 with P2Y12R (mean ± S.E., n = 3). **, p < 0.05 comparing level of basal P2Y12/NHERF1 interaction versus ADP-stimulated P2Y12/NHERF1 interaction (Mann-Whitney U test). C, agonist-dependent co-localization of P2Y12R and NHERF1 in human platelets. Human platelets were isolated and surface P2Y12 receptors were labeled with an anti-P2Y12 antibody (green). Platelets were stimulated in the absence (top panel) or presence (lower panel) of ADP (10 μM, 5 min). Endogenous NHERF1 was stained using an anti-NHERF1 antibody (purple). Co-localization is shown in the merged images (white). Images shown are representative of three independent experiments. Scale bar represents 10 μm.
nalized to an intracellular compartment and importantly showed partial co-localization with NHERF1 (Fig. 2C, lower panel). These data suggest that endogenous P2Y12Rs in human platelets do co-localize with endogenous NHERF1 in an agonist-dependent manner.

**Agonist-induced P2Y12R Internalization Is NHERF1 Dependent**—Having provided biochemical evidence that the P2Y12R is able to interact with NHERF1, we investigated the functional significance of these interactions in human 1321N1 cells stably expressing HA-tagged P2Y12 Rsi. Previous studies with the β2-AR and κ-opioid receptors have shown that although interactions with NHERF proteins are required for efficient recycling, they do not affect receptor internalization (5–7). Conversely, internalization of the PTH1R and the TPβ receptor has been shown to be inhibited by the presence of NHERF1 (7, 20). However, the role of NHERF proteins in the regulation of P2Y12R internalization and recycling is not known.

Targeted siRNA produced significant knock-down of NHERF1 expression in 1321N1 cells (84 ± 6%, n = 3; Fig. 3A). Previous reports have suggested endogenous NHERF1 stabilizes receptor expression at the cell surface (7, 20, 21). We found no obvious change in basal levels of cell surface P2Y12R expression following NHERF knockdown (data not shown). Unexpectedly we did find that reduced NHERF1 expression significantly impaired P2Y12R internalization (Fig. 3B).

In addition to regulation of GPCR traffic, NHERF1 is also implicated in the regulation of receptor signaling (22–24). To investigate the potential involvement of NHERF1 in G protein-mediated signaling downstream of P2Y12R activation, adenylyl cyclase activity was quantified through the measurement of cAMP accumulation in 1321N1 cells stably expressing P2Y12 Rs, both in the absence and presence of NHERF1.

Decreased NHERF1 expression did not significantly affect the ability of P2Y12R to negatively couple to adenylyl cyclase with the EC50 for ADP-stimulated P2Y12R activity 20.3 ± 2.8 and 19.3 ± 1.3 nm in scrambled and NHERF1 siRNA-treated cells, respectively, values comparable with those reported by other researchers (25, 26) (Fig. 3C). Although NHERF1 did not appear to affect acute P2Y12 signaling, it was possible that a reduction in cellular levels of NHERF1 could interfere with the process of receptor regulation. To investigate this possibility, desensitization and resensitization of the P2Y12R response was measured in the absence and presence of NHERF1. In control cells (Fig. 3D, control), exposure to ADP (10 μM) caused a robust inhibition of the forskolin-induced adenylyl cyclase activity. When cells were pre-treated with ADP (10 μM; 15 min) the P2Y12R was desensitized (ADP-PT). Following removal of the desensitizing ADP (+ADP/Apyr), the P2Y12R response was resensitized consistent with previous work from our laboratory (10, 14). P2Y12R desensitization was unaffected with previous work from our laboratory (10, 14). P2Y12R desensitization was unaffected by NHERF1 knockdown. Importantly, however, NHERF1 knockdown did prevent P2Y12R resensitization (Fig. 3D). The inability of P2Y12 Rs to resensitize in the absence of NHERF1 is consistent with our previous studies where we have shown that blockade of receptor internalization prevents P2Y12R resensitization (10). Taken together, these experiments provide further evidence for a specific role for NHERF1 in P2Y12R internalization.

**FIGURE 3. P2Y12R internalization is NHERF1-dependent.** 1321N1 cells stably expressing HA-P2Y12R were treated with control or NHERF1 siRNA. A, 1321N1 cell lysates derived from control or NHERF1 siRNA-treated cells were subjected to NHERF1 immunoblotting to show NHERF1 protein knock-down. Membranes were re-probed with an anti-α-tubulin antibody to ensure equal protein loading. B, control or NHERF1 siRNA-treated cells were challenged with ADP (10 μM; 0–60 min). Changes in cell surface receptor expression were evaluated by ELISA. C, P2Y12R activity in control and NHERF1 siRNA-treated cells. ADP-dependent inhibition of forskolin (1 μM; 10 min)-stimulated adenylyl cyclase activity was evaluated in control and NHERF1 siRNA-treated cells at a range of ADP concentrations. D, P2Y12R desensitization and subsequent desensitization were assessed by comparing agonist (ADP, 10 μM)-dependent inhibition of forskolin (1 μM; 10 min)-stimulated adenylyl cyclase activity before (control) and after pretreatment with either ADP alone (10 μM; 15 min; ADP-PT on graph) or after subsequent removal of desensitizing ADP with apyrase (0.2 units/ml; 15 min; ADP PT/Apyrase on graph). Data represent mean ± S.E. of 4 independent experiments; *, p < 0.05 compared versus control and #, p < 0.05 comparing ADP PT versus ADP/apyrase (Mann-Whitney U test).
The PDZ Ligand of P2Y_{12}R Is Required for Efficient Internalization of the P2Y_{12}R—Our recent studies showed that removal of the PDZ ligand of the P2Y_{12}R attenuated receptor internalization in CHO cells (9). Initial studies in which we stably transfected P2Y_{12}-PDZ∆ into 1321N1 cells again showed that removal of the PDZ ligand reduced agonist-induced P2Y_{12}R internalization (Fig. 4A). Given the importance in many GPCRs of the PDZ motif in stabilizing GPCR surface expression (4), we next sought to examine if there were any differences in the surface membrane localization of P2Y_{12}-PDZ∆ versus wild-type receptor. In the absence of agonist there was, however, no discernable difference in membrane localization as assessed by confocal microscopy (data not shown). Our recent study showed that although unable to undergo arrestin-dependent internalization the P2Y_{12}-PDZ∆ receptor still underwent clathrin-dependent internalization (9). We subsequently examined the ability of P2Y_{12}-PDZ∆ and wild-type receptor to co-localize with clathrin. Following agonist stimulation both the wild-type and P2Y_{12}-PDZ∆ receptors (receptors in green, Fig. 4B) colocalized to areas enriched in clathrin (purple, colocalization in white). Previous studies from ourselves (11) and others (27) have revealed that distinct populations of CCPs can sort GPCR cargo at the plasma membrane. We therefore compared the localization of wild-type and P2Y_{12}-PDZ∆ receptor following agonist addition. Cells stably expressing FLAG-P2Y_{12} were transiently transfected with either HA-tagged P2Y_{12}-PDZ∆ or HA-P2Y_{12} and the localization of each receptor was assessed following acute agonist stimulation (Fig. 4, C and D). It should be noted that prelabeling with anti-HA or anti-FLAG antibody did not promote receptor internalization and had no effect on the ability of the P2Y_{12}R to negatively couple to adenylyl cyclase (EC_{50} of ADP-stimulated P2Y_{12}R activity was 19.3 ± 2.3, 21.5 ± 2.8, and 18.9 ± 1.9 nM for control, + anti-HA, and + anti-FLAG, respectively). The contributions of FLAG (purple) or HA (green) to individual surface membrane or intracellular puncta were determined and plotted as shown. Importantly this analysis revealed there was minimal colocalization of P2Y_{12}-PDZ∆ and P2Y_{12} receptors at the cell membrane (Fig. 4C; note from the scatter plot the two distinct clusters of membrane spots stained either predominantly purple or predominantly green), suggesting that each receptor is sorted to distinct endocytic loci on the membrane surface. The analysis also revealed that, once internalized, the receptors were colocalized, as displayed by the single large cluster of spots stained more equally for each receptor. By way of a control and as previously reported HA-P2Y_{12} and FLAG-P2Y_{12} were colocalized at the cell membrane (Fig. 4D; note from the scatter plot that membrane spots are stained with varying levels of purple or green but no color predominates). Therefore for the P2Y_{12}R the absence of these last four amino acids targets the receptor to a distinct population of CCPs from the WT receptor.

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**FIGURE 4.** The PDZ ligand of the P2Y_{12}R is required for effective internalization and membrane sorting in 1321N1 cells. A, removal of the PDZ ligand of the P2Y_{12}R attenuates receptor internalization in 1321N1 cells. 1321N1 cells stably expressing HA-P2Y_{12}, wild-type (WT) or HA-P2Y_{12}-PDZ∆ were challenged with ADP (10 μM; 10 or 30 min). Changes in cell surface expression were measured by ELISA. Data are represented as mean ± S.E. (n = 5), *p < 0.05 comparing P2Y_{12} versus P2Y_{12}-PDZ∆ (Mann-Whitney U test). B, both the P2Y_{12}R and P2Y_{12}-PDZ∆ colocalize with clathrin at the cell membrane following receptor activation. 1321N1 cells stably expressing FLAG-P2Y_{12} were transiently transfected with either HA-tagged P2Y_{12} or HA-tagged pre-P2Y_{12}-PDZ∆ and preincubated with monoclonal anti-HA (HA-11) antibody or polyclonal anti-FLAG (H9262). Membraneous areas enriched in clathrin (purple) are indicated by purple arrows. The degree of receptor-clathrin colocalization following agonist addition can be seen in the overlay (colocalization in white). The scale bar represents 10 μm. Data shown are representative of three independent experiments. C and D, 1321N1 cells stably expressing FLAG-tagged P2Y_{12} were transiently transfected with either (C) HA-P2Y_{12}-PDZ∆ or (D) HA-P2Y_{12}-PDZ∆ and preincubated with monoclonal anti-HA (HA-11) antibody or polyclonal anti-FLAG (M2). Subsequently, cells were incubated at 37°C with ADP (10 μM; 5 min) and fixed. Agonist-induced accumulations of receptor (green) at or near the cell membrane are indicated by green arrows. Membranous areas enriched in clathrin (purple) are indicated by purple arrows. The degree of receptor-clathrin colocalization following agonist addition can be seen in the overlay (colocalization in white). The scale bar represents 10 μm. Data shown are representative of three independent experiments. C and D, 1321N1 cells stably expressing FLAG-tagged P2Y_{12} were transiently transfected with either (C) HA-P2Y_{12}-PDZ∆ or (D) HA-P2Y_{12}-PDZ∆ and preincubated with monoclonal anti-HA (HA-11) antibody or polyclonal anti-FLAG (M2). Subsequently, cells were incubated at 37°C with ADP (10 μM; 5 min) and fixed. Agonist-induced accumulations of receptor (green) at or near the cell membrane are indicated by green arrows. Membranous areas enriched in clathrin (purple) are indicated by purple arrows. The degree of receptor-clathrin colocalization following agonist addition can be seen in the overlay (colocalization in white). The scale bar represents 10 μm. Data shown are representative of three independent experiments. C and D, 1321N1 cells stably expressing FLAG-tagged P2Y_{12} were transiently transfected with either (C) HA-P2Y_{12}-PDZ∆ or (D) HA-P2Y_{12}-PDZ∆ and preincubated with monoclonal anti-HA (HA-11) antibody or polyclonal anti-FLAG (M2). Subsequently, cells were incubated at 37°C with ADP (10 μM; 5 min) and fixed. Agonist-induced accumulations of receptor (green) at or near the cell membrane are indicated by green arrows. Membranous areas enriched in clathrin (purple) are indicated by purple arrows. The degree of receptor-clathrin colocalization following agonist addition can be seen in the overlay (colocalization in white). The scale bar represents 10 μm. Data shown are representative of three independent experiments.
NHERF1 Can Regulate P2Y12R Internalization in the Absence of the PDZ Motif of the Receptor—Because endogenous NHERF proteins were shown to be involved in the regulation of P2Y12R internalization, we hypothesized that removal of the PDZ ligand of this receptor, which is predicted to interact with the PDZ domain containing proteins, would result in a loss of NHERF1 association and therefore result in defective receptor internalization. A GST fusion protein of the P2Y12 C-tail lacking the PDZ ligand (GST P2Y12 PDZΔ) was generated and tested for NHERF1 interaction. Although full-length P2Y12 C-tail bound NHERF1, the truncated P2Y12 C-tail failed to interact with NHERF1 suggesting that this in vitro interaction does indeed require an intact PDZ ligand (Fig. 5A). We subsequently tested if P2Y12-PDZΔ receptor internalization was NHERF1 dependent. Interestingly we found that, like the full-length receptor, P2Y12-PDZΔ internalization was inhibited in the absence of endogenous NHERF1 (Fig. 5B). The ability of NHERF1 siRNA to block internalization of P2Y12-PDZΔ indicated that, in contrast to the pulldown data (Fig. 5A) NHERF1 may be able to interact with P2Y12-PDZΔ independently of the PDZ ligand within intact cells. To further investigate the mechanism of P2Y12-PDZΔ internalization, we carried out co-immunoprecipitation experiments with the truncated receptors. Interestingly, we found that P2Y12-PDZΔ receptors retained the ability to interact with endogenous NHERF1 in an agonist-dependent manner, whereas under basal conditions there was little NHERF1 association (Fig. 5C, right-hand panel, second and third lanes). Taken together with the pulldown experiments, these data suggest that in the absence of agonist, the association of NHERF1 with P2Y12 requires an intact PDZ ligand, whereas the increased NHERF1 association that occurs upon agonist stimulation is able to continue in the absence of the PDZ-ligand found at the extreme C terminus of this receptor.

Agonist-dependent NHERF1 Association with the P2Y12R Requires Endogenous Arrestin—Because in our recent studies we discovered that an intact PDZ-binding motif was required for receptor/arrestin interaction in CHO cells (9) we next examined if this was also the case in 1321N1 cells. An siRNA sequence designed to target both arrestin-2 and arrestin-3 was used to inhibit arrestin expression in 1321N1 cells. Arrestin expression was evaluated using a purified polyclonal antibody against arrestin-2 (28). siRNA treatment did result in partial inhibition of arrestin expression (Fig. 5B). Importantly arrestin knockdown effectively attenuated the internalization of the full-length receptor with a minor effect on P2Y12-PDZΔ (Fig. 5B). Previous studies have shown that P2Y12R internalization is GRK and arrestin-dependent (11), whereas NHERF1 has been implicated in the regulation of arrestin function (7, 29–32) although there are conflicting data regarding the precise nature of this regulation. Given that P2Y12R internalization requires both arrestin and NHERF1, we hypothesized that these two adaptor complexes may be able to interact to bring about efficient receptor endocytosis.

We initially looked at arrestin dependence of the NHERF1/P2Y12R interaction in intact cells by co-immunoprecipitation in the presence and absence of arrestin siRNA. In the absence of agonist there was a level of basal NHERF1 association with WT P2Y12R, which increased when the cells were treated with ADP (Fig. 5C, left-hand panel, second and third lanes). However, in cells in which arrestin expression was inhibited with siRNA (Fig. 5C, left-hand panel, fourth and fifth lanes) there was a reduction in NHERF1 association with P2Y12 in the presence of ADP, suggesting that endogenous arrestin is required for NHERF1 recruitment to this receptor. Interestingly, the basal NHERF1/P2Y12R interaction appeared to be slightly increased in arrestin siRNA-treated cells suggesting there may be a level of competition between arrestin and NHERF proteins in the absence of agonist. The arrestin dependence of the P2Y12-PDZΔ/NHERF1 interaction was also studied by co-immunoprecipitation. In cells with reduced arrestin expression, NHERF1 continued to co-immunoprecipitate with P2Y12-PDZΔ (Fig. 5, right-hand panel, fourth and fifth lanes).

These data suggest a complex set of steps is required to regulate P2Y12R internalization. In the absence of agonist NHERF1 interacts with the P2Y12-R. Following agonist binding the receptor then interacts through its PDZ-binding motif with arrestin that in turn coordinates NHERF-dependent receptor internalization. In the absence of the PDZ-binding motif of this receptor NHERF1 still appears to be able to interact with P2Y12R and coordinate receptor internalization in an arrestin-independent manner.

NHERF1 and Arrestin Interact in Living Cells—Because our data suggested that arrestin appeared to regulate NHERF1/receptor association we next sought to determine whether these proteins were able to interact directly. To study NHERF1/arrestin interactions, GFP-tagged arrestin-2 and Myc-tagged NHERF1 were transiently overexpressed in HEK293 cells. Subcellular localization of potential NHERF1/arrestin interactions was visualized using a DuoLink in situ PLA. This assay works similarly to standard immunofluorescence detection, except that the secondary antibodies are attached to PLA probes that fluoresce only when the antibodies are in close proximity. A fluorescence signal indicates that two proteins within cells are separated by <40 nm. We found that in cells co-transfected with GFP-tagged arrestin-2 and Myc-tagged NHERF1 DuoLink fluorescence (shown in red) could be observed in a punctate distribution predominately at the cell surface, but also within the cytoplasm (Fig. 6A). Importantly the DuoLink PLA signal was absent in cells that were singly transfected with either GFP-Arrestin or Myc-NHERF1 alone.

To further confirm the imaging data, we also sought associations between arrestin and NHERF1 by co-immunoprecipitation in the absence of any cross-linking reagents. We found that in cells co-overexpressing both proteins, NHERF1 co-immunoprecipitated with GFP-arrestin-2 (Fig. 5B) consistent with recently published data (31). Taken together, these data show that arrestin is required for agonist-dependent NHERF1 recruitment to the P2Y12 receptor and suggest that a supercomplex between receptor, arrestin, and NHERF1 is required for efficient receptor internalization (Fig. 7).

DISCUSSION

In the present study we have described a novel role of NHERF in the regulation of GPCR internalization. This study shows that P2Y12R internalization is NHERF1-dependent likely
through an arrestin-dependent association with the extreme COOH terminus of this receptor. Whereas NHERF1 has previously been implicated in CCR5 internalization (33), this is the first report that NHERF1 expressed at endogenous levels is required for the internalization of a GPCR. The data presented here support recent reports of interactions between arrestin and NHERF1 (29, 31, 33) and furthermore, suggest that arrestin binding is required for NHERF1 recruitment to the activated receptor complex. In addition to providing insight into how the clinically relevant P2Y12R is regulated, these observations add
recently reported that for the P2Y12R an intact PDZ ligand tor recycling back to the cell surface (4). Indeed, we have for correct receptor trafficking, in particular for efficient recep-

ative of three independent experiments. The representative of three independent experiments. Data shown are represent-

tive of three independent experiments. The scale bar represents 10 μm.

another level of complexity to current models of NHERF-reg-

ulated GPCR function.

GPCR PDZ ligands are widely recognized as motifs critical for correct receptor trafficking, in particular for efficient recep-
tor recycling back to the cell surface (4). Indeed, we have recently reported that for the P2Y12,R an intact PDZ ligand (ETPM) is essential for the efficient recycling of this receptor to the cell surface in human platelets (9). We therefore set out to identify possible P2Y12,R binding partners that may interact

with the PDZ ligand of this receptor that may regulate its function.

In our initial studies we discovered that the PDZ-binding domain containing proteins NHERF1 and NHERF2 can inter-

act with the C-tail of this receptor, an association that in vitro appears to require the presence of the PDZ motif of this recep-
tor. Importantly we demonstrated in human platelets that P2Y12,R and NHERF1 colocalize following receptor stimulation. This is significant given that NHERF1 appears to be highly expressed in human platelets. NHERF1 is also reported to bind to endothelial cell-specific adhesion molecules in platelets via a type 1 PDZ ligand (34), an interaction that is suggested to be inhibitory for thrombus stability. The in vivo significance of the P2Y12/NHERF1 interaction in platelet function is still to be investigated.

In our subsequent studies, cell lines revealed the novel finding that endogenous NHERF1 expression is essentially required for P2Y12,R internalization while having no effect on acute receptor signaling (via Gt) or desensitization. The majority of previous studies have reported a role for NHERF1 in the regu-

lation of receptor signaling, recycling, or stabilization at the cell membrane (24, 35). Studies with the thromboxane (20), adenosine A2B (21), and the PTH1R (7, 30, 36) have also described a regulatory role for NHERF1 in GPCR internalization, however, these studies have all reported an inhibitory effect of NHERF1 on receptor internalization. The PTH1R has been shown to interact with NHERF1 and this interaction stabilizes the recep-
tor at the cell surface, thus inhibiting internalization. Further-

more, siRNA knockdown of NHERF1 in HEK293 cells has been shown to increase agonist-dependent PTH1R receptor inter-

nalization (7).

NHERF1 has been previously been implicated in the internal-

ization of some membrane proteins. The internalization of the membrane multidrug resistance protein 4 (37) requires endogenous NHERF1. In addition, a recent study with the GPCR CCR5 showed that overexpressed NHERF1 increased the rate of CCR5 receptor internalization (33), suggesting that NHERF1 may play a wider role in receptor endocytosis. There appears to be key differences in the mechanism of NHERF1-regulated CCR5 regulation versus the P2Y12 with the agonist-

independent CCR5/NHERF1 interaction, whereas P2Y12,NHERF1 association was increased by agonist addition. Interest-

ingly, the A2B and PTH1R, whose internalization is negatively regulated by NHERF1, have been reported to be basally associated with NHERF1, which dissociates upon ago-

nist activation (7, 21, 32, 36), hence an agonist-dependent associ-

ation or dissociation may provide a mechanism for differen-
tial regulation of endocytosis.

Our data suggests there is a basal association of NHERF1 with P2Y12,R and that this interaction appears to be dependent on the extreme C terminus PDZ ligand. The significance of the basal interaction is unknown although it is possible that constitutive NHERF1 association stabilizes the receptor at the cell surface in the absence of agonist (via interactions with the actin cytoskeleton) and reduces the level of constitutive receptor internalization by preventing arrestin binding, in a similar man-

ner to the PTH1R. Surprisingly removal of the PDZ ligand of the P2Y12,R that in vitro and under basal conditions reduced
NHERF1/Arrestin Regulate Receptor Internalization

The majority of previous studies have reported a role for NHERF1 in the regulation of receptor signaling, recycling, or stabilization at the cell membrane (24, 35) via an interaction with PDZ ligands present in the extreme C terminus. In contrast, we show that NHERF1-regulated P2Y12 R internalization can take place independently of the extreme C-terminal PDZ ligand. The apparent PDZ independence of the agonist-dependent NHERF1-P2Y12 association was unexpected, although the P2Y12 receptor PDZ ligand (ETPM) could be considered to be an atypical PDZ recognition motif. Furthermore, the C-terminal methionine (38) and the presence of the proline residue at position 341 might be predicted to lower the affinity for the NHERF PDZ domains. However, the PTHR, which has a similar PDZ ligand to P2Y12 (ETVM) does bind to NHERF1 in a PDZ-dependent manner. Possible explanations for these observations are that NHERF1 binds elsewhere on the receptor or that agonist-dependent interaction is indirect. For example, an internal PDZ ligand that regulates recycling has been described for the endothelin A receptor (39). Examination of the P2Y12 C terminus sequence reveals that there are potential internal ligands present (for example, residues 319–322 ATSL) that may be responsible for the increase in NHERF1 association upon receptor activation. The other possibility is that NHERF1 does not interact directly with the receptor and instead association is via an intermediary protein, such as arrestin. It should also be noted, however, that although the internalization of both the full-length and P2Y12 PDZΔ is NHERF1-dependent they localize to distinct populations of clathrin-coated pits likely due to differences in arrestin interaction (9). It should also be noted that the P2Y12 PDZ ligand may well be mediating interactions with other PDZ domain containing proteins, such as SNX27 as was recently reported for the β2-adrenoreceptor (8).

We report that in addition to NHERF1, endogenous β-arrestin is also required for P2Y12 R internalization. It has been recently reported that these scaffolding proteins are able to interact (31). Importantly in this study we show for the first time that a reduction in endogenous arrestin expression significantly attenuates agonist-dependent P2Y12/NHERF1 interaction. There is now a growing body of evidence that there is cross-regulation between these NHERF1 and arrestin, although some of these data are conflicting. Earlier studies with the PTH1R suggested that NHERF1 and arrestin were effectively negatively regulating each other (7, 29). A recent study, using a live cell imaging approach has also shown that arrestin recruitment to the PTH1R is delayed until NHERF1 dissociates from the receptor (32), providing further evidence for competition between these scaffold proteins for binding to the receptor. As discussed above, under basal conditions this also appears to apply to P2Y12. However, there are also recent reports that NHERF1 overexpression recruits arrestin to both the PTH1R (31) and CCR5 (33). In the present study we show that endogenous arrestin is required for the agonist-dependent NHERF1 interaction with P2Y12. Further evidence that arrestin recruitment is upstream of NHERF1 recruitment comes from the finding that normal receptor desensitization occurs in NHERF1 siRNA-treated cells, suggesting that the receptor/arrestin interaction remains intact in the absence of NHERF1. The potential mechanism for P2Y12 receptor internalization based on the findings reported here is outlined in Fig. 7.

In summary, this is the first report to show that the P2Y12 R is able to interact with NHERF1 in human platelets. Whether the mechanism described for NHERF1-regulated P2Y12 internalization can be extended to other GPCRs and how precisely NHERF1 interacts with the clathrin endocytic machinery remains to be determined, however, the data presented here suggest that the roles of NHERF1, like arrestin, are not only multifunctional but receptor-specific.

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