Role of the Rab11-Associated Intracellular Pool of Receptors Formed by Constitutive Endocytosis of the β Isoform of the Thromboxane A_2 Receptor (TPβ)†

Caroline Thériault, Moulay Driss Rochdi, and Jean-Luc Parent*

Service de Rhumatologie, Faculté de Médecine and Centre de Recherche Clinique-CHUS, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

Received December 17, 2003; Revised Manuscript Received March 12, 2004

ABSTRACT: Intracellular trafficking pathways of G protein-coupled receptors (GPCRs), following their agonist-induced endocytosis and their consequences on receptor function, are the subject of intense research efforts. However, less is known regarding their constitutive endocytosis. We previously demonstrated that the β isoform of the thromboxane A_2 receptor (TPβ) undergoes constitutive and agonist-induced endocytosis. Constitutive endocytosis of GPCRs can lead to the formation of an intracellular pool of receptors from which they can recycle back to the cell surface. In the present report, we show with the help of two TPβ mutants (TPβ-Y339A and TPβ-I343A) specifically deficient in constitutive endocytosis that this intracellular pool of receptors serves to maintain agonist sensitivity over prolonged receptor stimulation in HEK293 cells. Second messenger generation by the TPβ-Y339A and TPβ-I343A mutants was drastically reduced compared to the wild-type receptor as suggested by dose−response and time-course experiments of inositol phosphates production following agonist treatment, despite normal coupling between the receptors and the $G\alpha_q$ protein. Moreover, second messenger production after receptor activation was dramatically reduced when cells were pretreated with monensin, a recycling inhibitor. Receptor cell surface expression and endocytosis experiments further revealed that the small GTPase Rab11 protein is a determinant factor in controlling TPβ recycling back to the cell surface. Co-localization experiments performed by immunofluorescence microscopy indicated that both constitutive and agonist-triggered endocytosis resulted in targeting of TPβ to the Rab11-positive recycling endosome. Thus, we provide evidence that constitutive endocytosis of TPβ forms a pool of receptors in the perinuclear recycling endosome from which they recycle to the cell surface, a process involved in preserving receptor sensitivity to agonist stimulation.

The prostanoid thromboxane A_2 (TXA_2)[1] is synthesized mainly by activated platelets but also by monocytes/macrophages, and vascular and bronchial smooth muscle cells (*1*). TXA_2 mediates secretion, morphological changes, and aggregation of platelets. It is also known as a potent broncho- and vasoconstrictor as well as an inducer of vascular smooth muscle cell proliferation and hypertrophy (*2*). Numerous pathological states are associated with defects in TXA_2 synthesis or in its receptor function (*3*, *4*). These include a wide variety of cardiovascular, pulmonary, and kidney diseases (*5*−*8*) justifying the need to better understand the molecular mechanisms that regulate the actions of this prostanoid.

The TXA_2 receptor (TP) is encoded by a single gene that is alternatively spliced in the carboxyl terminus resulting in two isoforms, TPα (343 amino acids) and TPβ (407 amino acids), which share the first 328 amino acids (*9*, *10*). TP is a G protein-coupled rhodopsin-like receptor (GPCR). The major signaling pathway of TXA_2 and its receptor is via the activation of the $G\alpha_{q/11}$ family of G proteins (*4*). This leads to PLCβ activation resulting in an increased production of the second messengers inositol phosphates and diacylglycerol. As for other GPCRs, TP is subjected to a tight regulation by a diversity of cellular mechanisms (*3*, *11*−*18*). Among these, endocytosis of GPCRs is the focus of extensive efforts of numerous laboratories. Endocytosis is part of the desensitization process, thus permitting control of cell surface receptor expression as well as agonist sensitivity. It was also reported that endocytosis is involved in resensitization of GPCRs by bringing them near to an endosome-associated phosphatase (*19*). Dephosphorylation and subsequent recycling of receptors back to the plasma membrane contribute to resensitization of the cell response to agonist.

The majority of GPCRs undergo agonist-induced endocytosis. However, recent studies have demonstrated that several GPCRs including the PAR1 (*20*), CXCR4 (*21*), CCKA (*22*), thyrotropin (*23*), M_2 muscarinic (*24*), mGluR1a (*25*), $V1_A$ (*26*), CTR (*27*), mGluR5a and 5b (*28*) are constitutively endocytosed.

We previously demonstrated that TPβ, but not TPα, undergoes constitutive and agonist-induced endocytosis (*12*, *15*).

† This work was supported by a grant to J.-L.P. from the Canadian Institutes of Health Research. M.D.R. is supported by a doctoral fellowship from the CIHR. J.-L.P. is the recipient of a New Investigator Award from the CIHR.

* To whom correspondence should be addressed. Service de Rhumatologie, Faculté de Médecine, Université de Sherbrooke, 3001 12e Avenue Nord, Fleurimont, Qc, Canada, J1H 5N4. Phone: 819-564-5264. Fax: 819-564-5265. E-mail: jean-luc.parent@USherbrooke.ca.

[1] Abbreviations: GAP, GTPase-activating protein; EGFP, enhanced green-fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; PLC, phospholipase C; TP receptor, thromboxane A_2 receptor; TXA_2, thromboxane A_2.

10.1021/bi036268v CCC: $27.50
Published on Web 04/24/2004

During these studies, we identified a $YX_{2-3}\varphi$ motif (where X is any amino acid and φ is a bulky hydrophobic amino acid) in the carboxyl-terminal tail of TPβ as specifically responsible for constitutive endocytosis. Constitutively internalized TPβ appeared to accumulate in a perinuclear intracellular compartment (*15*). At that time, we proposed that constitutive endocytosis of TPβ likely helps to maintain an intracellular pool of receptors that recycle to the cell surface to preserve agonist sensitivity. In the present study, we used two receptor mutants, TPβ-Y339A and TPβ-I343A (*15*), specifically deficient in constitutive endocytosis to address this hypothesis. Experiments were also carried out to characterize the intracellular compartment where constitutively endocytosed TPβ accumulates.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents. $G\alpha_{q/11}$ polyclonal antibody and protein A-agarose were from Santa Cruz Biotechnology. The hemagglutinin (HA)-specific antibody was from Babco, whereas the Flag M1-specific monoclonal antibody was purchased from Sigma. Rhodamine Red goat anti-mouse antibody was from Molecular Probes. ECL reagents were purchased from Amersham Biosciences. The goat anti-mouse alkaline phosphatase-conjugated antibody and the alkaline phosphatase substrate kit were purchased from Bio-Rad. Polymerase chain reactions were done using the Expand High Fidelity PCR system from Roche Molecular Biochemicals. The stable thromboxane A_2 analogue U46619 was purchased from Cayman.

Cell Culture and Transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. Transient transfections of HEK293 cells grown to 75−90% confluence were performed using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Empty pcDNA3 vector was added to keep the total DNA amount added per plate constant.

Generation of Rab4 and Rab11 Constructs. The cDNA for Rab4 was obtained by reverse transcriptase-polymerase chain reaction on total RNA from HeLa cells using the primers 5′-GAGGAATTCATGTCCGAAACCTACG-3′ (Rab4F) and 5′-GAGCTCGAGCTAACAACCACACTCCTGAGC-3′ (Rab4R). The Rab11 cDNA was obtained by polymerase chain reaction on a human placenta cDNA library using the primers 5′-GAGGAATTCATGGGCACCCGCGACGACGAGTAC-3′ (Rab11F) and 5′-GAGCTCGAGTTAGATGTTCTGACAGCACTGCAC-3′ (Rab11R). All Rab cDNAs were digested with EcoRI and XhoI and ligated into pcDNA3 digested with the same restriction enzymes. The Rab11 cDNA was also subcloned from the pcDNA3 construct into pEGFP-C2 (Clontech, BD Biosciences) using EcoRI-ApaI. Integrity of the coding sequences of all the constructs was confirmed by dideoxy sequencing.

Total Inositol Phosphates Measurements. HEK293 cells were seeded at a density of 1.2×10^5 cells per well of 12-well plates. On the next day, cells were transfected with pcDNA3 alone (control), pcDNA3Flag-TPβ, or pcDNA3Flag-TPβ mutants (*12*) using FuGENE 6 according to the manufacturer's instructions. The following day, cells were metabolically labeled for 18−24 h with myo-[^{3}H]inositol at 4 μCi/mL in DMEM (high glucose, 0.5% BSA, 20 mM Hepes, pH 7.5, without inositol). On the day of the experiment, cells were washed once with phosphate-buffered saline (PBS) and then incubated in prewarmed DMEM (high glucose, without inositol) containing 0.5% BSA, 20 mM Hepes, pH 7.5, and 20 mM LiCl for 10 min. Thereafter, cells were stimulated with U46619 for indicated times and doses. Following stimulation, the medium was removed and the reactions were terminated by addition of 0.8 mL of ice-cold 0.4 M perchloric acid. Samples were collected in microfuge tubes and 0.35 mL of a 0.72 N KOH, 0.6 M $KHCO_3$ solution was added. Tubes were mixed then centrifuged for 5 min at 14 000 rpm in a microcentrifuge. Inositol phosphates were separated on AG 1-X8 resin (Bio-Rad Laboratories). Total labeled inositol phosphates were eluted with 1.5 M ammonium formate and counted by liquid scintillation. For experiments involving the recycling inhibitor monensin, cells were washed once with PBS and then pretreated for 30 min at 37 °C with 50 μM monensin in prewarmed DMEM supplemented with 0.5% BSA, 20 mM Hepes, pH 7.5, 20 mM LiCl. Following pretreatment, the medium was replaced with prewarmed DMEM containing 0.5% BSA, 20 mM Hepes, pH 7.5, 20 mM LiCl, 50 μM monensin and 3 μM U46619 or EtOH (control) for 60 min. Various pretreatment and stimulation conditions were assayed for monensin, and conditions observed not to affect cell viability were chosen.

Immunoprecipitations. HEK293 cells grown in 6-well plates were transfected with 2 μg/well of the pcDNA3, pcDNA3HA-TPβ, pcDNA3HA-TPβY339A, pcDNA3HA-TPβI343A, or pcDNA3HA-TPβR328Stop DNA constructs. Post-transfection (48 h), cells transfected with wild-type or mutant receptors were incubated in the presence or the absence of 500 nM U46619 for 10 min at 37 °C. Following stimulation, cells were harvested and rinsed twice with ice-cold PBS. Cells were then resuspended in 800 μL of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% IGEPAL, 0.5% deoxycholate, 0.1% SDS, 10 mM $Na_4P_2O_7$, 5 mM EDTA) containing protease inhibitors (10 nM chymostatin, 10 nM leupeptin, 9 nM antipain, and 9 nM pepstatin). After a 60-min incubation with rotation at 4 °C in lysis buffer, cell lysates were clarified by a 20-min centrifugation at 14 000 rpm at 4 °C. One microgram of HA-specific monoclonal antibody was added to the supernatant, and the reaction was carried out with rotation for 60 min at 4 °C. Thereafter, 50 μL of 50% protein-A-agarose preequilibrated in lysis buffer was added, followed by an overnight incubation with rotation at 4 °C. Samples were then centrifuged for 1 min at 3000 rpm in a microcentrifuge and washed three times with 1 mL of lysis buffer containing fresh protease inhibitors. Immunoprecipitated proteins were eluted by addition of 50 μL of SDS sample buffer followed by a 60-min incubation at room temperature. Initial lysates and immunoprecipitated proteins were analyzed by SDS−PAGE and then immunoblotting was performed with the indicated specific antibodies.

Internalization Assays. Quantification of receptor internalization was determined by ELISA as previously described (*12*, *15*) using transiently transfected HEK293 cells. Cells were plated out at 1.2×10^6 cells per 60-mm dish. On the following day, cells were transfected with pcDNA3 alone (control) or cotransfected with pcDNA3HA-TPβ and either pcDNA3HA-Rab4 or pcDNA3HA-Rab11. Cells were trans-

ferred to 24-well plates precoated with 0.1 mg/mL poly-L-lysine (Sigma) 24 h post-transfection at a density of 2×10^5 cells/well, and were maintained for an additional 24 h. On the day of the experiment, cells were washed once with PBS and then pretreated 30 min at 37 °C with 50 μM monensin in prewarmed DMEM supplemented with 0.5% BSA, 20 mM Hepes, pH 7.5, where indicated. Following pretreatment, the medium was replaced with prewarmed DMEM containing 0.5% BSA, 20 mM Hepes, pH 7.5, 50 μM monensin, and 1 μM U46619 or EtOH (control). Cells were then incubated for only 60 min at 37 °C because the presence of monensin for longer periods of time affected cell viability. Cells that were not subjected to any pretreatment were directly incubated in the presence or the absence of 1 μM U46619 at 37 °C for 120 min. After stimulation of the sample, the medium was removed and the cells were fixed in 3.7% formaldehyde/TBS (20 mM Tris, pH 7.5, 150 mM NaCl) for 5 min at room temperature. Cells were then washed three times with TBS and nonspecific binding blocked with TBS/1% BSA for 45 min. HA-specific monoclonal antibody was then added at a dilution of 1:1000 in TBS/1% BSA for 60 min. Following incubation with the primary antibody, cells were washed three times with TBS and blocked again in TBS/1% BSA for 15 min. Incubation with goat anti-mouse conjugated alkaline phosphatase (Bio-Rad) diluted 1:1000 in TBS/1% BSA was carried out for 60 min. The cells were washed three times with TBS and 250 μL of a colorimetric alkaline phosphatase substrate was added. Plates were then incubated at 37 °C until a yellow color appeared. A 100 μL aliquot of the colorimetric reaction was taken, stopped by the addition of 100 μL 0.1 N NaOH, and read at 405 nm using a Titertek Multiskan MCC/340 spectrophotometer. Cells transfected with pcDNA3 alone were studied concurrently to determine background.

Immunofluorescence Microscopy. HEK293 cells were seeded at a density of 1.2×10^6 cells per 60-mm plate. The next day, cells were transiently transfected with pcDNA3 alone (control) or cotransfected with pcDNA3Flag-TPβ or pcDNA3Flag-TPβY339A and pEGFP-C2-Rab11. On the following day, 2×10^5 cells were transferred onto coverslips and further grown overnight. Cells were incubated with Flag M1 antibody at a 1:500 dilution for 2 min at 4 °C, then 58 min at 16 °C in DMEM supplemented with 1% BSA and 1 mM $CaCl_2$. Cells were washed twice with ice-cold PBS/1 mM $CaCl_2$, then treated or not with 500 nM U46619 for 120 min at 37 °C in DMEM containing 0.5% BSA, 20 mM Hepes, pH 7.5, 1 mM $CaCl_2$. Cells were then fixed with 3% paraformaldehyde in PBS/1 mM $CaCl_2$ for 20 min at room temperature, washed with PBS/1 mM $CaCl_2$, and permeabilized with 0.1% Triton X-100 in PBS/1 mM $CaCl_2$. Nonspecific binding was blocked with 0.1% Triton X-100 in PBS/1 mM $CaCl_2$ containing 5% nonfat dry milk for 30 min at room temperature. Goat anti-mouse Rhodamine Red-conjugated secondary antibody (Molecular Probes) was then added at a dilution of 1:200 in blocking solution for 60 min at room temperature. The cells were washed 4 times with permeabilization buffer, with the last wash left at room temperature for 30 min. Finally, the cells were fixed with 3% paraformaldehyde as described above. Coverslips were mounted using Vectashield mounting medium (Vector Laboratories) and examined on a Nikon Eclipse TE2000-U inverted fluorescence microscope using a plan fluor 40×/0.75 objective. Image acquisition was done using a Hamamatsu Photonics C4742−95−12ER camera and Simple PCI high performance imaging software, and processed with Adobe Photoshop. The observer for these immunofluorescence experiments was blinded to the various indicated treatments and transfected constructs.

RESULTS

Our first goal in the present study was to demonstrate a role for constitutive endocytosis in the function of TPβ via second messenger generation experiments. To do so, we performed a dose−response experiment to measure total inositol phosphates production in HEK293 cells transiently transfected with Flag-tagged versions of TPβ or either of two mutants specifically deficient in constitutive endocytosis, TPβ-Y339A and TPβ-I343A (*15*). TPβ and the two TPβ-Y339A and TPβ-I343A receptor mutants have the same affinity for the agonist, as indicated by the binding of the TP antagonist [^{3}H]SQ29,548 that was displaced by U46619 with K_i values of 0.87 ± 0.17 μM (mean ± SE, $n = 4$) (*15*). Cell surface expression of the three receptors was adjusted by ELISA to levels corresponding to 0.8 ± 0.1 pmol of receptor/mg of protein (*12*). As shown in Figure 1A, the generation of inositol phosphates was always superior with TPβ than with the TPβ-Y339A and TPβ-I343A mutants following stimulation with concentrations of U46619 ranging from 100 nM to 10 μM. We then performed a time-course experiment of total inositol phosphates production in cells expressing the same constructs with a 3 μM U46619 stimulation for 0 to 60 min at 37 °C. Again, second messenger production was much more important for TPβ than for the two mutants specifically deficient in constitutive endocytosis (Figure 1B). Our data indicate that the two TPβ-Y339A and TPβ-I343A mutants are unable to sustain a signaling response analogous to the wild-type TPβ. Pretreatment of TPβ-expressing cells with 50 μM monensin for 30 min to prevent receptor recycling inhibited agonist-induced inositol phosphates production by 75% (Figure 1C). Taken together, our results strongly suggest that constitutive endocytosis of TPβ forms an intracellular pool of receptors that recycle to the cell surface with the fundamental role of preserving agonist sensitivity over prolonged receptor stimulation.

We next wanted to confirm that the differences observed in inositol phosphates generation were not a reflection of altered $G\alpha_q$-coupling of the mutant receptors. As seen in Figure 2 (upper panel), the same amount of endogenous $G\alpha_q$ protein was co-immunoprecipitated with TPβ, TPβ-Y339A, or TPβ-I343A in the absence or presence of U46619, showing that $G\alpha_q$-coupling of the two receptor mutants was the same as the wild-type receptor.

We then used TPβ carboxyl-terminal tail alanine mutants ranging from amino acids 338 to 344 (*15*) as a tool to demonstrate that the two TPβ-Y339A and TPβ-I343A mutations are not located in a receptor region involved in $G\alpha_q$ protein activation. Figure 3 shows that only the two mutants deficient in constitutive endocytosis displayed significantly reduced second messenger release. To further confirm this point, we co-immunoprecipitated the TPβ-R328Stop receptor mutant (*12*) with $G\alpha_q$ and observed that its coupling to $G\alpha_q$ was similar to that of TPβ (Figure 4A). Total inositol phosphates production measurement experi-

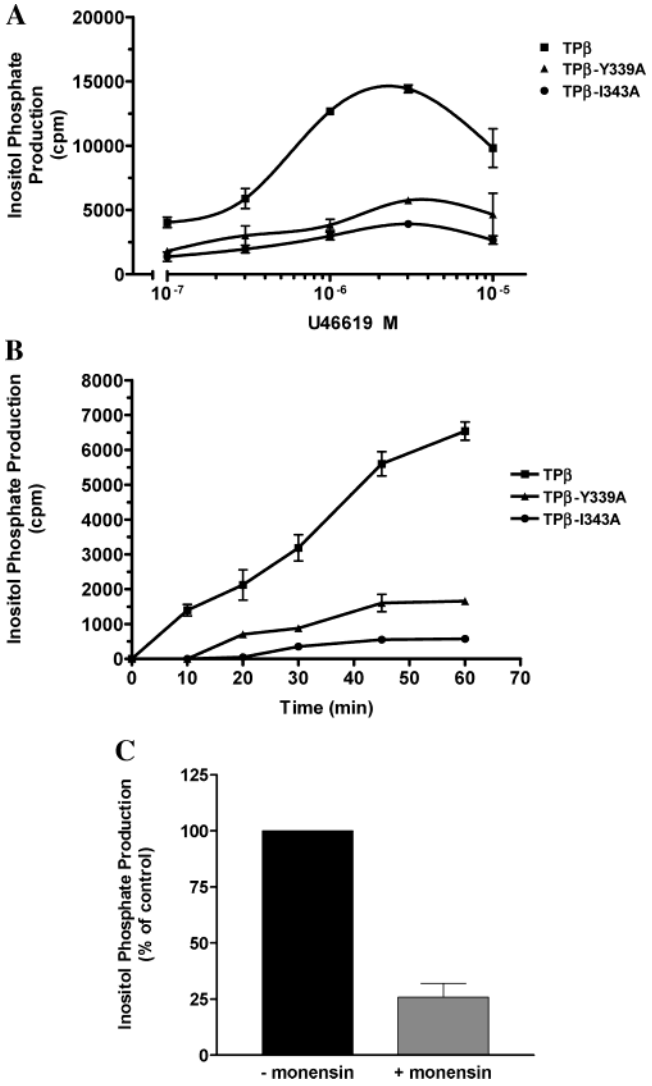

FIGURE 1: Total inositol phosphates generation following agonist stimulation of the TPβ, TPβ-Y339A, and TPβ-I343A receptors. HEK293 transiently transfected with either of the three receptors were metabollically labeled with *myo*-[^{3}H]inositol and then (A) stimulated with concentrations of U46619 ranging from 100 nM to 10 μM for 60 min, (B) exposed to 3 μM U46619 for a period of 0 to 60 min, or (C) pretreated with 50 μM monensin prior to a 60-min stimulation with 3 μM U46619 in the presence of monensin. Total [^{3}H]-inositol phosphates were isolated as described in Experimental Procedures, and measured by liquid scintillation counting. All values are mean ± SE from 3−5 separate experiments.

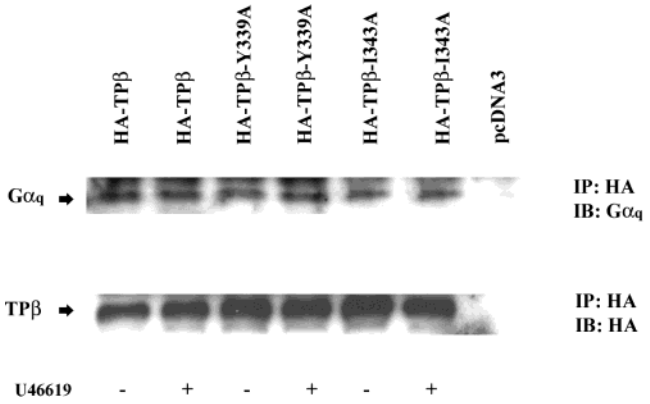

FIGURE 2: Co-immunoprecipitations of the TPβ, TPβ-Y339A, and TPβ-I343A receptors with the Gα_q protein. HEK293 cells transiently cotransfected with the indicated constructs were stimulated or not with 500 nM U46619 for 10 min and processed for immunoprecipitations as described under Experimental Procedures. Immunoprecipitations of receptors were carried out using a mouse anti-HA monoclonal antibody, and immunoblotting performed with the same antibody (lower panel) or with a Gα_q-specific polyclonal antibody (upper panel). Results are representative of four different experiments.

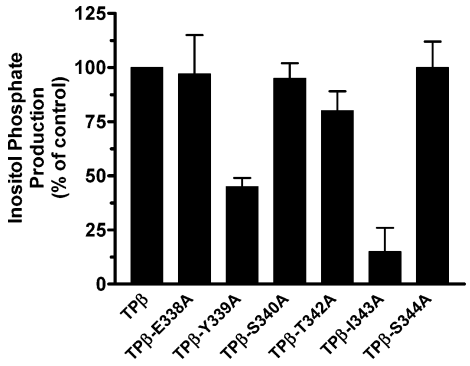

FIGURE 3: The TPβ-Y339A and TPβ-I343A mutations are not located in a receptor domain involved in Gα_q protein activation. HEK293 cells transiently transfected with either of the indicated TPβ receptor alanine mutants were metabollically labeled with *myo*-[^{3}H]inositol and then exposed to 3 μM U46619 for 60 min. Total [^{3}H]-inositol phosphates were isolated as described in Experimental Procedures and measured by liquid scintillation counting. Results are shown as a percentage of the wild-type receptor total inositol phosphates production. All values are mean ± SE from three separate experiments.

ment was carried out in HEK293 cells expressing equal levels of TPβ or TPβ-R328Stop stimulated with 3 μM U46619 for only 30 s to avoid contribution of TPβ constitutive endocytosis in the second messenger generation. As illustrated in Figure 4B, the inositol phosphates levels produced by each receptor were identical. Therefore, observations presented in Figures 2−4 prove that the distal portion of the TPβ carboxyl-terminal tail is not involved in the coupling to the Gα_q protein, indicating that the differences in second messenger generation reported in Figure 1 result from the inability of the receptor mutants to undergo constitutive endocytosis.

We previously observed that constitutively endocytosed TPβ accumulated in a perinuclear intracellular compartment from which they recycled back to the cell surface (*15*). Recently, a number of studies reported the involvement of the small GTPase Rab proteins in the trafficking of various membrane receptors. More particularly, Rab4 and Rab11 are known to regulate recycling of membrane components from the early and perinuclear recycling endosomes, respectively (*29*). Therefore, in the second part of our study, we were interested in determining the possible effects of Rab4 and Rab11 on agonist-induced endocytosis of TPβ. Coexpression of Rab11, but not of Rab4, with TPβ conferred a 2-fold increase in cell surface expression of the receptor (Figure 5A). Similarly, only Rab11 significantly affected agonist-induced endocytosis of TPβ (Figure 5B), and this effect was inhibited by monensin (Figure 5C). Thus, our results suggest that TPβ is recycled back to the cell surface by a Rab11-dependent pathway following constitutive and agonist-induced endocytosis.

We then assessed whether TPβ and Rab11 co-localized intracellularly in the perinuclear cell compartment where we observed accumulation of TPβ before (*15*). To distinguish

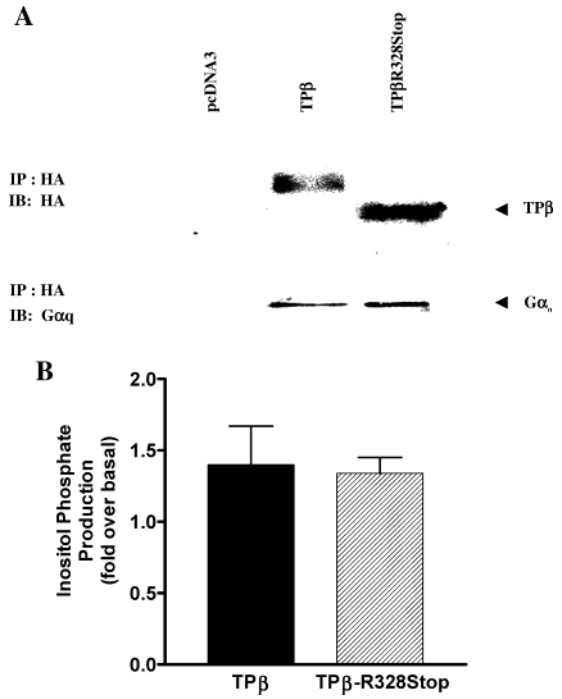

FIGURE 4: The distal region of the TPβ carboxyl-terminal tail is not involved in $G\alpha_q$ protein coupling. (A) HEK293 cells transiently expressing HA-TPβ or HA-TPβR328Stop were processed for immunoprecipitations as described under Experimental Procedures using a mouse anti-HA monoclonal antibody, and immunoblotting performed with the same antibody (upper panel) and with a $G\alpha_q$-specific polyclonal antibody (upper panel). Representative Western blots are shown. (B) HEK293 cells transiently transfected with either HA-TPβ or HA-TPβR328Stop were metabollically labeled with myo-[^{3}H]inositol and then stimulated with 3 μM U46619 for 30 s. Total [^{3}H]-inositol phosphates were isolated as described in Experimental Procedures, and measured by liquid scintillation counting. Results are mean $\pm$ SE from three separate experiments.

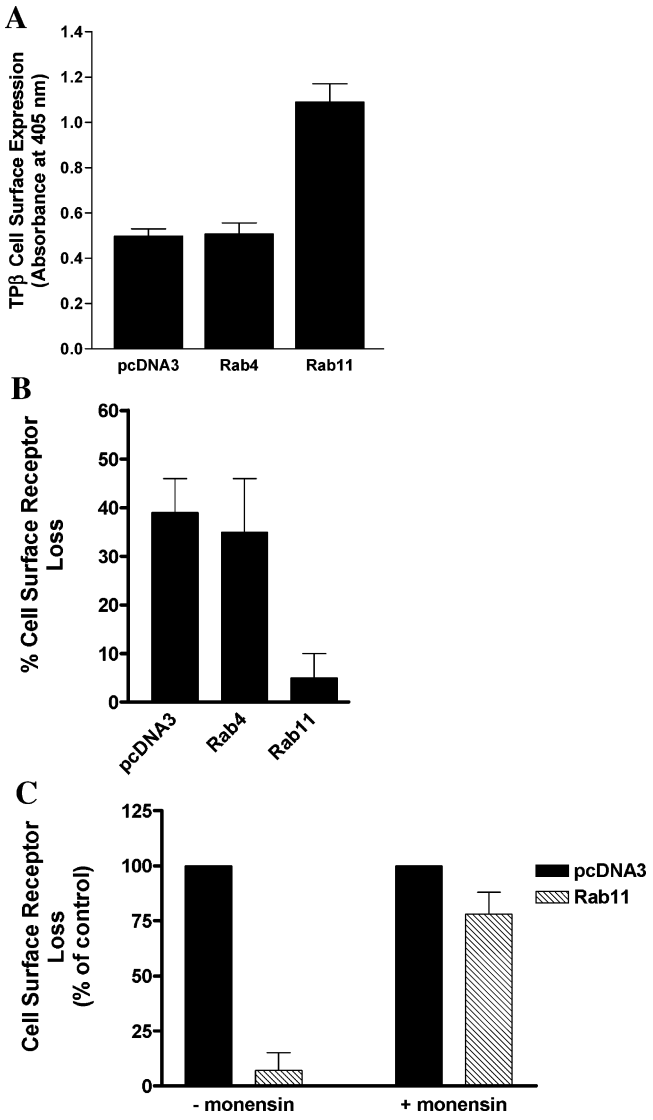

FIGURE 5: Rab11, but not Rab4, promotes recycling of TPβ to the cell surface. (A) Cell surface expression of HA-tagged TPβ was evaluated in HEK293 cells transiently cotransfected with pcDNA3-HATPβ and either pcDNA3, pcDNA3-Rab4, or pcDNA3-Rab11. (B) HEK293 cells transiently cotransfected with pcDNA3-HATPβ and either pcDNA3, pcDNA3-Rab4, or pcDNA3-Rab11 were subjected to a 1 μM U46619 stimulation for 120 min. (C) HEK293 cells were transiently cotransfected with pcDNA3-HATPβ and either pcDNA3 or pcDNA3- Rab11. Cells were pretreated with 50 μM monensin (a recycling inhibitor) or ethanol (control) for 30 min at 37 °C, and then stimulated with 1 μM U46619 for 60 min at 37 °C in the presence of monensin. Cell surface receptor expression was measured by ELISA as described in Experimental Procedures. Results are mean $\pm$ SE from five separate experiments.

between both types of endocytosis, we utilized the constitutive endocytosis-deficient TPβ-Y339A and TPβ-I343A mutants. This way, trafficking of the receptor following constitutive endocytosis could be studied in absence of agonist with wild-type TPβ, while trafficking specifically implicated in agonist-induced endocytosis could be assessed with TPβ-Y339A and TPβ-I343A in the presence of U46619. Rab11 was tagged with EGFP in its amino-terminal extremity (described under Experimental Procedures) while the Flag epitope-tagged receptors were detected by monoclonal Flag M1 primary antibody and goat anti-mouse Rhodamine Red-conjugated secondary antibody staining. Cells were preincubated with the anti-Flag antibody prior to endocytosis experiments to allow visualization of trafficking of only the receptors initially present at the cell surface (see Experimental Procedures) (*15*). There was an extensive co-localization of the receptor with Rab11 in the perinuclear region following 120 min of constitutive and agonist-promoted endocytosis of TPβ, whereas TPβ-Y339A and TPβ-I343A significantly co-localized with Rab11 only after agonist treatment (Figure 6).

DISCUSSION

TPβ was previously demonstrated to undergo constitutive (tonic) and agonist-induced endocytosis by our group (*12*, *15*). During these studies, we determined that a $YX_{2-3}\varphi$ (where X is any amino acid and φ is a bulky hydrophobic amino acid) motif found in the carboxyl-terminal tail of TPβ is specifically responsible for its constitutive endocytosis and that the receptor was recycled back to the cell surface after tonic internalization (*15*). Thus, our first goal in the present study was to demonstrate a role for constitutive endocytosis in the function of TPβ via second messenger generation experiments. With the use of two receptor mutants specifically defective in tonic endocytosis, TPβ-Y339A and TPβ-I343A (*15*), we showed that constitutive endocytosis of TPβ helps to maintain an intracellular pool of receptors that recycle to the cell surface to preserve agonist sensitivity during continuous stimulation. We confirmed by co-immunoprecipitations that the significant differences in second

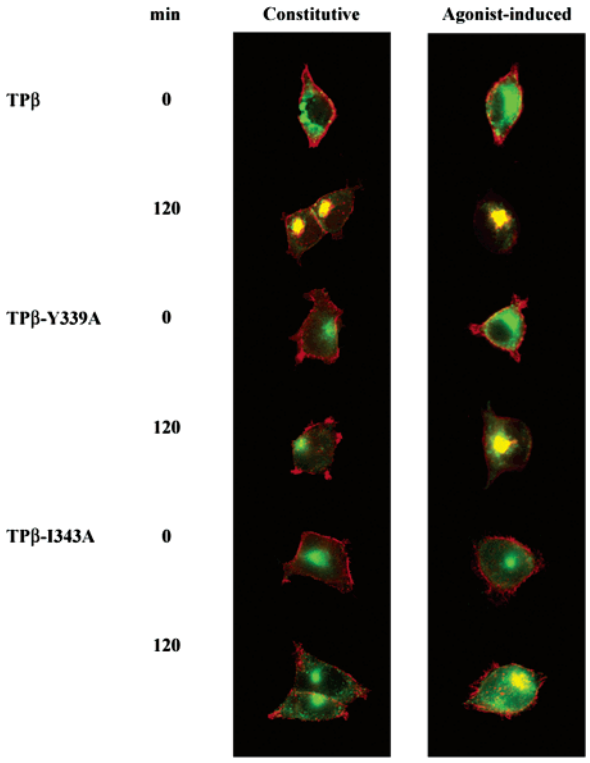

FIGURE 6: Targeting of TPβ to a Rab11-positive compartment. HEK293 cells transiently coexpressing EGFP-Rab11 and either Flag-TPβ, Flag-TPβ-Y339A, or Flag-TPβ-I343A were allowed to undergo constitutive or agonist-induced endocytosis for 120 min and processed for fluorescence microscopy as described under Experimental Procedures. Receptors and Rab11 are seen in red and green, respectively. Results are representative of three independent experiments.

messenger generation between the wild-type and the mutant receptors were not attributed to an impaired interaction between TPβ-Y339A or TPβ-I343A and the Gα_q protein. The fact that the same amount of Gα_q co-immunoprecipitated with the three receptors in the absence or the presence of the agonist U46619 was somewhat intriguing to us at first since we expected levels of receptor-associated Gα_q proteins to vary between nonstimulated and stimulated samples. However, supporting our data, Biddlecome et al. (*30*) suggested that the GAP activity of phospholipase Cβ1 on Gα_q leads to rapid hydrolysis of GTP on the Gα_q subunit so that the GTP-bound form of the G protein does not have time to completely dissociate from the receptor. Our co-immunoprecipitation results are further evidence of the existence of stable receptor/G protein interactions reported by other investigators (reviewed in ref *31*). With the help of various receptor mutants, we clearly showed that the distal portion of the carboxyl-terminal tail of TPβ is not involved in the coupling to, or the activation of, the Gα_q protein. This is in accordance with Gα protein-coupling domains on GPCRs which are mainly concentrated in regions proximal to the membrane in the third intracellular loop and the carboxyl-terminal tail (*32*). Therefore, an impaired coupling to the Gα_q protein cannot account for the lower levels of inositol phosphates production measured for TPβ-Y339A and TPβ-I343A. Treatment of cells expressing wild-type TPβ with monensin drastically reduced the receptor response to continuous stimulation, demonstrating that receptor recycling to the cell surface is necessary for maintaining its sensitivity to agonist exposure. Monensin is a well-known, widely used inhibitor of receptor recycling, but is also known as having a broad specter of actions. However, the fact that it blocks the Rab11-mediated effects (see below) strongly indicates that monensin inhibits receptor recycling in our system. Despite showing deficiency only in constitutive endocytosis, we cannot exclude that the TPβ-Y339A and TPβ-I343A mutants are not affected in other pathways. The Tyr339 and Ile343 residues form a $YX_{2-3}\varphi$ motif (*15*). The YXXφ amino acid motif can be involved in binding to various adaptor proteins. Experiments are underway in our laboratory to verify whether this motif in the TPβ receptor is responsible for interactions with such proteins.

Thus altogether, the results depicted in Figures 1−4 prove that constitutive endocytosis and recycling of TPβ are responsible for the differences in second messenger generation observed between the wild-type and mutant receptors. In the past few years, there has been a growing number of GPCRs reported to undergo agonist-independent endocytosis (*20*−*28*). Yet, very little is known regarding the function of this process in GPCR biology. Elegant experiments performed on the protease-activated receptor 1 (PAR1) showed that the unactivated receptor cycles tonically between the cell surface and an intracellular pool, providing an intracellular store of uncleaved receptors and allowing repopulation of the cell surface with uncleaved receptors after thrombin exposure without new receptor synthesis (*33*, *34*). PAR1 mutants that did not internalize in the absence of agonist were also shown to localize exclusively to the cell surface and to be defective in their ability to repopulate the cell surface with uncleaved receptors after thrombin exposure (*33*). This raised the following question: is the tonic internalization that is necessary for maintenance of the intracellular pool of PAR1 a specific mechanism of this receptor because of its peculiar way of activation? Here, we showed that the formation of an intracellular pool of TPβ following constitutive endocytosis, and its ensuing recycling to the cell surface, preserves agonist sensitivity in the context of sustained receptor stimulation. Thus, it seems like constitutive or tonic internalization of GPCRs could be an important regulatory mechanism for various members of this receptor family.

To characterize the intracellular compartment where the pool of constitutively endocytosed TPβ was formed, we performed experiments with the small GTPase Rab4 and Rab11 proteins, which are known to regulate recycling of receptors from the early and perinuclear recycling endosomes, respectively (*29*). Only Rab11 affected the levels of cell surface detection of TPβ both in unstimulated and agonist-stimulated cells. This Rab11 effect could be blocked by the recycling inhibitor monensin, showing that Rab11 was promoting recycling of the receptor at the cell surface. Immunofluorescence microscopy experiments revealed that TPβ and Rab11 co-localized extensively following both constitutive and agonist-induced endocytosis. Seachrist et al. (*35*) reported that the recycling of the β_2-adrenergic receptor back to the cell surface was controlled by Rab4. Rab11 overexpression was recently demonstrated to establish AT1A receptor recycling (*36*). Co-localization with Rab11 was observed for the β_2-adrenergic, the V2 vasopressin, SST3 somatostatin, AT1 angiotensin, and PAR2 receptors (*26*, *37*−*40*). Because of the robust Rab11 co-localization with

TPβ, and of its effect on the receptor recycling, Rab11 is likely the governing factor of TPβ recycling from the intracellular pool to the cell surface.

In summary, we demonstrated before that both constitutive and agonist-induced endocytosis of TPβ were dynamin-dependent, but only the latter was regulated by GRKs and arrestins (*12*, *15*). We report in the present study that constitutive and agonist-induced endocytosis both target TPβ to a pool of receptors formed in an intracellular compartment corresponding to the Rab11-positive perinuclear recycling endosome. Constitutive endocytosis of TPβ helps to maintain agonist sensitivity over prolonged receptor stimulation. Our present and previous observations (*12*, *15*) raise the possibility of a biologically important functional determinant between the two isoforms of the TP receptor. TPα is the predominantly expressed isoform in platelets at the protein level (*41*). A sudden burst of TP receptor activation in response to TXA_2 generation without sustained receptor response may be relevant to platelet physiology. On the other hand, preservation of sensitivity to agonist exposure for TPβ, which is expressed in endothelial cells, could be relevant to situations such as angiogenesis.

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BI036268V