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Agents that elevate cyclic AMP induce receptor phosphorylation primarily at serine 331 in HEK 293 cells overexpressing human thromboxane receptor α

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Abstract

Human embryonic kidney (HEK) 293 cells stably transfected with the His-tagged thromboxane receptor α (TPα) were used to study the phosphorylation and desensitization of the receptor induced by prostaglandin E_1 (PGE_1) or forskolin. These agents are known to increase the intracellular level of cyclic AMP (cAMP) and activate cAMP-dependent protein kinase (PKA). Pretreatment of cells with either agent significantly attenuated Ca^{2+} release induced by the agonist [1*S*-[1α,2α(*Z*),3β(1*E*,3*S*),4α]]-7-[3-[3-hydroxy-4-(4-indophenoxy)-1-butenyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid (I-BOP). These agents also induced concentration-dependent phosphorylation of TPα as demonstrated by increased ^{32}P-labeling of the receptor from cells prelabeled with $^{32}P_i$. To facilitate the identification of the intracellular domains involved in phosphorylation, glutathione *S*-transferase (GST)-intracellular domain fusion proteins were used as substrates for purified PKA. It was found that only the C-terminal tail fusion protein could serve as a substrate for PKA. To identify the specific serine/threonine residues in the C-terminal tail that are involved in phosphorylation, various alanine mutants of these residues were checked for their ability to serve as substrates. Ser-331 was found to be involved in PKA-mediated phosphorylation. The S331A mutant receptor overexpressed in HEK 293 cells was not phosphorylated significantly following stimulation by PGE_1 or forskolin, indicating that Ser-331 was the major site of phosphorylation. Furthermore, cells overexpressing the mutant receptor became responsive to I-BOP-induced Ca^{2+} mobilization even after pretreatment with PGE_1 or forskolin. These results indicate that Ser-331 is the primary site responsible for the phosphorylation and desensitization of the human TPα induced by agents that activate PKA.

Keywords: Thromboxane A_2; Receptor; Protein kinase A; Cyclic AMP; Prostaglandin E_1; Phosphorylation; Desensitization

1. Introduction

TXA_2, a product of prostaglandin endoperoxide isomerization catalyzed by thromboxane synthase [1], exhibits potent and diverse biological activities. It induces platelet aggregation and vascular and respiratory smooth muscle contraction [2]. It also stimulates mitogenesis and the hypertrophy of vascular smooth muscle cells [3]. In addition, its mimetics induce apoptosis of immature thymocytes and may regulate thymocyte differentiation and development [4]. These actions are transduced by cell surface receptors identified with a variety of structural ligands [5]. Although such a pharmacological study suggests diversity among TPs, a single gene encoding a member of the heptahelical superfamily of GPCRs has been cloned [6]. Two isoforms, derived from alternate splicing of the carboxyl-terminal tail of the receptor, have been identified [7]. Human TPα mRNA encodes a protein of 343 amino acids with a molecular mass of 37 kDa. However, purified native TPα exhibits a molecular mass of 55–57 kDa in human platelets and other animal tissues,

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Abbreviations: cAMP, cyclic AMP; cGMP, cyclic GMP; DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; GSH, glutathione; GST, glutathione-*S*-transferase; HEK, human embryonic kidney; NTA, nitrilotriacetic acid; PCR, polymerase chain reaction; PGD_2 and PGE_1, prostaglandin D_2 and E_1, respectively; PGI_2, prostacyclin; PKA, cAMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; TP, thromboxane receptor; and TXA_2, thromboxane A_2.

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indicating that the receptor is most likely a glycoprotein [8,9]. Human TPβ mRNA encodes a protein of 406 amino acids and is identical in sequence to that reported for TPα for the first 328 amino acids. A difference in the C-terminal tails of the two isoforms has been shown to account for differential responses to agonist-induced receptor internalization [10] and to prostacyclin-mediated receptor desensitization [11].

It is known that protein kinase-mediated phosphorylation of the GPCRs modulates their functions, including desensitization [12]. GPCR kinases are thought to be responsible for agonist-induced homologous desensitization of the receptors [13], whereas second messenger kinases such as cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG) or protein kinase C are believed to be involved in the heterologous desensitization of the receptors [14]. Activation of these protein kinases by exogenous agonists generally leads to phosphorylation of the intracellular domains and subsequent desensitization of the receptors. The third intracellular loop and the C-terminal tail have been highlighted as the most important structural features involved in phosphorylation and desensitization [15]. Recent studies on the TP have confirmed that the C-terminal tail plays an important role in phosphorylation, desensitization, and internalization [10,11,16,17].

Studies on the regulation of platelet function by prostaglandins date back to the first report by Kloeze [18], who demonstrated that PGE_1 was a potent inhibitor of platelet aggregation. The inhibitory activity of PGE_1 on platelet aggregation was subsequently associated with an elevation in platelet cAMP levels [19] and could be duplicated with PGD_2 and PGI_2 [20,21]. In fact, PGI_2 appears to be the most potent inhibitor of platelet aggregation induced by TXA_2 [21]. The counter actions of PGI_2 and TXA_2 lead to the proposal that the levels of these two eicosanoids may control vascular homeostasis [22]. The mechanism of interaction between cAMP-elevating prostaglandins and TXA_2 has been attributed to PKA-mediated phosphorylation of the TP [23]. However, the precise site of phosphorylation remains to be determined. In this report, we utilized HEK 293 cells overexpressing human TPα and demonstrated that Ser-331 in the C-terminal tail is the primary site of phosphorylation induced by agents that elevate intracellular levels of cAMP. A separate report describing phosphorylation of the C-terminal tail of the TPα by agents that elevate intracellular levels of cGMP has been published [24].

2. Materials and methods

2.1. Materials

pcDNA3 expression vector was from Invitrogen. PGEX-2T plasmid vector was from Pharmacia Biotech. T4 DNA ligase and *Vent* DNA polymerase were from New England Biolabs, Inc. *Pfu* DNA polymerase was from Stratagene. HEK 293 cells were obtained from the American Type Culture Collection. *Taq* DNA polymerase, *Eco*RI, *Bam*HI, heat-inactivated fetal bovine serum, and antibiotic–antimycotic and geneticin-selective antibiotic (G418) were from GIBCO-BRL. The QIAprep Spin Plasmid Miniprep Kit, the QIAquick PCR Purification Kit, the QIA Quick Gel Extraction Kit, and Effectene transfection reagent were from QIAGEN. [1*S*-[1α,2α(*Z*),3β(1*E*,3*S*),4α]]-7-[3-[3-hydroxy-4-(4-indophenoxy)-1-butenyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid (I-BOP), PGE_1, and SQ29,548 were from Cayman Chemical. $^{32}P_i$ (500 mCi/mL) and [γ-^{32}P]ATP (10 Ci/mmol) were from ICN Pharmaceuticals, Inc. Fura-2/AM was purchased from the Calbiochem-Novabiochem Corp. Horseradish peroxidase-linked protein A was obtained from Transduction Laboratories. Oligonucleotide primers were synthesized by Integrated DNA Technologies. DMEM, Ni^{2+}-NTA agarose, GSH agarose, PMSF, forskolin, leupeptin, soybean trypsin inhibitor, benzamidine, purified bovine heart catalytic subunit of PKA (50 units/μg), and other chemicals were from the Sigma Chemical Co. The ECL$^+$ western blotting detection system was obtained from Amersham. Rabbit antiserum against the N-terminal sequence (MWPNGSSLGPCFRPTN) of human TPα was prepared as described previously [25].

2.2. Subcloning of C-terminal His-tagged human TPα cDNA

His-tagged human TPα cDNA was amplified from pBacPak8-TPα plasmid DNA by PCR using *Vent* DNA polymerase as described previously [26]. The oligonucleotides used for PCR were designed based on the amino terminal sequence (5′-CGGGATCCATGTGGCCCAACGGCAGTTC-3′) and the carboxyl terminal sequence (5′-ATAGAATTCTCA*ATGGTGATGGTGATGGTG*CTGCAGCCCGGAGC-3′) with extra *Bam*HI and *Eco*RI sites on the ends, respectively, and six histidinyl residues on the C-terminus. The PCR product was subcloned into the mammalian expression vector pcDNA3 at *Bam*HI and *Eco*RI sites. The insertion of the cDNAs was confirmed by DNA sequencing.

2.3. Site-directed mutagenesis of TPα and its C-terminal tail

Site-directed mutagenesis was carried out by the Megaprimer method as described previously [24].

2.4. Expression of the His-tagged human wild-type and mutant TPα in HEK 293 cells

HEK 293 cells were grown in 90% DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), gentamicin, and antibiotic–antimycotic at 37° in

a humidified atmosphere of 95% air and 5% CO_2. Cells were subcultured every 3 days after becoming confluent by the use of 0.25% trypsin with 1 mM EDTA and plated at a density of 1×10^5 cells/mL.

To create cell lines stably expressing wild-type and mutant TPα, pcDNA3 expression vector containing the cDNAs of the wild-type and mutant His-tagged TPα were transfected into HEK 293 cells using Effectene transfection reagent according to the directions of the manufacturer. Briefly, 5×10^5 cells were plated per 60-mm dish in 5 mL of complete medium the day before transfection. One microgram of DNA was first mixed with DNA-condensation buffer to a total volume of 150 μL; then 8 μL of Enhancer was added. After incubation at room temperature for 5 min, 25 μL of Effectene transfection reagent was added to the DNA–Enhancer mixture, mixed, and incubated at room temperature. After 15 min, 1 mL of cell culture medium was added to the transfection complexes. This solution was mixed and added to 60-mm dishes containing HEK 293 cells washed with culture medium. Cells were incubated with the complexes at 37° and 5% CO_2 for 2 days to allow for gene expression. To isolate permanent transfectants, G418-resistant cells were selected in complete culture medium containing 500 μg/mL of G418 as described previously [25]. Expression of the wild-type and the mutant TPα was monitored by western blotting analysis using a rabbit antiserum against the N-terminal sequence of the TPα as described previously [25].

2.5. Preparation of HEK 293 cell membranes

HEK 293 cell membranes were prepared as described previously [25].

2.6. Measurement of intracellular calcium release

Intracellular calcium release was measured by fluorescence excitation of cells loaded with the fluorescent probe Fura-2/AM as described previously [25].

2.7. Western blotting analysis

Cell membranes (50 μg) prepared from HEK 293 cells expressing the His-tagged wild-type and mutant TPα were subjected to SDS–PAGE on 10% polyacrylamide gels. The proteins were then electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes, and western blotting analysis was carried out as described previously [25].

2.8. Expression of the intracellular domain specific peptides of TPα as GST-fusion proteins

The DNA sequences encoding the three intracellular loops and C-tail were generated by PCR and cloned into the pGEX-2T vector at *Bam*HI and *Eco*RI sites. Both cloning and expression of various fusion proteins were carried out as described previously [24].

2.9. In vitro phosphorylation of the intracellular domains of the TPα

In vitro phosphorylation experiments with the intracellular domain specific peptides of the TPα were carried out as described previously [24]. Reactions were performed in 50 μL of 20 mM Tris–HCl, pH 7.4, buffer containing 5 mM $MgCl_2$, 20 μg of GST-fusion peptide, and 1 μg of purified catalytic subunit of PKA. The reaction was initiated by the addition of [γ-^{32}P]ATP (0.1 mM, 1000 cpm/pmol). After incubation for 30 min at 30°, GSH-agarose (20 μL of 50% suspension) was added, and the incubation was allowed to continue for another 20 min. The GSH-agarose was precipitated by centrifugation (1000 *g* for 5 min at room temperature), washed six times with buffer (1 mL each), and then treated with SDS–PAGE loading buffer, boiled, and subjected to 10% SDS–PAGE. The ^{32}P-labeled peptide was detected by autoradiography.

2.10. In vivo phosphorylation of wild-type and mutant TPα

HEK 293 cells (1×10^6/mL) stably expressing His-tagged wild-type or mutant TPα were washed once with phosphate-free DMEM and labeled with $^{32}P_i$ (100 μCi/mL) in the phosphate-free medium for 1.5 hr at 37° as described previously [24]. The cells were then exposed to buffer alone or to I-BOP (100 nM) for 10 min. Reactions were terminated by the addition of ice-cold PBS buffer. After washing three times with ice-cold PBS buffer, cells were scraped in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μg/mL of leupeptin, 10 μg soybean trypsin inhibitor, 1 mM benzamidine, and 0.5 mM PMSF) and incubated for 30 min at 0°. Cell debris was removed by centrifugation at 10,000 *g* for 15 min at 4°. The His-tagged receptor was isolated by adding 10 μL of 50% Ni^{2+}-NTA agarose beads. After washing six times with lysis buffer, the beads were suspended in SDS-loading buffer, boiled, and subjected to 10% SDS–PAGE. The ^{32}P-labeled receptor was detected by autoradiography.

3. Results

PGE_1, PGD_2, and PGI_2 are known to inhibit platelet aggregation induced by TXA_2 [18–21]. These agents are also known to elevate intracellular levels of cAMP and to activate PKA. To facilitate studies on the molecular mechanism of receptor regulation induced by these agents,

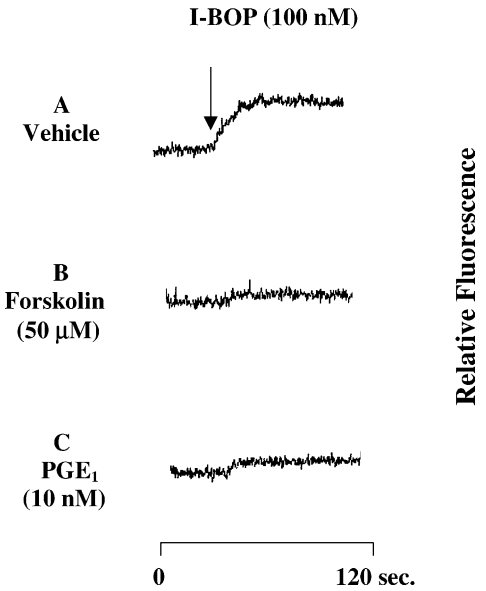

Fig. 1. Desensitization of the Ca^{2+} response induced by forskolin or PGE_1 in HEK 293 cells overexpressing the TPα. HEK 293 cells (1×10^6/mL) stably transfected with pcDNA-TPα-$(His)_6$ were treated with vehicle (A) or 50 μM forskolin (B) or 10 nM PGE_1 (C) for 10 min before being challenged with 100 nM I-BOP for 2 min. The intracellular Ca^{2+} response was examined as described under Section 2. A representative experiment of two qualitatively similar results is shown.

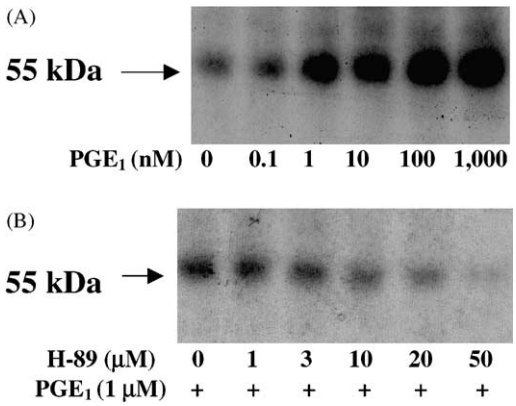

Fig. 2. PGE_1-induced phosphorylation of the TPα in overexpressing HEK 293 cells and its inhibition by a PKA inhibitor, H-89. HEK 293 cells (1×10^6/mL) stably transfected with pcDNA-TPα-$(His)_6$ were metabolically labeled with ^{32}P and then treated with increasing concentrations of PGE_1 (A) for 10 min or with increasing concentrations of H-89 for 10 min before stimulation of phosphorylation with 1 μM PGE_1 (B) for 10 min as indicated. The phosphorylated His-tagged receptor was isolated by Ni^{2+}-NTA agarose and separated using 10% SDS–PAGE as described under Section 2. A representative experiment of two qualitatively similar results is shown.

we employed HEK 293 cells overexpressing TPα-$(His)_6$ as a model system to examine receptor phosphorylation and desensitization. When HEK 293 cells expressing TPα-$(His)_6$ were pretreated with an agent that elevates intracellular cAMP such as PGE_1 or forskolin, subsequent stimulation of the cells with a receptor agonist resulted in desensitization of the receptor, as shown in Fig. 1. Cells pretreated with vehicle responded well to 100 nM I-BOP in releasing Ca^{2+}. When cells were pretreated with 10 nM PGE_1 or 50 μM forskolin for 10 min and then washed, they became refractory to I-BOP-induced Ca^{2+} release. To examine if the receptor was phosphorylated and desensitized, HEK 293 cells expressing TPα-$(His)_6$ were challenged with PGE_1 or forskolin, and the phosphorylated receptor was isolated by Ni^{2+}-NTA agarose and subjected to SDS–PAGE. Fig. 2(A) indicates that PGE_1 mediates receptor phosphorylation in a concentration-dependent manner. Near maximal phosphorylation was seen at 100 nM PGE_1. Similar concentration-dependent phosphorylation of the receptor was also observed with 2–50 μM forskolin (data not shown). To determine whether receptor phosphorylation was mediated by PKA, cells were treated with a PKA-specific inhibitor, H-89, before challenge with PGE_1. Fig. 2(B) shows that receptor phosphorylation was inhibited by H-89 in a concentration-dependent manner. Almost complete inhibition was observed at 50 μM H-89. To identify the sites of phosphorylation among the intracellular domains, the GST-intracellular loop (GST-IL) fusion proteins were examined as substrates for PKA. Fig. 3 indicates that only the C-terminal tail was phosphorylated to a significant extent by the purified catalytic subunit of PKA.

There are six Ser/Thr residues (Ser-324, Thr-325, Ser-329, Ser-331, Thr-337, and Ser-340) in the C-terminal tail. Ser-Ser and Ser/Thr-Thr/Ser residue pairs are separated by at most two amino acids. Ser and Thr to alanine mutations were introduced into each of these pairs alone and in combination as shown in Fig. 4 (mC3-mC9). Among these C-terminal tail mutants, only mutants having unaltered Ser-329/Ser331 (mC3, mC5, and mC7) were found to be phosphorylated. To narrow down the site of phosphorylation and to confirm the above findings, mutants having a single unaltered site of Ser/Thr (mP1, mP2, mP3, and mP4) were generated and tested as substrates. Only mutant mP4 having unaltered Ser-331 was found to be a substrate, as shown in Fig. 5. The results indicate that Ser-331 may be the primary site of receptor phosphorylation in intact cells following PKA activation by PGE_1 or forskolin. To verify that Ser-331 is the potential site of receptor phosphorylation induced by PGE_1 or forskolin in intact cells, HEK 293

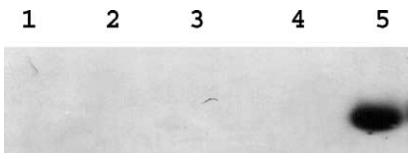

Fig. 3. *In vitro* phosphorylation of the cytoplasmic domains of the TPα by purified PKA. Each GST-intracellular loop fusion protein (20 μg) was used as a substrate for the purified PKA (1 μg). The kinase reaction and the isolation of labeled fusion proteins were carried out as described under Section 2. Lane 1: GST; lane 2: GST-1st intracellular loop; lane 3: GST-2nd intracellular loop; lane 4: GST-3rd intracellular loop; and lane 5: GST-C-terminal tail. A representative experiment of three qualitatively similar results is shown.

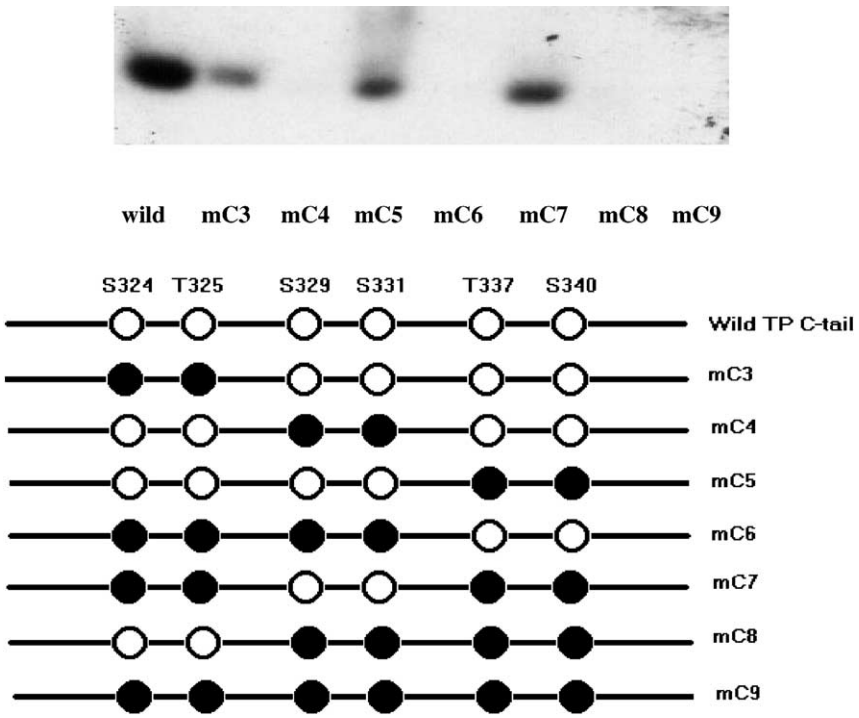

Fig. 4. *In vitro* phosphorylation of the various double mutants of the C-terminal tail of the TPα. Various mutants of the C-terminal tail were constructed by site-directed mutagenesis as described under Section 2. The dark circles indicate the specific amino acid residue being mutated to an alanine. Each GST-C-terminal tail mutant fusion protein (20 μg) was used as a substrate for purified PKA, as described in the legend of Fig. 3. The kinase reaction as well as the isolation and the SDS–PAGE analysis of GST fusion proteins were carried out as described under Section 2. A representative experiment of two qualitatively similar results is shown.

cells stably transfected with pcDNA3-TPα-$(His)_6$ having an S331A mutation were generated and used for further studies. Fig. 6 shows that PGE_1 or forskolin at two different concentrations induced phosphorylation of the receptor in HEK 293 cells, as shown above. However, these two agents did not induce significant phosphorylation of the S331A mutant receptor. To determine whether the mutant receptor still undergoes PGE_1- or forskolin-induced receptor

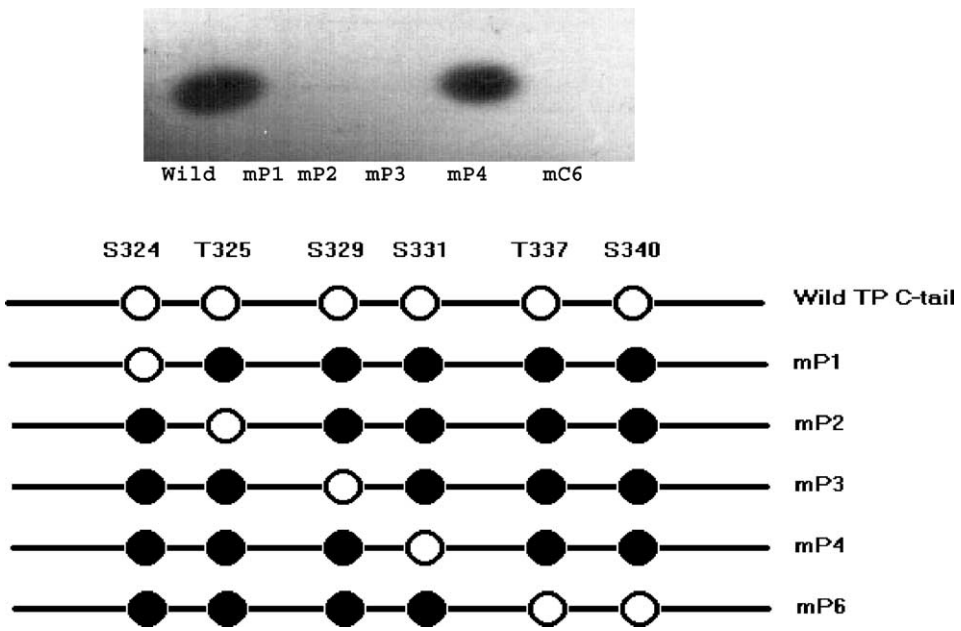

Fig. 5. *In vitro* phosphorylation of the multiple site mutants of the C-terminal tail of the TPα. Various multiple site mutants of the C-terminal tail were constructed by site-directed mutagenesis as described under Section 2. Other reaction conditions and the SDS–PAGE analysis of the fusion protein were carried out as described in the legends of Figs. 3 and 4. A representative experiment of two qualitatively similar results is shown.

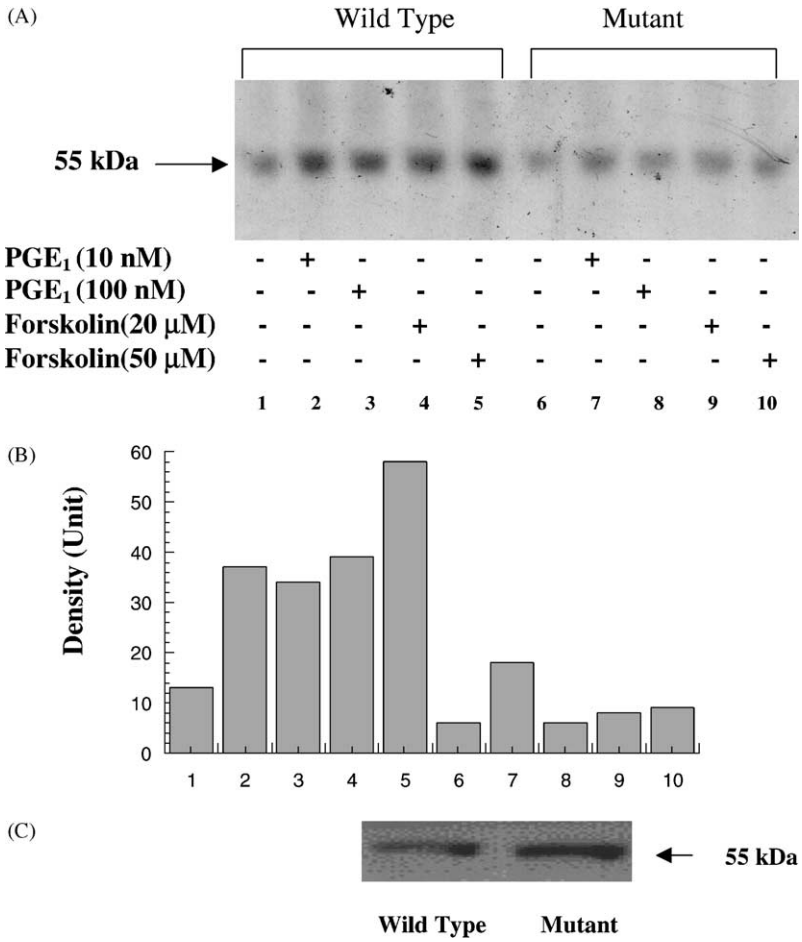

Fig. 6. *In vivo* phosphorylation of the wild-type and the S331A mutant TPα induced by PGE_1 or forskolin in TPα overexpressing HEK 293 cells. HEK 293 cells (1×10^6/mL) expressing comparable amounts of the wild-type or the S331A mutant receptor as determined by western blotting were metabolically labeled with ^{32}P and then treated with PGE_1 or forskolin at the indicated concentrations. The phosphorylated His-tagged receptor was isolated by Ni^{2+}-NTA agarose and separated using 10% SDS–PAGE as described under Section 2. (A) Autoradiogram of the levels of phosphorylation in the wild-type and in the mutant receptor induced by PGE_1 or forskolin. (B) Densitometric analysis of the levels of phosphorylation in panel A. (C) Western blot analysis of the expression of the wild-type and the mutant receptor in HEK 293 cells. A representative experiment of two qualitatively similar results is shown.

desensitization, HEK 293 cells expressing the S331A mutant receptor were treated with PGE_1 or forskolin before stimulation with I-BOP. Fig. 7 demonstrates that PGE_1 (100 nM) or forskolin (50 μM) pretreatment of cells expressing the wild-type receptor inhibited I-BOP induced Ca^{2+} release, as demonstrated earlier. However, pretreatment of cells expressing the S331A mutant receptor with vehicle did not attenuate I-BOP induced Ca^{2+}. In fact, I-BOP triggered more rapid Ca^{2+} response in cells expressing the mutant receptor than in those expressing the wild-type receptor. Pretreatment of the cells expressing the mutant receptor with 100 nM PGE_1 or 50 μM forskolin did not abolish I-BOP-induced Ca^{2+} release, although some degree of attenuation was observed as compared with vehicle-treated cells.

4. Discussion

Cross-talk between receptors is a primary mechanism responsible for their heterologous desensitization. Indeed, cross-talk occurs between TP and prostacyclin receptor (IP) or PGE receptor (EP) or PGD receptor (DP) in platelets [18–21]. Prestimulation of TP by the agonist U-46619 enhances IP agonist iloprost-induced generation of cAMP in platelets [27]. Similarly, U-46619 also augmented subsequent iloprost-mediated formation of cAMP in a PKC-dependent manner in the megakaryoblastic cell line MEG-01 [28]. In view of the interplay between TXA_2 and PGI_2 or PGE_1 or PGD_2, the intracellular signaling processes induced by any of these cAMP-elevating prostaglandins must have significant influence on TP. For example, platelets pretreated with any of these prostaglandins will not aggregate upon stimulation with a TP agonist [18–21]. We have also confirmed such a desensitization process in HEK 293 cells overexpressing TPα. We employed PGE_1 to study this interplay between receptors because PGE_1 is an agonist for the EP as well as the IP [29]. PGE_1 has been used frequently in the analysis of IP because of the instability of PGI_2. Pretreatment of these cells with PGE_1 or an adenylate cyclase activator, forskolin, significantly attenuated Ca^{2+} mobilization induced by

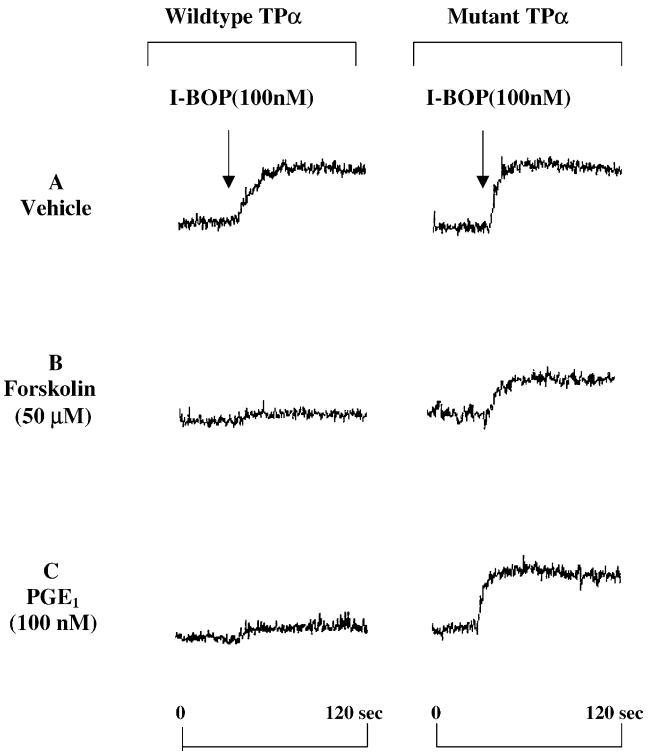

Fig. 7. Desensitization of the Ca^{2+} response induced by forskolin or PGE_1 in HEK 293 cells overexpressing the wild-type or the S331A mutant TPα. HEK 293 cells (1×10^6/mL) expressing comparable amounts of the wild-type or the S331A mutant receptor were treated with vehicle (A), 50 μM forskolin (B), or 100 nM PGE_1 (C) for 10 min before Ca^{2+} mobilization was initiated by 100 nM I-BOP for 2 min. The intracellular Ca^{2+} response was examined as described under Section 2. A representative experiment of two qualitatively similar results is shown.

I-BOP, indicating that the TP signal transduction process was hampered by the addition of these cAMP-elevating agents. The effect of PGE_1 and forskolin on the signal transduction system of TP can be attributed to the TP phosphorylation induced by these agents. Either agent can induce TP phosphorylation in a concentration-dependent manner. The receptor phosphorylation appeared to be a PKA-mediated process since the specific PKA inhibitor H-89 attenuated phosphorylation induced by PGE_1. The site of receptor phosphorylation was localized exclusively to the C-terminal tail of the receptor since the GST-C-terminal tail fusion protein was the only substrate for PKA among all of the GST-intracellular domain fusion proteins. The precise Ser/Thr residues in the C-terminal tail involved in phosphorylation were determined by a site-directed mutagenesis approach. It was found that Ser-331 was the primary site of phosphorylation. To demonstrate that this serine residue is involved in phosphorylation in intact cells, HEK 293 cells overexpressing the S331A mutant receptor were challenged with PGE_1 or forskolin. It appears that the receptor was no longer phosphorylated significantly as found in the wild type, indicating that Ser-331 was the major site of phosphorylation. That Ser-331 is involved primarily in PKA-mediated phosphorylation and desensitization was supported further by Ca^{2+} mobilization studies in which HEK 293 cells overexpressing S331A mutant TPα became responsive to I-BOP-induced Ca^{2+} release even after pretreatment of the cells with PGE_1 or forskolin. The lack of desensitization of the cells transfected with the mutant receptor strengthens the contention that phosphorylation at Ser-331 is critical for subsequent desensitization of the receptor induced by PGE_1 or forskolin.

While this study was being completed, Walsh *et al.* [11] reported that the α, but not the β, isoform of the human TP was a target for PGI_2-mediated desensitization. They have identified Ser-329 in TPα as being responsible for IP-mediated phosphorylation and desensitization. The site of phosphorylation is different from that reported in this study. Ser-329 was selected for the site-directed mutagenesis study because the PKA recognition motif was found in the sequence surrounding this residue. Other sites were not considered and studied in that report [11]. Strictly speaking, Ser-331 in the sequence of $RPRS^{329}LS^{331}L$ is a consensus sequence (RXXS/T) for phosphorylation by both PKA and PKG [30]. We have demonstrated recently in both *in vitro* and *in vivo* studies that Ser-331 is the major site of phosphorylation induced by agents that activate PKG [24]. Ser-331 appears more likely to be the common site for both PKA- and PKG-mediated receptor phosphorylation because Ser-331 but not Ser-329 is a consensus residue in the TPα of all species. This would support the involvement of a common mechanism for maintaining vascular homeostasis in mammalian systems.

Heterologous desensitization of the TP can be achieved not only by PKA-mediated phosphorylation but also by phosphorylation induced by other protein kinases. Previous reports have indicated that platelet TP can indeed serve as a substrate for PKA, PKC, and PKG [31,32]. Specifically, Kinsella *et al.* [31] showed that PKC and PKA caused phosphorylation of a fusion protein containing the latter third of the TP. This finding suggested that phorbol 12-myristate 13-acetate (PMA) or cAMP may inhibit TXA_2-induced TP activation through phosphorylation of the receptor. The site of receptor phosphorylation was explored in a few studies. Spurney and Coffman [16] first demonstrated that the C-terminus of the TP contributed to coupling and desensitization in a mouse mesangial cell line. Subsequently, Spurney [17] showed that the C-terminal tail of the mouse TP was involved not only in agonist but also in PMA-induced phosphorylation and desensitization. The S321A/S322A/S328A (equivalent to Ser-324, Ser-325, and Ser-331 in humans) mutant receptor was found to be attenuated as compared with the wild type in phosphorylation and desensitization induced by the agonist U-46619 and by PMA. Walsh *et al.* [11] demonstrated that the α, but not the β, isoform of the human TP was involved in PGI_2-mediated desensitization, indicating that the difference in C-terminal tail structures was responsible for PKA-mediated phosphorylation and desensitization. This group has further identified Ser-329 as being the

site of phosphorylation induced by PGI_2. With regard to cGMP, Wang *et al.* [32] reported that 8-Br-cGMP induced activation of PKG, which resulted in increased phosphorylation of the C-terminal tails of human TPs. We determined the precise location of the phosphorylation site to be at Ser-331 [24]. These studies indicate that the TP can be phosphorylated and heterologously desensitized by various agents that elevate intracellular levels of cyclic nucleotides and diacyl glycerol.

In addition to receptor phosphorylation, it has been recognized recently that G-protein phosphorylation may also be an important regulatory mechanism [33]. Manganello *et al*. [34] reported that cAMP induced PKA-dependent phosphorylation of the TP-associated $G\alpha_{13}$. The consequence of such a phosphorylation may be the functional uncoupling of the signal transduction system by altering the stability of the heterotrimer or by tightening its association with the receptor. It appears that cAMP-elevating agents may induce phosphorylation of the TP as well as its associated $G\alpha_{13}$, resulting in uncoupling of the receptor–G-protein complex in either situation.

In summary, the present results provide a molecular basis of cross-talk between the TP and the IP or the EP and a mechanism responsible for the desensitization of the TP. Characterization of the molecular events that underlie TP receptor desensitization in platelets and other cell types is of great importance in our understanding the mechanism of vascular homeostasis and the pathological relevance of this receptor in clinical syndromes of platelet-dependent vascular occlusion such as myocardial infarction and stroke.

Acknowledgments

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