Two Thromboxane A_2 Receptor Isoforms in Human Platelets

Opposite Coupling to Adenylyl Cyclase with Different Sensitivity to Arg^{60} to Leu Mutation

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Abstract

Thromboxane A_2 (TXA_2) receptor is a key molecule in hemostasis as its abnormality leads to bleeding disorders. Two isoforms of the human TXA_2 receptor have been cloned; one from placenta and the other from endothelium, here referred to as TXRα and TXRβ, respectively. These isoforms differ only in their carboxyl-terminal tails. We report that both isoforms are present in human platelets. The two isoforms expressed in cultured cells show similar ligand binding characteristics and phospholipase C (PLC) activation but oppositely regulate adenylyl cyclase activity; TXRα activates adenylyl cyclase, while TXRβ inhibits it. The Arg^{60} to Leu mutant of TXRα, which has been shown to impair PLC activation (Hirata, T., A. Kakizuka, F. Ushikubi, I. Fuse, M. Okuma, and S. Narumiya. 1994. *J. Clin. Invest.* 94: 1662–1667), also impairs adenylyl cyclase stimulation, whereas that of TXRβ retains its activity to inhibit adenylyl cyclase. These findings suggest that the pathway linked to adenylyl cyclase inhibition might be involved in some of the TXA_2-induced platelet responses such as shape change and phospholipase A_2 activation which remain unaffected in the patients with this mutation. (*J. Clin. Invest.* 1996. 97:949–956.) Key words: G-proteins • phospholipase C • phospholipases A • platelet activation • alternative splicing

Introduction

Platelet activation is a complex network of interdependent biochemical processes through which platelets undergo a sequence of responses: shape change, aggregation, and secretion. All of these responses can be induced by a number of physiological agonists such as thrombin, ADP, platelet-activating factor and thromboxane A_2 (TXA_2)[1] (1). TXA_2 is a major arachidonic acid metabolite in platelets that binds to the specific membrane receptor to initiate these processes (2). The TXA_2 receptor is linked via a guanine nucleotide-binding protein (G protein) of the G_q class to phospholipase C (PLC), which hydrolyses phosphoinositides to the two potent stimulatory second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (3, 4). These molecules affect divergent pathways of platelet activation; IP_3 causes increases in cytoplasmic free calcium and diacylglycerol causes activation of protein kinase C. However, whether this pathway contributes to all of the TXA_2-induced platelet responses remains undefined. Some studies using the radiolabeled TXA_2 analogs as ligands demonstrated two classes of binding sites in platelets, and indicated that they might represent the receptor subtypes (5–8). They further suggested that the two putative subtypes of the receptor may independently mediate shape change and aggregation (8, 9). This suggestion has been supported by the reports that platelet shape change and aggregation can be differentiated by several TXA_2 analogs. For example, in some species, the TXA_2 agonist U46619 induces platelet shape change and an increase in calcium without aggregation (10). In addition, the TXA_2 analog S-145 prevents aggregation and secretion by U46619 but itself induces shape change (11). One model consistent with these observations is that the TXA_2 receptor-mediated signaling consists of two separate receptor-effector systems mediating distinct functional responses; one linked to PLC activation resulting in platelet aggregation and secretion, and the other mediating an increase in cytosolic calcium and platelet shape change (12).

We have recently identified Arg^{60} to Leu mutation in the first cytoplasmic loop of the TXA_2 receptor in a bleeding disorder characterized by defective platelet aggregation responses to TXA_2 and its analogs (13). The platelets of the patients with this mutation were impaired in PLC activation. Consistently, the Arg^{60} to Leu mutant of TXRα expressed in cultured cells exhibited impaired agonist-induced PLC activation. Their platelets, however, exhibited shape change and phospholipase A_2 (PLA_2) activation when stimulated by a TXA_2 agonist (14, 15). These findings favor the hypothesis that shape change and PLA_2 activation are not mediated by PLC activation but by other pathways independent of PLC activation and not affected by this mutation. A cDNA encoding a TXA_2 receptor has initially been cloned from human placenta and the deduced 343–amino acid sequence indicates that it belongs to the superfamily of G protein-coupled receptors (16). Recently, a cDNA encoding a receptor isoform of 407 amino acids has been isolated from human endothelium (17). These

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; G protein, guanine nucleotide-binding protein; GR32191, [1R-[1α(Z), 2β,3β,5α]]-(+)-7-[5-[[(1,1′biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid; HBS, Hepes-buffered saline; I-BOP, [1S-[1α,2α(Z), 3β(1E,3S*),4α]]-7-[3-[3-hydroxy-4-[4-iodophenoxy]-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; IP_3, inositol 1,4,5-triphosphate; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PLC, phospholipase C; RT, reverse transcription; S-145, 5Z-7-(3-*endo*-phenylsulphonylaminobicyclo[2.2.1]hept-2-*exo*-yl)heptenoic acid; STA_2, 9,11-epithio-11,12-methano-TXA_2; TXA_2, thromboxane A_2; U46619, 15S-hydroxy-11α,9α(epoxymethano)prosta-5Z,13E-dienoic acid.

two isoforms, here referred to as TXRα and TXRβ, are generated by alternative splicing from a single gene and differ only in their carboxyl-terminal domains; the 15 amino acid sequence in the carboxyl end of TXRα is replaced by a 79–amino acid sequence in TXRβ. The existence of multiple isoforms could account for the separate effector systems linked to the TXA_2 receptor, provided both isoforms are present in platelets and divergent in their signaling pathways.

Here we report the identification of two isoforms of the human TXA_2 receptor in platelets. The two isoforms expressed in cultured cells showed the same ligand binding characteristics and agonist-induced activation of PLC but distinct effects on adenylyl cyclase activity; TXRα activated adenylyl cyclase, while TXRβ inhibited it. Since the patients with the Arg^{60} to Leu mutation of the TXA_2 receptor showed some of the TXA_2-induced platelet responses, we have examined the effects of this mutation on these pathways to explore their possible involvement in those responses. We show that the Arg^{60} to Leu mutation selectively impairs the pathways linked to PLC activation and to adenylyl cyclase stimulation but not that linked to adenylyl cyclase inhibition. It is, therefore, possible that the pathway linked to adenylyl cyclase inhibition is involved in the platelet responses remaining unaffected in these patients.

Methods

Materials. 9,11-epithio-11,12-methano-TXA_2 (STA_2) was a generous gift from Ono Pharmaceuticals Co. Ltd. (Osaka, Japan). 5Z-7-(3-*endo*-phenylsulphonylaminobicyclo[2.2.1]hept-2-*exo*-yl) heptenoic acid (S-145) and its tritium derivative [^{3}H]S-145 were generous gifts from Shionogi Research Laboratories (Osaka, Japan). [1S-[1α, 2α(Z), 3β(1E, 3S*), 4α]]-7-[3-[3-hydroxy-4-[4-iodophenoxy]-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (I-BOP) was obtained from Cayman Chemical Co. (Ann Arbor, MI). [1R-[1α(Z), 2β, 3β, 5α]]-(+)-7-[5-[[(1, 1′-biphenyl)-4-yl]methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid (GR32191) was kindly supplied by Dr. R. A. Coleman (Glaxo Research Ltd., Ware, England). *Myo*-[2-^{3}H]inositol (19.1 Ci/mmol) and cAMP [^{125}I] assay system were obtained from Amersham Corp. (Arlington Heights, IL), pertussis toxin from Funakoshi Co. Ltd. (Tokyo, Japan), forskolin from Sigma Chemical Co. (St. Louis, MO) and RO-20-1724 from Paesel (Frankfurt/M, Germany). All other materials were obtained from the sources as described previously (13).

Amplification of platelet mRNA. Total RNA was extracted from platelets by the acid guanidium-phenol-chloroform method (18). 1 μg of total RNA was reverse-transcribed (RT) in a volume of 20 μl as described previously (19). For a control, reactions were carried out under the same conditions without reverse transcriptase. 2 μl of the reaction mixture was used for amplification by polymerase chain reaction (PCR) in a final volume of 20 μl, containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10% dimethylsulfoxide, 0.25 mM dNTPs, 1 U Taq Polymerase (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 20 pmol of each primer. After denaturation at 94°C for 2 min, 35 cycles (94°C for 1 min, 62°C for 1 min, 72°C for 1.5 min) were followed by a final elongation step for 3.5 min at 72°C. Primers used are based on the sequences of the TXA_2 receptor isolated from placenta (16). One pair of primers include TX30 (5′-CCGCCATGGCCTCAGAGCGCTAC-3′) corresponding to nucleotides (nt) 371-393, as numbered in the published sequence (16), and 3T4 (5′-GGGGTGCCCGCGTTTCACAT-3′; nt 1884–1903). Other pairs include 7S1 (5′-CTGCTCATCTACTTGCGCGT-3′; nt 868–887) and 3T4, or 7S1 and 3T2 (5′-CAGGGTCAAAGAGCATGCAA-3′; nt 1787–1806). PCR products were electrophoresed, excised, purified using Geneclean II (Bio 101 Inc., Vista, CA), and blunt-end-ligated to pBluescript II SK+ (Stratagene, La Jolla, CA). Plasmid subclones were sequenced by the dideoxy chain termination method.

Genomic PCR. Genomic DNA was prepared from white blood cells of the patient T.T. (13) as described (20). A 1146 bp fragment that includes the portion encoding peptide-β was amplified by PCR, using primers I2 (5′-GTCCAGGCTGACAGCTCTCC-3′) corresponding to intronic nt -29 to -10 upstream from the second coding exon (21) and 3T4. PCR was done as described in RT-PCR except that 0.1 μg genomic DNA was used instead of RT reaction mixture as a template. PCR products were subcloned and sequenced.

Expression of wild-type and mutant TXA_2 receptor isoforms. For transient expression, cDNAs encoding the two isoforms were inserted into the pCMX expression vector under the control of the cytomegalovirus promoter (22) and COS-m6 were transfected in 15-cm dishes with 25 μg of plasmid DNA by calcium phosphate precipitation (23). The cells were grown in DME with 10% fetal calf serum and harvested at 72 h after transfection for ligand binding assays or at 24 h to be trypsinized and plated in 6-well plates for phosphatidylinositol (PI) assay. For stable expression, cDNAs encoding the wild-type and mutant isoforms were inserted into the mammalian expression vector pEF-BOS (24) with a blasticidin S-resistance marker (Funakoshi Co. Ltd.) and introduced into Chinese hamster ovary (CHO) cells by the calcium phosphate method. Stable transformants were cloned by selection in the α-modified minimal essential medium containing 10% fetal calf serum and 15 ng/ml blasticidin S (Funakoshi Co. Ltd.).

Ligand binding studies. Tansfected COS-m6 cells or CHO cells were cultured in 15 cm dishes to confluency and harvested as described previously (25). Cells were washed once and suspended at 1×10^7 cells/ml in a buffer containing 20 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$. Binding assays were performed essentially as described (26). Briefly, cells (100 μl) were incubated in a total volume of 200 μl at 30°C for 1 h. In the [^{3}H]S-145 saturation experiments, nine different concentrations of the radioligand (0.125–32 nM) were used. In the displacement experiments, 1 nM of [^{3}H]S-145 and different concentrations of the unlabeled competitors were employed. The reaction was terminated by the addition of 4 ml of ice-cold 10 mM Tris (pH 7.4) and immediate vacuum filtration through GF/C filters (Whatman International Ltd., Maidstone, England) followed by four additional 4-ml washes with the same buffer. The radioactivity on the filter was measured by scintillation counting. Nonspecific binding was determined in the presence of a 1,000-fold excess of unlabeled S-145.

Measurement of PI hydrolysis. Transfected COS-m6 cells or CHO cells were cultured in 6-well plates to 90% confluency in the medium described above and then incubated for 20 h in inositol-free DME with 10% dialyzed fetal calf serum containing 1 μCi/ml *myo*-[2-^{3}H]inositol. The cells were washed with Hepes-buffered saline (HBS) and preincubated with 10 mM LiCl in HBS at 37°C for 10 min. Then various concentrations of STA_2 were added to each medium. After 5 or 30 min incubation, the medium was removed and the reaction was terminated by 1 ml of 5% trichloroacetic acid. The generation of IP_3 or total inositol phosphate was measured as described (27).

cAMP assays. CHO cells stably expressing wild-type or mutant isoforms were cultured in 24-well plates and treated with or without 100 ng/ml pertussis toxin for 12 h. They were then washed once with HBS and preincubated for 10 min in HBS containing 0.1 mM RO-20-1724 at 37°C. Cells were then stimulated for 10 min with I-BOP at the indicated concentrations with or without 1 μM forskolin. The reaction was terminated by the addition of an equal volume (0.5 ml) of 10% trichloroacetic acid. Quantification of cAMP was performed by a radioimmunoassay using an Amersham cAMP assay kit.

Results

Identification of two isoforms of the human TXA_2 receptor in platelets. cDNAs encoding two isoforms of the human TXA_2

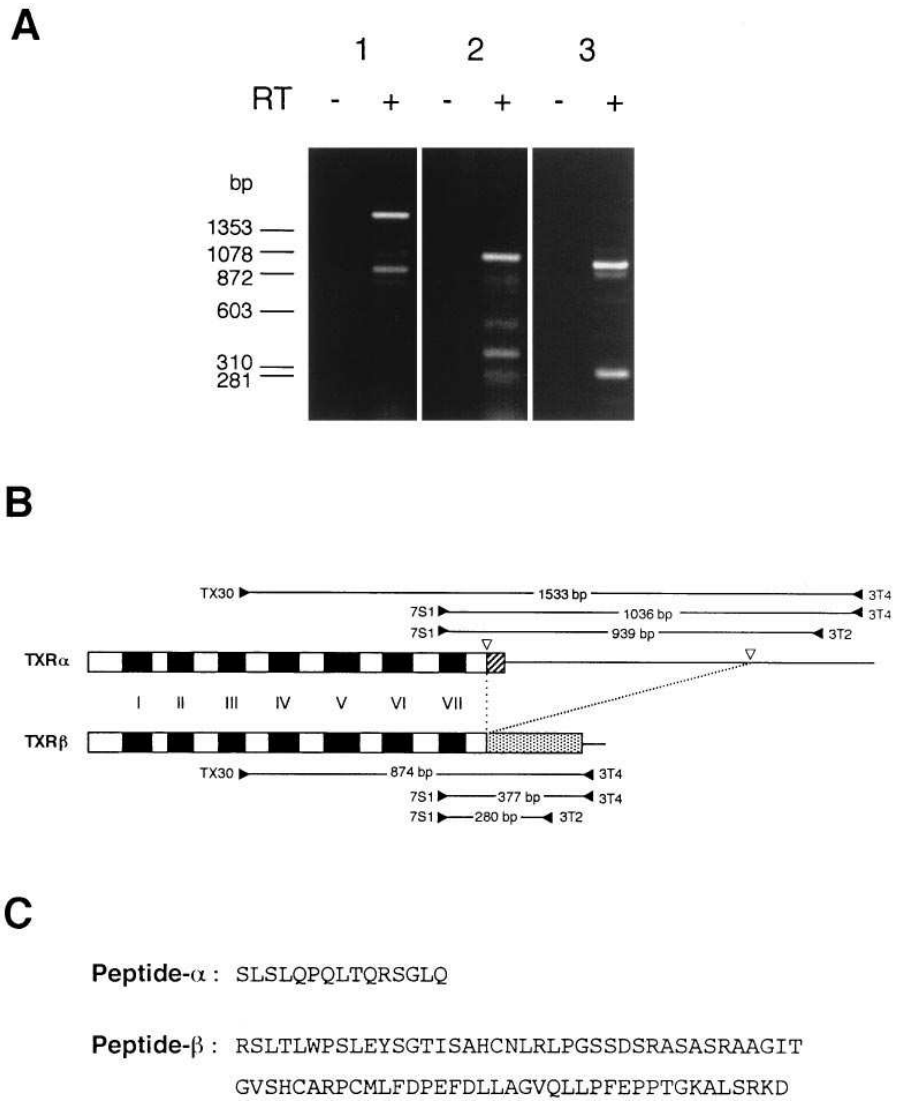

Figure 1. PCR detection of the two isoforms of the human TXA_2 receptor in platelets. (*A*) Ethidium bromide staining of PCR products. Platelet RNA was used as a template after the incubation in the presence (+) or absence (−) of reverse transcriptase (RT). PCR products were analyzed on 2% agarose gels. Two bands are identified in each PCR reaction using primers TX30 and 3T4 (reaction 1), 7S1 and 3T4 (reaction 2), and 7S1 and 3T2 (reaction 3). The larger and the smaller bands in each reaction correspond to TXRα and TXRβ mRNAs, respectively. (*B*) Schematic representation of the two isoforms of the TXA_2 receptor showing the primers used in PCR. Putative transmembrane domains I-VII are shown by closed boxes and different carboxyl-terminal tails by a hatched box (peptide-α) and a dotted box (peptide-β). Splicing sites are indicated by inverted triangles. The positions of primers in each cDNA and the expected sizes of PCR products are shown. (*C*) Amino acid sequences of peptide-α and peptide-β. Peptide-α and peptide-β contain 15 and 79 amino acids, respectively.

receptor have already been cloned (16, 17). These are schematically represented in Fig. 1 *B*. Comparison of two cDNAs revealed that TXRβ has a deletion of a 659-bp sequence that comprises the distal portion of the cytoplasmic carboxyl tail and the following 3′ untranslated region of TXRα. Deletion of this sequence results in another reading frame downstream in TXRβ. Consequently, a 15 amino acid carboxyl-terminal sequence of TXRα (peptide-α) is replaced with a new 79 amino acid sequence (peptide-β) in the carboxyl end of TXRβ (Fig. 1 *C*). Peptide-α contains four serine and threonine residues as potential phosphorylation sites, whereas peptide-β contains 14 residues. To determine whether the two receptor isoforms exist in human platelets, RT-PCR was carried out. Two fragments were amplified using a sense primer TX30 located in the third transmembrane domain and an antisense primer 3T4 in the 3′ untranslated domain shared by the two isoforms (Fig. 1 *A*, lane *1*). The amplification of these fragments depended strictly on cDNA synthesis, indicating that the fragments were derived from RNA and not from contaminated genomic DNA. Sequence analyses confirmed that the larger band corresponds to TXRα and the smaller band TXRβ. Additional PCR reactions using different pairs of primers (7S1+3T4 and 7S1+3T2) each yielded two bands of the expected size (Fig. 1 *A*, lanes *2* and *3*), further confirming the presence of the two isoforms in platelets.

Ligand binding characteristics of the two TXA_2 receptor isoforms. To investigate functional differences between the two isoforms, TXRα and TXRβ were transiently expressed in COS-m6 cells or stably expressed in CHO cells. The two isoforms expressed in CHO cells displayed indistinguishable, high-affinity binding of the TXA_2 receptor antagonist [^{3}H]S-145 with a K_d value of 2.1 nM for both TXRα and TXRβ (Fig. 2 *A*). Scatchard analyses using [^{3}H]S-145 revealed a single class of binding sites on both receptor isoforms. Similar results were obtained on transfected COS-m6 cells (data not shown). Because previous studies using I-BOP, a TXA_2 agonist, and GR32191, a TXA_2 antagonist, detected two classes of binding sites in platelets (8, 12), binding of these ligands to the two isoforms were studied by testing their abilities to displace specific [^{3}H]S-145 binding. I-BOP displaced specific [^{3}H]S-145 binding to the two isoforms similarly (Fig. 2 *B*). GR32191 also showed similar displacement for the two isoforms (Fig. 2 *C*). Furthermore, GR32191, which identified reversible and irreversible binding sites in platelets (12), bound only irreversibly to both

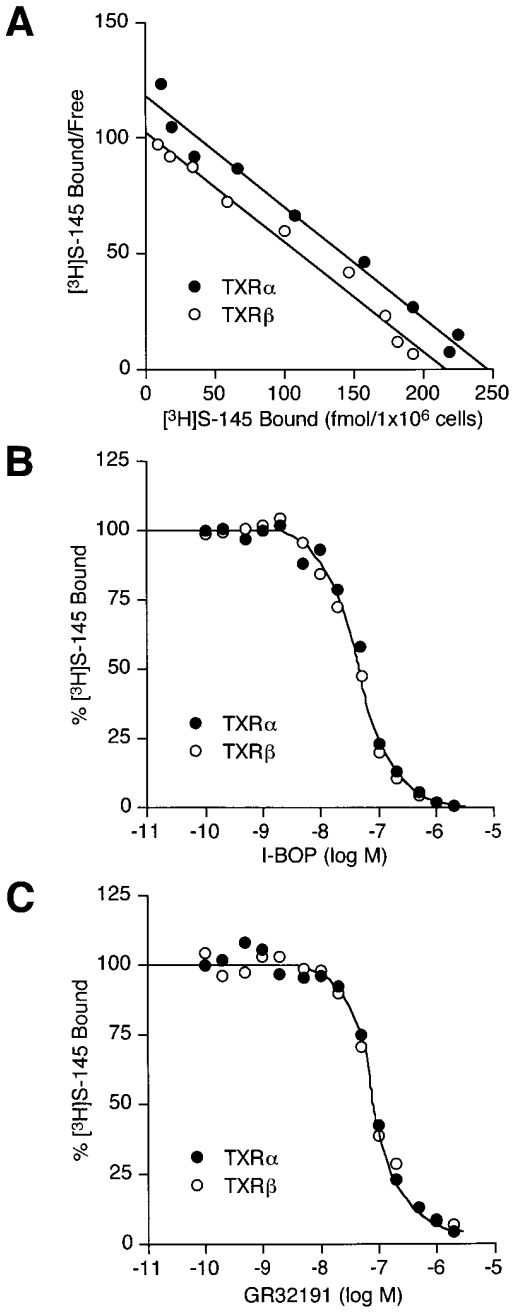

Figure 2. Ligand binding characteristics of the two TXA_2 receptor isoforms. (*A*) Scatchard analyses of TXRα (●) or TXRβ (○). Specific [^{3}H]S-145 binding to CHO cells stably expressing TXRα or TXRβ was determined. TXRα exhibited an affinity for [^{3}H]S-145 (K_d = 2.1 nM) which was comparable to that of TXRβ (K_d = 2.1 nM). Both cell lines expressed a similar number of receptors (B_{max} = 246.8 and 216.3 fmol / 1 × 10^6 cells for TXRα and TXRβ, respectively). Data shown are representative of three independent experiments, each performed in duplicate. (*B* and *C*) Displacement of specific [^{3}H]S-145 binding to TXRα (●) or TXRβ (○) by I-BOP (*B*) and GR32191 (*C*). Both ligands displaced specific [^{3}H]S-145 binding to CHO cells stably expressing TXRα or TXRβ similarly. Each curve is representative of three independent experiments, each performed in duplicate.

isoforms (data not shown). Thus, the two isoforms at least under the present experimental conditions do not correspond to the subtypes shown previously in platelets.

PLC activation by the two TXA_2 receptor isoforms. We next investigated whether the different carboxyl-terminal tails affect the PLC activation. Untransfected COS-m6 cells did not show any significant increase in inositol phosphate production to the TXA_2 agonist STA_2 (data not shown). Stimulation of cells expressing TXRα or TXRβ by STA_2 induced an increase in inositol phosphate production with almost the same concentration-response curves (Fig. 3). Since we had previously shown that the Arg^{60} to Leu mutation of TXRα impairs its agonist-induced activation of PLC, we asked whether this mutation also affects PLC activation mediated by TXRβ. Stable transformants expressing a similar number of wild-type or mutant TXRβ were chosen for this experiment. Mutant TXRβ exhibited an unaltered binding affinity for [^{3}H]S-145, compared to that of wild-type TXRβ (Fig. 4 *A*). When the two cell lines were examined for the activation of PLC, mutant TXRβ was found to be impaired in its ability to produce agonist-induced PI hydrolysis (Fig. 4 *B*). These results indicate that the mutation impairs PLC activation both in TXRα and TXRβ. Thus, the Arg^{60} in the first cytoplasmic loop appears to be essential for PLC activation.

Activation and inhibition of adenylyl cyclase by the two TXA_2 receptor isoforms. Previous studies have shown possible involvement of the adenylyl cyclase pathway in TXA_2-mediated platelet responses, although the results are conflicting (28). Therefore, we examined effects of the difference of the carboxyl-terminal tail on adenylyl cyclase activation in cells expressing the two isoforms. As shown in Fig. 5, I-BOP increased the level of cAMP in cells expressing TXRα in a concentration-dependent fashion above 10 nM, while no increase was

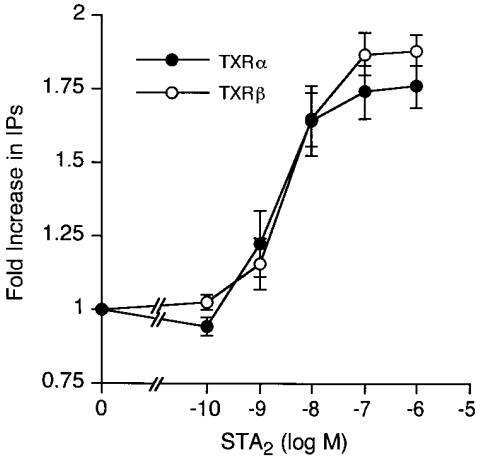

Figure 3. STA_2-induced PLC activation in COS-m6 cells expressing the two TXA_2 receptor isoforms. COS-m6 cells transiently expressing TXRα (●) or TXRβ (○) were incubated with STA_2 for 30 min and total inositol phosphate production was measured. Both isoforms are expressed in a similar number (190.1 pmol and 145.7 pmol / 1 × 10^6 cells for TXRα and TXRβ, respectively). The data are presented as fold increases, where the values from the cells in the absence of STA_2 were chosen as the reference values. Data points are mean±SE from 3 replicate wells from a single experiment. Where not shown, an SE bar is smaller than the symbol. The results were independently confirmed in two additional experiments. The EC_{50} values for activation through TXRα and TXRβ were 2.3 and 3.9 nM, respectively.

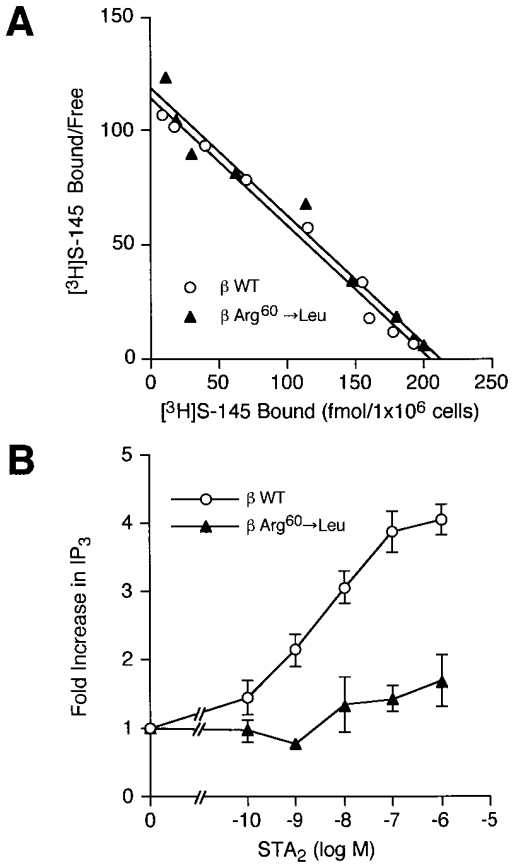

Figure 4. Functional properties of wild-type and mutant TXRβ stably expressed in CHO cells. (*A*) Scatchard analyses of wild-type (○) and mutant (▲) TXRβ. Wild-type and mutant TXRβ exhibited similar affinities for [^{3}H]S-145 (K_d = 1.8 nM for both wild-type and mutant TXRβ). Both cell lines expressed a similar number of receptors (B_{max} = 204.9 and 212.2 fmol / 1 × 10^6 cells for wild-type and mutant TXRβ, respectively). (*B*) STA_2-induced IP_3 accumulation in CHO cells expressing wild-type (○) and mutant (▲) TXRβ. CHO cells were incubated with STA_2 for 5 min and IP_3 production was measured. Data points are mean±SE from three replicate wells from a single experiment. The results were independently confirmed in two additional experiments. The data are presented as fold increases, where the values from the cells in the absence of STA_2 were chosen as the reference values.

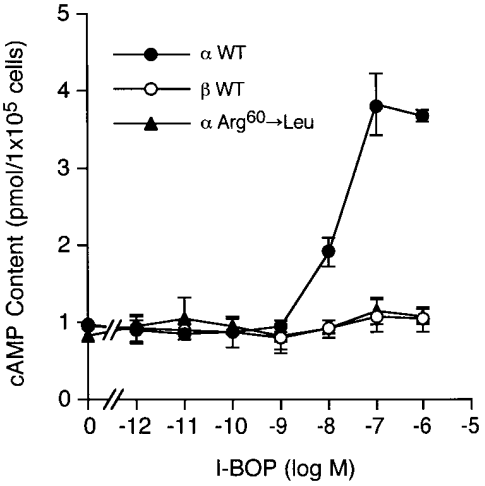

Figure 5. Adenylyl cyclase stimulation by wild-type and mutant TXRα. CHO cells expressing TXRα (●), TXRβ (○) or the Arg^{60} to Leu mutant of TXRα (▲) were incubated with various concentrations of I-BOP and the cAMP contents in the cells were measured as described in Methods. Data points are mean±SE from 3 replicate wells from a single experiment. The results were independently confirmed in 2 additional experiments. The EC_{50} value for activation through TXRα was 17 nM.

found in those expressing TXRβ. We next examined whether the two isoforms can inhibit adenylyl cyclase. I-BOP inhibited the forskolin-induced cAMP generation in cells expressing TXRβ but not in cells expressing TXRα (Fig. 6 *A*). This inhibition was sensitive to pertussis toxin treatment (Fig. 6 *C*), suggesting that it was mediated by the interaction of the receptor with G_i/G_o. Since pertussis toxin did not affect adenylyl cyclase stimulation by TXRα (data not shown), it seems that each isoform couples to a separate G protein in addition to a common G protein of the G_q class.

Effects of the Arg^{60} to Leu mutation on adenylyl cyclase activity. The finding that the TXA_2 receptor mediates pathways other than that linked to PLC led us to examine the effect of the Arg^{60} to Leu mutation on these pathways, since the patients with this mutation show some platelet responses such as shape change and PLA_2 activation despite the defective activation of PLC when stimulated by a TXA_2 agonist. Before addressing this question, we examined the genomic sequence corresponding to peptide-β, that had not been analyzed in our previous study (13), by genomic PCR in one of the patients, and confirmed that it bears no mutation. The Arg^{60} to Leu mutation, which had been shown to impair PLC activation by TXRα, also impaired adenylyl cyclase activation by this isoform (Fig. 5). In contrast, as shown in Fig. 6 *B*, the mutant TXRβ was capable of inhibiting adenylyl cyclase above the I-BOP concentration of 1 pM. This inhibitory effect was also sensitive to pertussis toxin treatment (Fig. 6 *C*). Taken together, the Arg^{60} to Leu mutation affects the pathways linked to PLC activation and to adenylyl cyclase stimulation but not that linked to adenylyl cyclase inhibition. The existence of a pathway which is not affected by the mutation may be related to the partial response to TXA_2 in these patients.

Discussion

We have shown here that the two isoforms of the human TXA_2 receptor exist in platelets and that these isoforms mediate distinctive spectra of transduction pathways. Although the two isoforms similarly activated PLC, they differently regulated adenylyl cyclase activity; TXRα activated adenylyl cyclase, while TXRβ inhibited it. Previous studies using some TXA_2 analogs identified two classes of binding sites in platelets and suggested the possibility that the receptor subtypes corresponding to these two classes are present and mediate separate functional responses of platelets. The presence of the two TXA_2 receptor isoforms in platelets first led us to compare their binding properties in relation to these studies. However, the expression studies in CHO cells and COS cells revealed no difference between the two; both I-BOP and GR32191 displaced specific [^{3}H]S-145 binding to the isoforms similarly, and GR32191 irreversibly bound to both isoforms. These results suggest that the two isoforms do not correspond to the two

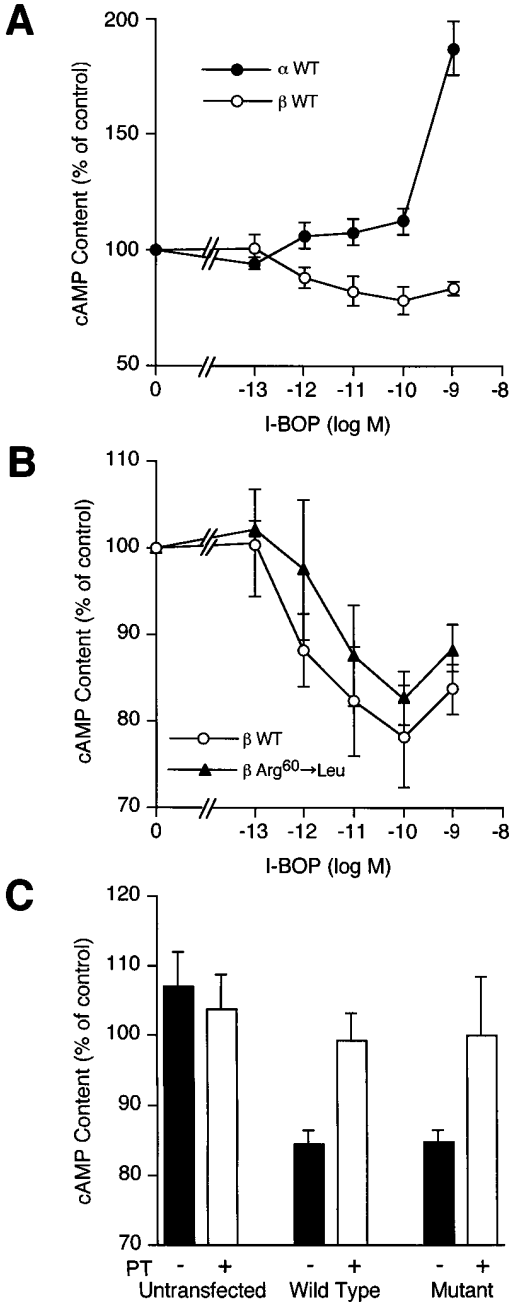

Figure 6. Adenylyl cyclase inhibition by wild-type and mutant TXRβ. (*A* and *B*) I-BOP-induced inhibition of forskolin-induced cAMP accumulation in CHO cells expressing TXRα (●), TXRβ (○) or the Arg^{60} to Leu mutant of TXRβ (▲). Cells were incubated with 1 μM forskolin and various concentrations of I-BOP and the cAMP contents were measured. The data are presented as percentages of the control values obtained from the forskolin-stimulated cells in the absence of I-BOP. Data points are mean±SE from three replicate wells from a single experiment. The results were independently confirmed in 2 additional experiments. The EC_{50} values for activation through wild type and Arg^{60} to Leu mutant of TXRβ were 0.93 and 4.1 pM, respectively. (*C*) Effects of pertussis toxin on the I-BOP-induced inhibition of forskolin-induced cAMP accumulation. Cells were cultured in the presence (+) or absence (−) of pertussis toxin (PT) for 12 h, then incubated with 1 μM forskolin and 100 pM I-BOP and the cAMP contents were determined. Data points are mean±SE from six replicate wells from a single experiment. The results were independently confirmed in two additional experiments.

classes of binding sites for some ligands found in intact platelets, unless they behave differently in platelets.

The identification of the pathway linked to adenylyl cyclase inhibition made us to wonder how this pathway is involved in TXA_2-induced platelet responses. Both PLC activation and inhibition of adenylyl cyclase can lead to platelet activation. Given the lower concentrations required for adenylyl cyclase inhibition than for PLC activation, the two pathways might act independently rather than synergistically. We have found that they are distinguished by the effects of the Arg^{60} to Leu mutation; PLC activation is attenuated by this mutation, whereas adenylyl cyclase inhibition is not. This mutation has been identified in a bleeding disorder characterized by defective platelet aggregation responses to TXA_2 and its agonists (13). Previous study demonstrated that despite the impaired activation of PLC, the patients' platelets showed shape change (14) and PLA_2 activation (15), implying the existence of the pathways leading to these responses that are not affected by the mutation. As our studies showed that the inhibition of adenylyl cyclase is unaffected by the mutation, the pathway represented by this parameter might be implicated in shape change and PLA_2 activation. This pathway appears to be mediated by G_i/G_o because it is sensitive to pertussis toxin treatment. This is in accordance with our previous finding that the purified TXA_2 receptor is functionally reconstituted with both G_{i2} and G_q in phospholipid vesicles (29). Consistent with the idea that this G_i/G_o-linked pathway is involved in PLA_2 activation, TXA_2 stimulates mitogen-activated protein kinase via G_i in some types of cells and activates PLA_2 (30, 31). Thus, the present study suggests an intriguing possibility that the TXA_2 receptor isoforms separately mediate different kinds of activation responses in human platelets, although we cannot completely exclude the possibilities that other effector systems linked to the mutated receptor or those mediated by other receptor(s) than the mutated receptor evoke these responses.

Our experiments have also shown stimulation of adenylyl cyclase by one isoform of the TXA_2 receptor. Ishikawa and Ogawa (32) previously found that STA_2 elevated cAMP level in platelets at high concentrations to cause the inhibitory effect on aggregation. Based on this finding, they speculated that this pathway could negatively regulate platelet activation to attenuate or terminate responses. However, the stimulation they observed was not suppressed by a TXA_2 antagonist, ONO-3708. Because ONO-3708 completely displaced the radioligand binding to the cloned TXA_2 receptor (16), it is doubtful that the stimulation observed by these authors is mediated by the TXA_2 receptor we discuss here. Given the fact that stimulation of adenylyl cyclase is observed at relatively high agonist concentrations, the physiological significance of this pathway remains unclear.

Production of multiple isoforms by alternative splicing has been reported in G protein–coupled receptor family (33–38). Two regions of divergence that result from alternative splicing have been identified: the third cytoplasmic loop and the carboxyl-terminal tail. Those that have alternatively-spliced carboxyl tails include several members: the neurokinin-1 receptor (36), the prostaglandin E receptor EP_3 subtype (37), the monocyte chemoattractant protein-1 receptor (38), and the TXA_2 receptor. Alternative splicing of the EP_3 receptor creates isoforms which exhibit distinct functional properties, including different efficiency in activation of a G protein (37), different specificity in coupling to G proteins (39) and different sensitiv-

ity to desensitization (40). The present study suggests that the carboxyl-terminal tails of the two TXA_2 receptor isoforms, like those of the bovine EP_3 receptor (39), determine the specificity of G protein coupling. Furthermore, the distinctive effects of the Arg^{60} to Leu mutation suggested that the interaction of the receptor with different G proteins is distinctly regulated by different domains of the receptor; this residue appears to be important for the interaction of the receptor with G_q and possibly with G_s, but not with G_i/G_o.

Taken together, the data presented here support the existence of multiple signaling pathways via the two TXA_2 receptor isoforms in platelets, which could interact each other to coordinate platelet activation processes. Comparison of the effects of the Arg^{60} to Leu mutation on these pathways with the responses observed in the platelets of the patients with this mutation implicates the possible relationship between the TXRβ-G_i pathway and the platelet shape change and PLA_2 activation. This hypothesis should be rigorously tested in future studies.

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