Role of C-terminal Serines in Desensitization and Phosphorylation of the Mouse Thromboxane Receptor*

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To investigate the role of C-terminal hydroxyamino acids in desensitization of the receptor for thromboxane A₂ (TxA₂), we created a mutant TxA₂ receptor (TP receptor) in which serines at positions 321, 322, and 328 were replaced with either alanine or glycine. Mutant and wild type receptors were expressed in a mesangial cell line, and clones expressing similar numbers of receptors were studied. Affinity and specificity of TxA₂ binding to the mutant receptor were identical to wild type receptors. In contrast, TxA₂-induced inositol trisphosphate generation by the mutant receptor was enhanced compared with the wild type. Prior treatment with the TxA₂ agonist U46619 reduced subsequent U46619-induced increase in inositol trisphosphate generation by both receptors; however, the extent of desensitization was significantly reduced in the receptor mutant. Protein kinase C (PKC) inhibitors attenuated TxA₂-induced desensitization of wild type receptors, but had little effect on TxA₂-induced desensitization of mutant receptors. Pretreatment with the phorbol ester phorbol 12,13-dibutyrate (PDBu) (100 nM) decreased subsequent responsiveness of wild type but not mutant TP receptors. U46619-induced desensitization of wild type receptors was associated with enhanced phosphorylation of receptor proteins. This agonist-specific phosphorylation of the TP receptor was largely prevented by inhibitors of PKC. Treatment with 100 nM PDBu increased phosphorylation of both wild type and mutant TP receptors, but the extent of phosphorylation of the receptor mutant was reduced compared with the wild type. Increasing the concentration of PDBu from 100 nM to 1 μM PDBu reduced responsiveness of both mutant and wild type receptors without enhancing phosphorylation of either of the receptor proteins. These data suggest that 1) phosphorylation of C-terminal serines contributes to agonist-specific desensitization of the TP receptor, 2) PKC-induced desensitization of TP receptors is caused, in part, by phosphorylation of C-terminal serines, and 3) desensitization of TP receptors by PKC is complex and involves mechanisms that may not require direct phosphorylation of receptor proteins.

Thromboxane A₂ (TxA₂)¹ is a labile lipid mediator with potent platelet aggregating and vasoconstrictor actions that have been implicated in thrombotic and vasospastic disorders affecting the heart, lungs, kidneys, and peripheral vascular system (1–3). Its effects are mediated by activating specific cell surface receptors and are subject to regulatory controls (4–6). Prior exposure to TxA₂ results in decreased receptor responsiveness (4, 6). This loss of receptor responsiveness or desensitization has been extensively studied in the cAMP-coupled β₂-adrenergic receptor and the phosphodiesterase-coupled receptor rhodopsin. In these receptor systems, desensitization is largely caused by direct phosphorylation of receptors at serine and threonine residues, often in the C terminus (reviewed in Dohlman et al. (7)). Receptor phosphorylation is mediated by both receptor specific kinases and general kinase systems such as protein kinase A and C (8). These kinase systems provide a mechanism for regulating receptor activity through negative feedback loops (homologous desensitization) as well as for modulating receptor responsiveness through cross-talk between different receptor systems (heterologous desensitization).

The receptor for TxA₂ belongs to the large superfamily of heptahelical G protein-coupled receptors (9–11). In most cell systems, TP receptors couple to phospholipase C through pertussis toxin insensitive G proteins (12, 13). More recently, two isomers of the human TP receptor have been described (14) which, in addition to coupling to phospholipase C, oppositely regulate adenyl cyclase activity (15). Previous studies suggest that desensitization of TP receptors is mediated, in part, through activation of PKC (4, 6). PKC-induced desensitization may involve direct phosphorylation of C-terminal domains of the TP receptor because 1) truncation of the C terminus attenuates desensitization mediated by direct activation of PKC by phorbol esters (16), 2) PKC can phosphorylate C-terminal sequences of the TP receptor in vitro (5), and 3) phorbol ester-induced activation of PKC causes phosphorylation of the human TP receptor isoforms in the intact cell (17). While the C terminus of the TP receptor contains several potential PKC phosphorylation sites, it is not known if phosphorylation of these domains contributes to TP receptor desensitization. We therefore constructed a mutant TP receptor (mutant 1) in which serines with favorable phosphorylation motifs for PKC (positions 321, 322, and 328) were replaced with either alanine or glycine. Both mutant and wild type receptors were epitope-

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¹ The abbreviations used are: TxA₂, thromboxane A₂; TP receptor, TxA₂ receptor; [I]iBOP, [15-(1a, 2β,5-2Z,3a-(1E,3S/4S)-7-[3-(3-hydroxy-4-p-iodophenox)-1-butenyl]-7-oxa-bicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; U46619, (15S)-hydroxy-11Z,5Z,(epoxy)methano/prost-5Z,13E-dienoic acid; SQ29548, (1S)-1a,2β,3Z,4S,4aS)-7-(3-(2-(phenyl-amino)-carbonyl)hydrizinomethyl)-7-oxa-bicyclo[2.2.1]heptan-2-yl]-5-heptenoic acid); PKC, protein kinase C; KRB, Krebs-Ringer buffer; PDBu, phorbol 12,13-dibutyrate; G protein, guanine nucleotide regulatory protein; IP₃, inositol triphosphates; PI, phosphoinositide; DMEM, Dulbecco’s modified Eagle’s medium; PCR, polymerase chain reaction; HEK, human embryonic kidney; HA, hemagglutinin; ET, epitope-tagged.
tagged to permit rapid isolation of receptor proteins and assessment of their phosphorylation state. As an additional control, we compared desensitization of mutant 1 with another mutant receptor (mutant 2) in which a C-terminal serine lacking a consensus motif for phosphorylation by PKC (position 339) was replaced with glycine. Using these constructs, we investigated the role of C-terminal serines in regulating TP receptor responsiveness by direct phosphorylation of receptor proteins.

MATERIALS AND METHODS

Isolation and Mutagenesis of a Genomic Clone Encoding the Mouse TP Receptor—A genomic clone encoding the mouse TP receptor was isolated as described previously (16). We subcloned a XhoI/ApaI fragment of the genomic clone containing the complete amino acid-encoding region into the mammalian expression vector pcDNA 3 (Invitrogen, San Diego, CA). Mutagenesis of the wild type receptor was performed using the polymerase chain reaction (PCR) (18). To change serines at positions 321, 322, and 328 to either alanines or glycine, we replaced nucleotides at positions 961, 964, and 982 with guanines using the primer pairs encompassed nucleotides 952–997 (GGCCGCGTTCGTCGTTGGCAGTCTGCGATCGGGCCGCTTGGCTGTTGGCCTGGCCG) and 1030–1009 (TCGTTGAGCTCTGACGTTGACATGCTGCGGTCATGGTGAC) of the mouse TP receptor cDNA (11). To change serine at position 339 to glycine, we replaced the nucleotide at position 1015 with guanine using the primer pairs encompassed nucleotides 790–809 (GGACGACTTGGTGCAACACAGAC) and 1030–1001 (CCCTCAGGTTGACGATCGGGCCGCTTGGCCG) of the mouse TP receptor cDNA (11). PCR products were cloned into the TA cloning vector (Invitrogen) containing our genomic clone. Mutant constructs were sequenced using the dideoxy method (19) to confirm the desired mutations.

Epitope-tagged TP receptors were created using PCR to insert the 12CA5 epitope (20) at both the N and C termini of the TP receptor as follows. For the N-terminal epitope, we used primer pairs encompassed nucleotides −2−18 (GGCTAGGACGATCGGGCCGCTTGGCTGTTGGCCTGGCCG) and 1035−1009 (TCGTTGAGCTCTGACGTTGACATGCTGCGGTCATGGTGAC) of the mouse TP receptor cDNA (11). The primer encompassing nucleotides −12−18 was 12CA5 epitope sequence. For the C-terminal epitope, we used primer pairs encompassed nucleotides 970−809 (GGACGACTTGGTGCAACACAGAC) and 1035−1009 (GACGTGCTTCTAGCGCTATCGTGGCAGTCGTTGGCGATCGGGCCGCTTGGCCG) of the mouse TP receptor cDNA (11), where the primer encompassing nucleotides −12−18 contained the 12CA5 epitope sequence. The PCR products were the vectors containing either the wild type or mutant TP receptor lacking three C-terminal serines. PCR products were cloned into the TA cloning vector (Invitrogen) using the manufacturer's directions. To create epitope-tagged receptors, we took advantage of an unique SfiI restriction site located at nucleotide 955 (11) in the C terminus of the TP receptor. Appropriately sized SfiI/ApaI fragments were isolated by polyacrylamide gel electrophoresis (19) from the TA cloning vector containing our PCR products. These SfiI/ApaI fragments were subcloned into the mammalian expression vector pcDNA 3 (Invitrogen) containing our genomic clone. Mutant constructs were sequenced using the dideoxy method (19) to confirm the desired mutations.

Culture and Transfection of a Mouse Mesangial Cell Line—Mouse mesangial cells derived from SV40 transgenic mice (21) were obtained from American Type Culture Collection (Rockville, MD). Cells were grown and subcultured as described previously (16). To create cell lines stably expressing wild type or mutant TP receptors, our pcDNA 3 expression vector containing either the wild type or mutant constructs was transfected into SV40-transformed mouse mesangial cells by the calcium-phosphate method (19). To isolate permanent transfectants, G418-resistant cells were selected in complete medium containing 500 mg/ml G418. Following G418 selection, individual clones were screened for DNA binding as described below.

Culture and Transfection of Human Embryonic Kidney (HEK) Cells—HEK293 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in 75% Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) (all from Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 90% air and 5% CO2. HEK293 cell lines were cultured every 9 days by becoming confluent using a 0.25% trypsin with 1 mM EDTA (Life Technologies, Inc.). Cell viability was assessed by standard dye exclusion techniques (0.1% trypsin blue) and was always greater than 95%.

For transfection, HEK293 cells were plated in 100-mm dishes and grown to approximately 80% confluency. Cells were then transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s directions. For each transfection we used 8 μg of DNA and 24 μl of LipofectAMINE based on preliminary experiments that suggested these amounts optimized the level of receptor expression and transfection efficiency (~40%). Briefly, either DNA or LipofectAMINE was mixed in 0.8 ml of DMEM. The DNA and LipofectAMINE solutions were then combined, mixed, and incubated at room temperature. After 30 min of incubation, the LipofectAMINE/DMEM mixture was added to the 100-mm dishes containing washed HEK293 cells. Following an overnight incubation, the LipofectAMINE/DMEM solution was replaced with DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). HEK293 cells were studied 48 h following transfection.

Ligand Binding Assays—Whole cell ligand binding assays were performed as described previously (16) using the stable radiolabeled thromboxane receptor antagonist 3H-labeled SQ29548 (22) (NEN Life Science Products) and the following unlabeled compounds: SQ29548 (Squibb Institute, Princeton, NJ), the thromboxane agonists U46619 (23) (Cayman Chemicals, Ann Arbor MI), or [127I]BOP (24) (Cayman Chemicals), the inactive thromboxane metabolite thromboxane B2 (Advanced Magnetics). Equilibrium binding data were analyzed by the method of Scatchard (25) to give estimates of the maximal number of specific binding sites (Bmax) and apparent equilibrium dissociation constant for each inhibitor (Ki). Protein concentration used in the binding assays was determined using the method of Bradford (26).

Measurement of [3H]Inositol Phosphate Generation—Inositol phosphates were measured as described previously using anion exchange chromatography (16). For the desensitization experiments, cells were pretreated for 10 min with the indicated concentrations of agonists, or their vehicles, in 2 ml Krebs-Ringer buffer (KRB) containing 118 mM NaCl, 4.6 mM KCl, 24.9 mM NaHCO3, 1 mM KH2PO4, 11.1 mM glucose, 1.1 mM MgSO4, 1.0 mM CaCl2, 5 mM HEPES, and 0.1% bovine albumin, pH 7.4, at 37 °C. After desensitization, cells were washed three times with KRB and then incubated in 2 ml of KRB for 4 min before adding 2 μl lithium chloride to a final concentration of 20 mM. This 4 min-time period allowed inositol phosphate levels to return to baseline following treatment with U46619 (data not shown). One minute after adding the lithium chloride solution, cells were stimulated with the indicated concentrations of U46619 or its vehicle for 2 min. The reaction was stopped, and samples were processed as described previously (16). Immunoprecipitation of Epitope-tagged Tyr Receptors—Forty-eight hours following transfection, cells were washed with KRB without KH2PO4 and then incubated in KRB without KH2PO4 but containing 0.1% bovine serum albumin (fraction V) and 0.1–0.2 μCi of [32P]Pi. After 90 min, cells were stimulated with agonist for 10 min at the indicated concentrations. The reaction was stopped by washing the cells with ice cold Dulbecco’s phosphate-buffered saline and then scraping the cells into a 1.5-ml tube containing ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 100 mM sodium lactate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride. The lysate was transferred to a 1.5 ml microcentrifuge tube and then rocked for 30 min at 4 °C. Insoluble material was removed by centrifugation at 10,000 × g for 4 min. One
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Results of two to six experiments per compound.

| Compounds       | Wild type | Mutant 1 | Mutant 2 |
|-----------------|-----------|----------|----------|
| IBOP            | 2 ± 2 nM  | 3 ± 1 nM | 2 ± 2 nM |
| SQ29548         | 6 ± 1 nM  | 5 ± 1 nM | 6 ± 2 nM |
| U46619          | 143 ± 45 nM | 130 ± 31 nM | 129 ± 9 nM |
| TxB2            | 12 ± 4 μM | 10 ± 3 μM | 10 ± 2 μM |
| Prostaglandin E2| 2 ± 1 μM  | 3 ± 3 μM | 8 ± 2 μM |

RESULTS

The Absence of C-terminal Serines of the TP Receptor Does Not Affect TxA2 Binding—We used PCR to create 2 mutant TP receptors as described under “Materials and Methods.” As shown in Fig. 1, serines at positions 321, 322, and 328 were changed to either alanine or glycine in mutant 1 (shown by asterisks). The serine at position 339 was changed to glycine in mutant 2 (shown by the arrow head). These hydroxy amino acid residues are highly conserved in the mouse, rat, and human TP receptor.

milliliter of supernatant was transferred to a 1.5-ml microcentrifuge tube, and 10 μg of 12CA5 monoclonal antibody (Boehringer Mannheim) were added. After rocking at 4 °C for 1 h, 70 μl of 20% (v/v) protein A-Sepharose was added, and the samples rocked at 4 °C for 1 h. The protein A-Sepharose was washed twice in lysis buffer supplemented with 0.1% ammonium sulfate and once in lysis buffer without ammonium sulfate. SDS-sample buffer (100 μl) was added to the pellet and boiled for 10 min. Proteins were separated on 12% polyacrylamide gel with 0.1% ammonium sulfate and once in lysis buffer without ammonium sulfate. SDS-sample buffer (100 μl) was added to the pellet and boiled for 10 min. Proteins were separated on 12% polyacrylamide gel with 0.1% SDS as described by Laemmli (28). After drying the gels, phosphorylated proteins were detected by autoradiography.

Statistical Analysis—Data are presented as the mean ± S.E. of the mean. For comparisons between two groups, statistical significance was assessed using an unpaired t test. For comparisons between more than two groups, statistical analysis was performed by analysis of variance followed by Bonferroni’s procedure for multiple pairwise comparisons (29).

PKC-induced Desensitization of TP Receptors Is Attenuated by the Absence of C-terminal Serines—To investigate the role of C-terminal serines in desensitization of the TP receptor, mesangial cells expressing wild type or mutant TP receptors were incubated with vehicle or 1 μM U46619 prior to washing and rechallenge with 1 μM concentrations of the TxA2 agonist. As shown in Table III, IP3 generation in vehicle-treated cells tended to be increased in mutant 1 compared with wild type receptors or mutant 2. Prior incubation with U46619 reduced subsequent TxA2-induced increases in IP3 generation by both wild type receptors and mutant 2. Pretreatment with U46619 also caused some reduction in IP3 generation by mutant 1, but this decrease in responsiveness did not reach statistical significance.

In order to normalize for differences in the baseline response, data for cells pretreated with U46619 were expressed as the percent response in vehicle treated cells (Table III). When the data are normalized in this fashion, there was significantly less desensitization in cells expressing mutant 1 compared with wild type receptors. These data suggest that desensitization of IP3 responses is attenuated by the absence of C-terminal serines at positions 321, 322, and 328 of the mouse TP receptor. The similar pattern of desensitization in wild type receptors and mutant 2 also suggests that homologous desensitization of TP receptors is not affected by the absence of the serine at position 339.

Previous studies from this laboratory (16) found that the absence of the C-terminal 22 amino acids of the mouse TP receptor attenuates both agonist-specific and PKC-induced desensitization. Because this truncation mutant and mutant 1 both lack serines at positions 321, 322, and 328, we determined the role of PKC in the attenuated homologous desensitization observed with mutant 1. For these experiments, mesangial cells expressing wild type or mutant TP receptors were incubated with vehicle or 1 μM U46619 in the presence or absence of the kinase inhibitor staurosporine (200 nM) (30) prior to washing and rechallenge with 1 μM concentrations of the TxA2 agonist. As seen in Fig. 2, staurosporine attenuated homologous desensitization in clones expressing the wild type TP receptor. In contrast, staurosporine had little effect on subsequent responsiveness of mutant 1 following exposure to a desensitizing stimulus. These data suggest that a staurosporine-sensitive kinase contributes to homologous desensitization of wild type, but not mutant, TP receptors.

To further investigate the role of C-terminal domains in PKC-induced desensitization of the TP receptor, clones ex-
pressing wild type receptors or mutant 1 were incubated with either 10 nM, 100 nM, or 1 μM concentrations of the PKC-activator PDBu prior to washing and stimulation with 1 μM U46619. Results of these experiments are shown in Fig. 3. Pretreatment with 10 nM PDBu had little effect on responsiveness of either the wild type or mutant TP receptor. In contrast, 100 nM PDBu significantly reduced TxA2-induced PI hydrolysis by the wild type receptor, but had little effect on PI hydrolysis in clones expressing the mutant 1. 1 μM PDBu reduced responsiveness of both wild and mutant TP receptors to a similar extent. These data suggest that C-terminal serines at positions 321, 322, and 328 of the TP receptor regulate the extent of desensitization caused by activation of PKC.

**PKC-induced Phosphorylation of TP Receptors Is Attenuated by the Absence of Serines 321, 322, and 328**—To determine if homologous desensitization of wild TP receptors was associated with direct phosphorylation of receptor proteins, cells were transfected with wild type TP receptors that had been tagged with the 12CA5 epitope. Two days following transfection, cells were loaded with 32P, prior to stimulation with 1 μM U46619 and immunoprecipitation of receptor proteins as described under “Materials and Methods.” For our initial studies, we transfected mouse mesangial cells with our epitope-tagged receptor; however, despite preclearing of the lysate and stringent wash conditions, we continued to have high backgrounds which made interpretation of the autoradiograms difficult. We therefore changed to HEK293 cells for our immunoprecipitation studies since these cells have been used successfully by other investigators to study direct phosphorylation of eicosanoid receptors (17, 31). Using HEK293 cells, we successfully visualized phosphorylated receptor proteins as shown in Fig. 4. Stimulation of transfected HEK293 cells with 1 μM U46619 caused phosphorylation of a broad band at ~44 kDa. The band was not seen in cells transfected with the wild type receptor (panel A) or when the immunoprecipitation was performed in the presence of the hemagglutinin peptide, which is the epitope recognized by the 12CA5 antibody (panel B), suggesting that the ~44 kDa band represents the TP receptor.

To determine whether HEK293 cells were a suitable model system for studying TP receptor regulation, we transiently transfected cells with the wild type receptor or mutant 1. Two days following transfection, cells were incubated with either vehicle, 1 μM U46619 or 100 nM PDBu, prior to washing and rechallenge with 1 μM concentrations of the TxA2 agonist. As shown in Table IV and Table V, IP3 generation in vehicle-treated cells was increased in mutant 1 compared with wild type receptors, similar to the pattern of TP responsiveness in our mesangial cell clones. Prior treatment with either U46619 or PDBu reduced subsequent TxA2-induced increases in IP3 generation in HEK293 cells expressing wild type receptors or mutant 1. However, when data were normalized for differences in the baseline response, there was significantly less desensitization in cells expressing mutant 1 compared with wild type receptors (Tables IV and V). These data suggest that the pattern of TP receptor responsiveness and desensitization is similar in HEK293 cells transiently transfected with TP receptors and in our mouse mesangial cell clones stably expressing TP receptors.

### Table III

Homologous desensitization of IP3 responses in a mesangial cell line

|                         | Pretreated with vehicle | Pretreated with U46619 | Percent baseline response* (cells pretreated with U46619) |
|-------------------------|-------------------------|------------------------|----------------------------------------------------------|
|                         | Wild type (n = 18)      | 154 ± 15               | 86 ± 10                                                   |
|                         | Mutant 1 (n = 15)       | 242 ± 26               | 183 ± 38                                                  |
|                         | Mutant 2 (n = 12)       | 164 ± 31               | 75 ± 6                                                    |

* Data for cells pretreated with U46619 were normalized for differences in the base-line response by expressing the data as the percent response in vehicle-treated cells.

\[ p < 0.025 \] versus vehicle.

\[ p < 0.05 \] versus mutant 1.

\[ p < 0.05 \] versus vehicle.

![FIG. 2. Effect of staurosporine on homologous desensitization.](image)

Clones expressing wild type receptors or mutant 1 were incubated with either vehicle or 1 μM U46619 in the presence or absence of 200 nM staurosporine. After 10 min, cells were washed and then rechallenged with 1 μM U46619. IP3 generation was measured in cells rechallenged with TxA2 agonist. Staurosporine attenuated homologous desensitization in clones expressing the wild type TP receptor but had little effect on responsiveness in clones expressing the mutant receptor. Experiments were performed in triplicate, and data points are the mean of 20 experiments. *p < 0.05 versus U46619 in the absence of staurosporine; *p < 0.01 versus vehicle.

![FIG. 3. Effect of phorbol esters on TP receptor responsiveness.](image)

Clones expressing wild type receptors or mutant 1 were incubated with either 10 nM, 100 nM, or 1 μM concentrations of PDBu prior to washing and stimulation with 1 μM U46619. IP3 generation was measured in cells stimulated with U46619. Pretreatment with 10 nM PDBu had little effect on TP receptor responsiveness. In contrast, 100 nM PDBu significantly reduced U46619-induced PI hydrolysis by the wild type receptor but had little effect on responsiveness of the receptor mutant. 1 μM PDBu reduced responsiveness of both wild and mutant TP receptors. Experiments were performed in triplicate, and data points are the mean of 4–11 experiments. *p < 0.05 versus vehicle.
Phosphorylation of the TP Receptor

FIG. 4. U46619-induced phosphorylation of the TP receptor. HEK293 cells were transfected with either wild type TP receptors (WT) or TP receptors that had been tagged with the 12CA5 epitope (ET). Two days following transfection, 32P-loaded cells were stimulated with 1 μM U46619 for 10 min followed by immunoprecipitation of receptor proteins as described under "Materials and Methods." Stimulation with 1 μM U46619 enhanced phosphorylation of a broad band at ~44 kDa. The band was not seen in cells transfected with the wild type receptor (panel A) or when the immunoprecipitation was performed in the presence of the HA peptide which is the epitope recognized by the 12CA5 antibody (panel B), suggesting that the ~44-kDa band represents the TP receptor.

**TABLE IV**

Homologous desensitization of IP₃ responses in HEK293 cells

Cells were pretreated for 10 min with 1 μM U46619, washed, and then rechallenged with 1 μM U46619 as described under "Materials and Methods." Experiments were performed in duplicate, n = number of experiments.

| IP₃ (% increase above control) | Percent baseline response* (cells pretreated with U46619) |
|-------------------------------|-----------------------------------------------------------|
| Pretreated with vehicle       | Pretreated with U46619                                    |
| Wild type (n = 11)            |                                                            |
| 58 ± 9                      | 17 ± 4                                                    |
| Mutant #1 (n = 11)           |                                                            |
| 141 ± 29                    | 106 ± 38                                                  |

* Data for cells pretreated with U46619 were normalized for differences in the base-line response by expressing the data as the percent response in vehicle-treated cells.

We next determined the role of PKC in direct phosphorylation of TP receptor proteins. For these studies, cells were incubated with 1 μM U46619 in the presence or absence of the specific PKC inhibitor GF109203X (1 μM) (32). As seen in Fig. 5, GF109203X inhibited, but did not prevent, phosphorylation of TP receptor proteins. Inhibition of TP receptor phosphorylation by GF109203X suggests that phosphorylation of the mouse TP receptor is mediated to a large extent by PKC. In data not shown, similar results were obtained with the general kinase inhibitor staurosporine (200 μM) (30).

To study the role of C-terminal serines in agonist-induced phosphorylation of the TP receptor, we created an epitope-tagged mutant receptor lacking serines at positions 321, 322, and 328 of the mouse TP receptor (ET mutant 1). Cells expressing epitope-tagged wild type receptors or ET mutant 1 were loaded with 32P, prior to stimulation with 1 μM U46619 and immunoprecipitation of receptor proteins as described under "Materials and Methods." Basal phosphorylation of wild type and mutant receptors was similar, and these data were combined for the densitometric analysis. As seen in Fig. 6, U46619-induced phosphorylation of the TP receptor was reduced in cells transfected with ET mutant 1 compared with cells transfected with wild type receptors. The apparent difference in agonist-induced phosphorylation of ET mutant 1 was not due to decreased expression of the receptor mutant, because in experiments performed in parallel, TXA₂ binding was similar in cells transfected with either wild type receptors or ET mutant 1 (325 ± 14 fmol/mg of protein (WT) versus 314 ± 13 fmol/mg of protein (ET mutant 1); P = NS, n = 3 experiments). Thus, the absence of serines 321, 322, and 328 attenuates both agonist-induced phosphorylation and homologous desensitization (Table III) of TP receptors, suggesting that phosphorylation of one or more of these serine residues contributes to agonist-specific desensitization of the mouse TP receptor. Although U46619-

![FIG. 4](image-url)

![FIG. 5](image-url)

![TABLE IV](table-url)

![TABLE V](table-url)
induced phosphorylation of ET mutant 1 was reduced compared with the wild type, there was still a significant increase in phosphorylation compared with its basal phosphorylation state (see Fig. 6). This U46619-induced phosphorylation of ET mutant 1 suggests that the TP receptor can be phosphorylated at residues other than serine 321, 322, or 328.

To study the role of PKC in direct phosphorylation of C-terminal serines, cells expressing epitope-tagged wild type receptors or ET mutant 1 were loaded with 32P, prior to stimulation with 100 nM PDBu and immunoprecipitation of receptor proteins as described under "Materials and Methods." As in the U46619-induced phosphorylation experiments, basal phosphorylation of wild type and mutant receptors was similar, and these data were combined for the densitometric analysis. Phosphorylation of wild type receptors. The level of phosphorylation of either the wild type receptor or ET mutant 1 was not significantly different at the 100 nM and 1 μM concentrations of PDBu. These data suggest that increasing PDBu from 100 nM to 1 μM does not result in significant increases in TP receptor phosphorylation.

**DISCUSSION**

TxA2 plays a key role in diseases affecting the heart, lungs, kidneys, and peripheral vascular system (1–3). Its effects are regulated by both PKC-dependent and PKC-independent mechanisms (4, 6). Previous studies have suggested that C-terminal domains are required for regulation of the TP receptor by PKC (17), perhaps by direct phosphorylation of the receptor protein (5, 17). In the present study, we investigated the role of the C-terminal serines in the rapid regulation of TP receptor phosphorylation of wild type receptors. The level of phosphorylation of either the wild type receptor or ET mutant 1 was not significantly different at the 100 nM and 1 μM concentrations of PDBu.
responsive. We found that TP receptors lacking serines at positions 321, 322, and 328 in the C terminus are resistant to both U46619- and PKC-induced desensitization. Attenuated desensitization of the receptor mutant is associated with decreased phosphorylation of receptor proteins compared with wild type receptors. Taken together, these data suggest that both agonist-specific and PKC-induced desensitization of the TP receptor are mediated, at least in part, by phosphorylation of C-terminal hydroxyamino acids.

The absence of the C-terminal serines at positions 321, 322, and 328 inhibited, but did not prevent, U46619-induced phosphorylation of the TP receptor. These findings indicate that the TP receptor is phosphorylated in an agonist-dependent fashion at residues other than serines 321, 322, and 328. It is likely that this agonist-dependent phosphorylation of mutant 1 is mediated by either receptor specific kinases or general kinase systems such as PKC. With regard to the latter hypothesis, the specific PKC inhibitor GF109203X markedly attenuated, but did not prevent, U46619-induced phosphorylation of the TP receptor. Similar results were found using the more general kinase inhibitor staurosporine. Thus, the TP receptor is phosphorylated in an agonist-dependent manner by a staurosporine- and GF109203X-resistant kinase. These findings are consistent with the notion that receptor specific kinases may contribute to desensitization of the mouse TP receptor. This family of kinases has been shown to play a major role in regulating receptor responsiveness in other receptor systems (7, 8). Although the consensus motif for phosphorylation by receptor-specific kinases is not fully elucidated, serine or threonine residues flanked by acidic amino acids (D/ES/T or D/EXS/T) appear to be preferred substrates for G protein-coupled receptor kinases (33). Motifs of this configuration are found in the third intracellular loop of the mouse, rat, and human TP receptors (9–11) and might play a role in regulating TP receptor responsiveness. Indeed, preliminary studies from this laboratory suggest that phosphorylation of the TP receptor by G protein-coupled receptor kinases may contribute to TP receptor regulation (34).

The absence of serines 321, 322, and 328 prevented desensitization of TP receptors by 100 nM PDBu. In contrast, 1 μM PDBu caused desensitization of wild type and mutant receptors to a similar extent. One possibility to explain this observation is that 1 μM PDBu enhances phosphorylation of domains of the TP receptor outside of the C-terminal 22 amino acids. Indeed, hydroxyamino acids in the proximal C terminus as well as the second and third intracellular loops have favorable motifs for phosphorylation by PKC (11, 35). Moreover, the absence of serines 321, 322, and 328 inhibits, but does not prevent, phosphorylation of the TP receptor by 100 nM PDBu. We therefore determined if treatment with 1 μM PDBu further enhances TP receptor phosphorylation compared with 100 nM PDBu. In these experiments, increasing PDBu from 100 nM to 1 μM did not significantly enhance phosphorylation of either wild type or mutant TP receptors. While we cannot exclude the possibility that small increases in receptor phosphorylation were not detected by our assay system, it seems more likely that 1 μM PDBu caused phosphorylation of other substrates leading to attenuation of TP receptor signaling. In this regard, PKC might: 1) phosphorylate and inactivate other downstream components of the signaling cascade or 2) phosphorylate and activate other regulatory mechanisms. Evidence for each of these possibilities has been demonstrated in other receptor systems. For example, activation of PKC by phorbol esters has been shown to phosphorylate and inactivate phospholipase-β (36). Moreover, G protein-coupled receptor kinase 2 can be phosphorylated by PKC in vitro resulting in 2-fold activation of the enzyme toward its substrate (37). Thus, PKC might regulate multiple components of the signaling cascade.

U46619-stimulated IP₃ generation was higher in mutant 1 compared with either wild type receptors or mutant 2. A possible explanation for this enhanced PI hydrolysis is that general kinase systems may inhibit TP receptor responsiveness under basal conditions. The absence of C-terminal serines might attenuate this inhibition by these kinase systems. In support of this hypothesis, we previously found that PKC inhibitors tended to enhance TP receptor responsiveness in cells that express an endogenous receptor for TxA₂ (6). Alternatively, the absence of regulatory serines may result in more sustained PI hydrolysis in mutant 1 compared with wild type receptors. In this regard, agonist-induced elevations in IP₃ levels are prolonged in other receptor systems following removal of C-terminal regulatory domains (38). Further studies will be needed to determine if either of these possibilities contributes to enhanced PI hydrolysis by TP receptors lacking serines 321, 322, and 328.

Although the PKC inhibitor GF109203X did not completely prevent U46619-induced phosphorylation of the mouse TP receptor, agonist-induced phosphorylation was markedly reduced in the presence of the PKC inhibitor. In contrast to our findings, Habib et al. (17) found that phosphorylation of human TP receptors was only modestly affected by GF109203X in HEK293 cells expressing TP receptor isoforms. Possibilities to explain these differing results include: 1) species specific differences in TP receptor regulation or 2) methodological differences between the studies. With regard to the latter, Habib et al. (17) immunoprecipitated human TP receptors using isoform-specific anti-peptide antibodies from cells stably expressing high levels of TP receptors. In contrast, we studied phosphorylation of 12CA5-tagged TP receptors in a transient transfection system expressing more modest levels of TP receptor proteins. While it is not known if these methodologic differences can account for the differing results, both these studies were performed in artificial expression systems that might affect normal regulatory mechanisms. It will therefore be important to determine whether results using overexpression systems are generalizable to cells that endogenously express receptors for TxA₂.

Mutating serine at position 339 did not affect signaling or desensitization of the mouse TP receptor. While we did not directly study the effects of this mutation on receptor phosphorylation, serine 339 does not have a favorable motif for phosphorylation by PKC, protein kinase A, protein kinase G, or calcium-calmodulin-dependent protein kinases (34). In addition, this amino acid residue is not flanked by acidic amino acids, which tend to be favored sites for phosphorylation by G protein-coupled receptor kinases (33). It therefore seems unlikely that serine 339 is an important target for phosphorylation by protein kinases.

In summary, the present studies indicate that the absence of serines at positions 321, 322, and 328 inhibits both U46619- and PKC-induced desensitization of the TP receptor. Agonist-specific desensitization is associated with enhanced phosphorylation of receptor proteins. Both U46619-induced desensitization and phosphorylation are attenuated by either PKC inhibitors or the absence of serines 321, 322, and 328. PKC-induced desensitization caused by 100 nM PDBu is also associated with increased phosphorylation of receptor proteins. This PKC-mediated phosphorylation was inhibited by deleting serines, 321, 322, and 328. Finally, increasing the concentration of PDBu from 100 nM to 1 μM PDBu reduced responsiveness of wild type receptors and mutant receptors lacking serines 321, 322, and 328 without enhancing phosphorylation.
of either of the receptor proteins. These data suggest that 1) phosphorylation of C-terminal serines contributes to both agonist-specific and PKC-dependent desensitization of the TP receptor, and 2) PKC-induced desensitization of the TP receptor is complex and involves both phosphorylation of C-terminal serines as well as mechanisms that may not require direct phosphorylation of receptor proteins.

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