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Effect of Receptor Number on Desensitization of the Mouse Thromboxane Receptor

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ABSTRACT. Desensitization of G-protein coupled receptors limits the physiologic effects of an agonist. Short-term desensitization mechanisms are critically dependent on receptor phosphorylation by protein kinases. The effectiveness of these regulatory mechanisms might be limited by substrate (receptor) availability. To investigate the role of receptor number in the desensitization of G-protein coupled receptors, we transfected a mouse mesangial cell line with a genomic clone encoding the mouse thromboxane A_2 (TxA_2) receptor and obtained cell lines that expressed low (≈250–500 fmol/mg protein) or high (2500–4000 fmol/mg protein) levels of TxA_2 receptors. Activation of TxA_2 receptors stimulated phosphoinositide (PI) hydrolysis and increased intracellular calcium ($[Ca^{2+}]_i$) levels. Prior exposure to the TxA_2 agonist (15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid (U46619) reduced subsequent (15*S*)-hydroxy-11α,9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid-induced increases in inositol trisphosphates and intracellular calcium levels by ≈50% in clones expressing low numbers of TxA_2 receptors, but had little effect on TxA_2 receptor responsiveness in clones expressing high receptor numbers. Failure of TxA_2 receptors to desensitize caused sustained increases in intracellular calcium levels and phosphoinositide hydrolysis. Thus, homologous desensitization of TxA_2 receptors is attenuated in cells expressing high levels of receptors for TxA_2. These data suggest that receptor number plays a key role in the short-term regulation of G-protein coupled receptors. BIOCHEM PHARMACOL **55**;8: 1271–1281, 1998. © 1998 Elsevier Science Inc.

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Exposure of receptors on intact cells or tissues to an agonist often leads to diminished receptor responsiveness to subsequent agonist exposure. This loss of receptor responsiveness (desensitization) is mediated by a variety of mechanisms occurring over time periods ranging from minutes to days [1, 2]. Rapid mechanisms of receptor regulation include: (1) internalization or sequestration of receptors into an intracellular compartment away from the cell surface, and (2) impaired coupling of the receptor to its effector systems. Uncoupling of the receptor from its effector systems is thought to be triggered by phosphorylation of the receptor by general kinase systems as well as by receptor specific kinases [2, 3]. In the case of receptor specific kinases, the phosphorylated receptor interacts stoichiometrically with a second group of protein cofactors that inactivate the receptor, presumably by steric mechanisms [3]. These enzymatic reactions are crucial for terminating the effects of agonists and may be affected by alterations in the amount or activity of the regulatory enzymes or protein cofactors as well as by the availability of receptor substrate.

In the present study, we investigated short-term regulation of receptors for TxA_2.† This labile lipid mediator is a potent platelet-aggregating and vasoconstrictor eicosanoid that has been implicated in the pathogenesis of diseases affecting the heart, lungs, kidneys, and peripheral vascular system [4–6]. Its effects are mediated by activating specific cell surface receptors. In most cell systems, receptor activation stimulates PLC through pertussis toxin insensitive G-proteins [7, 8]. Alternative splicing also has been shown to produce two isoforms of the human TxA_2 receptor [9], which, in addition to coupling to PLC, oppositely regulate adenylyl cyclase activity [10]. These physiologic effects of TxA_2 are tightly regulated. Studies from this laboratory [11]

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† *Abbreviations:* TxA_2, thromboxane A_2; [I]BOP, [15-(1α,2β(5*Z*), 3α-(1*E*,3*S*)4α)]-7-[3-(3-hydroxy-4-(*p*-iodophenoxy)-1-butenyl)-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptenoic acid; U46619, (15*S*)-hydroxy-11α, 9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid; SQ29548, [1*S*-(1α,2β(5*Z*), 3β,4α)-7-(3-((2-((phenyl-amino)-carbonyl)hydrazino) methyl- 7-oxabicyclo-(2.2.1)heptan-2-yl)-5-heptenoic acid]; K_d, dissociation constant; B_{max}, maximal number of specific binding sites; K_i, dissociation constant for competitive inhibitors; PKC, protein kinase C; PLC, phospholipase C; KRB, Krebs–Ringer buffer; PDBu, phorbol 12,13-dibutyrate; G-protein, guanine nucleotide regulatory protein; IP1, inositol monophosphates; IP2, inositol biphosphates; IP3, inositol trisphosphates; $[Ca^{2+}]_i$, intracellular calcium; PI, phosphoinositide; PGE_2, prostaglandin E_2; TxB_2, thromboxane B_2; PCR, polymerase chain reaction; and 4α-DD, 4α-phorbol 12,13-didecanoate.

and by other investigators [12] have found that desensitization of TxA_2 receptors is mediated, at least in part, through activation of PKC, perhaps through direct phosphorylation of the receptor protein. In support of this hypothesis, Kinsella *et al.* [13] demonstrated that PKC can phosphorylate C-terminal sequences of the TxA_2 receptor *in vitro*. This suggests that the receptor for TxA_2 may be a substrate for PKC *in vivo* and that negative feedback loops involving protein kinases may regulate responsiveness of TxA_2 receptors.

Although some investigators have postulated that the amount of receptor substrate may determine the extent of receptor desensitization [14], there is little experimental evidence to support this hypothesis in the literature. To investigate the effect of varying the level of receptor substrate on desensitization of TxA_2 receptors, we permanently transfected a mouse mesangial cell line with a genomic clone encoding the mouse TxA_2 receptor, and obtained cell lines that expressed TxA_2 receptors at high or low levels. Exposure of clones to TxA_2 agonists caused rapid increases in PI hydrolysis and $[Ca^{2+}]_i$ levels. Desensitization of TxA_2 receptors occurred promptly in cells expressing low numbers of TxA_2 receptors, but was attenuated in clones with higher receptor numbers. Failure of cells to desensitize caused sustained increases in $[Ca^{2+}]_i$ levels and inositol phosphate generation. These data suggest that homologous desensitization of TxA_2 receptors is attenuated by increasing the abundance of receptors for TxA_2.

MATERIALS AND METHODS

Mesangial Cell Cultures

Mouse mesangial cells derived from SV40 transgenic mice [15] were obtained from the American Type Culture Collection. Cells were grown in 75% Dulbecco's Modified Eagle medium (DMEM) and 25% F-12 nutrient medium (HAMS) supplemented with 5% heat-inactivated fetal bovine serum, 14 mM of HEPES, penicillin (100 units/mL), and streptomycin (100 μg/mL) (all from Life Technologies) at 37° in a humidified atmosphere of 95% air and 5% CO_2. Mesangial cells were subcultured every week after becoming confluent, using 0.25% trypsin with 1 mM of EDTA (Life Technologies), and plated at a density of 2–5 $\times$ 10^5 cells/mL. Cell viability was assessed by standard dye exclusion techniques (0.1% trypan blue) and was always greater than 95%.

Isolation and Expression of a Genomic Clone Encoding the Mouse TxA_2 Receptor

A genomic clone encoding the mouse TxA_2 receptor was isolated as previously described [16] using the following strategy. PCR primers were prepared based on the published sequences of the mouse TxA_2 receptor cDNA [17]. The primer pairs encompassed nucleotides 534-553 (CTCTT GGTGCTTCCTGACAC) and 972-953 (CTGGAGCT GTGAACTGAACC) of the TxA_2 receptor cDNA [17]. A PCR product of the appropriate size was amplified from total RNA isolated from mouse lung, and its sequence was found to be homologous to previously described TxA_2 receptors [17]. Using this partial cDNA as a probe, we detected a single distinct band on Southern blotting of mouse genomic DNA. We used a homologous probe to screen a genomic DNA library made from 129/Ola mouse DNA. Three identical clones were isolated, and portions of one of the clones were sequenced and found to contain the complete coding sequences for the TxA_2 receptor protein. We subcloned a *XhoI*/*ApaI* fragment of our genomic clone containing the complete amino acid encoding regions into the mammalian expression vector pcDNA 3 (Invitrogen), and transfected this construct into mouse mesangial cells by the calcium-phosphate method [18]. G418 resistant cells were selected in complete medium containing 500 μg/L G418. Following G418 selection, individual clones were screened for TxA_2 binding as described below.

Ligand Binding Assays

Whole cell ligand binding assays were performed as previously described [11, 16] using the stable radiolabeled thromboxane receptor antagonist [^{3}H]SQ29548 [19] (New England Nuclear) and the following unlabeled compounds SQ29548 (Squibb Institute), the thromboxane agonists U46619 [20] (Cayman Chemicals) or [^{127}I]BOP [21] (Cayman), the inactive thromboxane metabolite TxB_2 (Advanced Magnetics Inc.), or PGE_2 (Advanced Magnetics). Equilibrium binding data were analyzed by the method of Scatchard [22] to give estimates of the B_{max} and apparent equilibrium K_d by fitting the data to a nonlinear model using the ENZFITTER computer program (Elsevier–Biosoft). For the competitive binding assays, data were analyzed by the method of Cheng and Prusoff [23] to calculate the dissociation constant for each inhibitor (K_i). Protein concentration used in the binding assays was determined using the method of Bradford [24].

Measurement of [^{3}H]Inositol Phosphate Generation

Inositol phosphates were measured as previously described using anion exchange chromatography [11, 16]. For the desensitization experiments, cells were pretreated for 10 min with the indicated concentrations of agonists, inhibitors, or their vehicles in 2 mL of KRB at 37°. After desensitization, cells were washed three times with KRB and then incubated in 2 mL of KRB for 4 min before adding 2 M of 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 thrombin or their vehicle for 2 min.

TABLE 1. Responsiveness of mouse mesangial cell clones

	Receptor number (fmol/mg protein)	IP generation (% increase above control)			Peak $[Ca^{2+}]_i$ (nM)
		IP1	IP2	IP3	
Clone I	264 ± 52	38 ± 22	90 ± 20	91 ± 17	263 ± 21
Clone II	517 ± 29	76 ± 17	197 ± 36*	231 ± 38†	375 ± 42†
Clone III	2288 ± 257	76 ± 19	242 ± 41†	237 ± 7†	427 ± 55†
Clone IV	3704 ± 205	92 ± 24	225 ± 23†	242 ± 22†	481 ± 107†
Non-transfected	Negligible‡	No response	18 ± 9	17 ± 11	No response

Cells were stimulated with 10 μM of U46619, and PI hydrolysis was measured 2 min later. Data points are the means ± SEM of 4–16 experiments per clone. Basal levels of IP generation are presented in Table 2.

*,†Significantly different from clone I (clone expressing ≈250 fmol/mg protein) at: *$P < 0.05$ or †$P < 0.025$.

‡Small amounts of specific TxA_2 binding were detected in some assays (less than 20 fmol/mg of protein).

The reaction was stopped, and samples were processed as previously described [11, 16].

Cytosolic Calcium Measurements

$[Ca^{2+}]_i$ levels were measured in confluent cells by fluorescence excitation of cells loaded with the fluorescent probe fura 2 acetoxymethyl ester (fura 2-AM, Sigma) as previously described [11, 16]. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz *et al.* [25] using the following formula:

$$[Ca^{2+}]_i = K_d \frac{(R - R_{min})\, S_{f2}}{(R_{max} - R)\, S_{b2}}$$

K_d is the dissociation constant of the Ca^{2+}–fura 2 complex, and 224 nM was employed in these calculations [25]. R is the fluorescence emission ratio derived by dividing the fluorescence at an excitation wavelength at 340 nm by the fluorescence excitation wavelength at 380 nm. S_{f2} and S_{b2} is the fluorescence at an excitation wavelength of 380 nm for Ca^{2+}-free dye (S_{f2}) and for Ca^{2+}-bound dye (S_{b2}). R_{max} and R_{min} are the maximal and minimal fluorescence emission ratios, respectively. R_{max} and S_{b2} were determined experimentally at 37° using 1 μM of fura 2 dissolved in a solution of the following composition designed to mimic intracellular ionic conditions: 115 mM of KCl, 20 mM of NaCl, 1 mM of $MgCl_2$, 10 mM of HEPES, 2 mM of $CaCl_2$, pH 7.1. This solution was supplemented with 10 mM of EGTA to obtain R_{min} and S_{f2}.

Statistical Analysis

Data are presented as the means ± SEM. Statistical significance was assessed using a paired or unpaired *t*-test, as indicated.

RESULTS

Characteristics of TxA_2 Receptors in Transfected Mesangial Cells

TxA_2 binding was negligible in nontransfected mesangial cells (Table 1). We therefore screened for clones expressing our genomic construct, using radioligand binding assays as described in Materials and Methods. Four clones were identified that stably expressed receptors for TxA_2. By Scatchard analysis [22] the clones expressed a single TxA_2 binding site at densities of ≈250, ≈500, ≈2500, and ≈4000 fmol/mg of protein, respectively. As shown in Fig. 1, the [^{3}H]SQ29548 binding site displayed the specificity appropriate for a TxA_2 receptor. Unlabeled SQ29548 displaced the radiolabeled compound with a K_i of 6 ± 1 nM. The TxA_2 receptor agonists [^{127}I]BOP and U46619 had K_i values of 3 ± 2 and 134 ± 14 nM, respectively. In contrast, PGE_2 and the inactive TxA_2 metabolite TxB_2 displaced the radioligand only at much higher concentra-

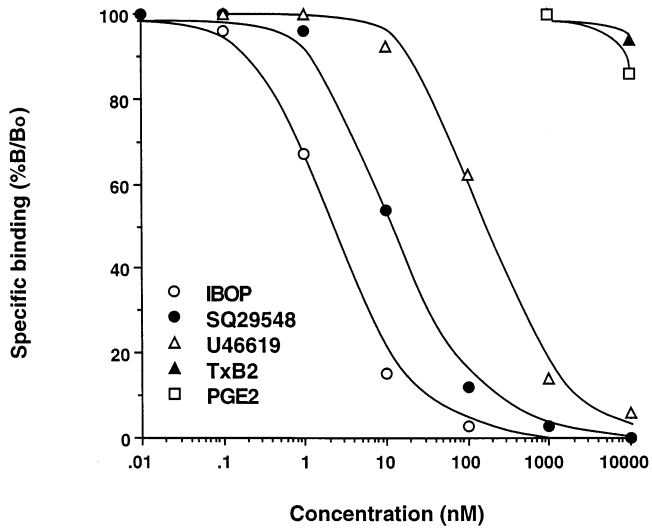

FIG. 1. Inhibition of specific [^{3}H]SQ29548 binding to stably transfected mesangial cells. A mouse mesangial cell line was transfected with our genomic construct containing the complete coding sequences for the TxA_2 receptor protein. Clones stably expressing the TxA_2 receptor were isolated as described in Materials and Methods. In these clones, unlabeled SQ29548 displaced the radiolabeled compound with a K_i of 6 ± 1 nM. The TxA_2 receptor agonists [^{127}I]BOP and U46619 had K_i values of 3 ± 2 and 134 ± 14 nM, respectively. PGE_2 and the inactive TxA_2 metabolite TxB_2 displaced the radioligand only at much higher concentrations. Data are percent specific binding in the presence (B) or absence (B_o) of each competitor. Thromboxane binding by individual clones is presented in Table 1. Experiments were performed in duplicate, and data points are the means of 4 experiments.

tions. The rank order potency of binding of these compounds was similar to cells that endogenously express receptors for TxA_2 [26].

Effect of TxA_2 Receptor Number on Signal Transduction

The TxA_2 receptor agonist U46619 (10 μM) caused rapid increases in inositol phosphates and $[Ca^{2+}]_i$ levels in all four transfected cell lines but had little effect on PI hydrolysis or $[Ca^{2+}]_i$ levels in nontransfected mesangial cells, as shown in Table 1. When TxA_2 receptor number was increased from ≈250 to ≈500 fmol/mg of receptor protein, inositol phosphate generation induced by the TxA_2 agonist increased proportionally with receptor number. However, increases in TxA_2 receptor number above ≈500 fmol/mg of protein did not cause further enhancement of PI hydrolysis. A similar pattern was found for TxA_2-induced increases in $[Ca^{2+}]_i$ levels (Table 1). These data suggest that: (1) the transfected TxA_2 receptor couples to PLC, as would be expected of endogenous receptors for TxA_2 [11, 26], (2) increasing TxA_2 receptor number results in proportional increases in PLC activity up to a certain threshold value (≈500 fmol/mg of protein), and (3) above this threshold value, further increases in receptor number do not result in further increases in second messenger generation, a finding consistent with the results of other investigators in the β-adrenergic receptor system [27].

The pattern of inositol phosphate responses and calcium responses was different depending on the TxA_2 receptor number. Figure 2 shows the time course of PI hydrolysis in clones I and III, expressing TxA_2 receptors at a density of either ≈250 or ≈2500 fmol/mg of protein, respectively. As shown in panel A, PI hydrolysis increased promptly following exposure to TxA_2 agonist and then stabilized at ≈60% (1P3), ≈70% (IP2) and ≈30% (IP1) of the peak levels in cells expressing ≈250 fmol TxA_2 receptors/mg of protein (clone I). A similar pattern for U46619-induced PI hydrolysis was seen in cells expressing ≈500 fmol TxA_2 receptors/mg protein (clone II). Panel B shows results for cells expressing ≈2500 fmol TxA_2 receptors/mg of protein (clone III). In these cells, U46619 induced a rapid rise in inositol phosphate levels that was sustained throughout the time period studied. After 10 min of exposure to a TxA_2 agonist, IP3, IP2, and IP1 levels remained at ≈70, ≈80, and ≈50% of the peak levels, respectively. This sustained pattern of U46619-induced PI hydrolysis was also seen in cells expressing ≈4000 fmol TxA_2 receptors/mg of protein (clone IV).

To determine the effect of TxA_2 receptor number on U46619-induced increases in $[Ca^{2+}]_i$ levels, we monitored $[Ca^{2+}]_i$ levels while cells were perfused continuously with the TxA_2 agonist. Representative results of these studies are shown in Fig. 3. In cells expressing low numbers of TxA_2 receptors (clones I and II), $[Ca^{2+}]_i$ levels increased ≈15 sec after the application of U46619, peaked in ≈20–30 sec, and then returned slowly toward baseline, leveling off at ≈40% of the peak levels 4–5 min after the application of

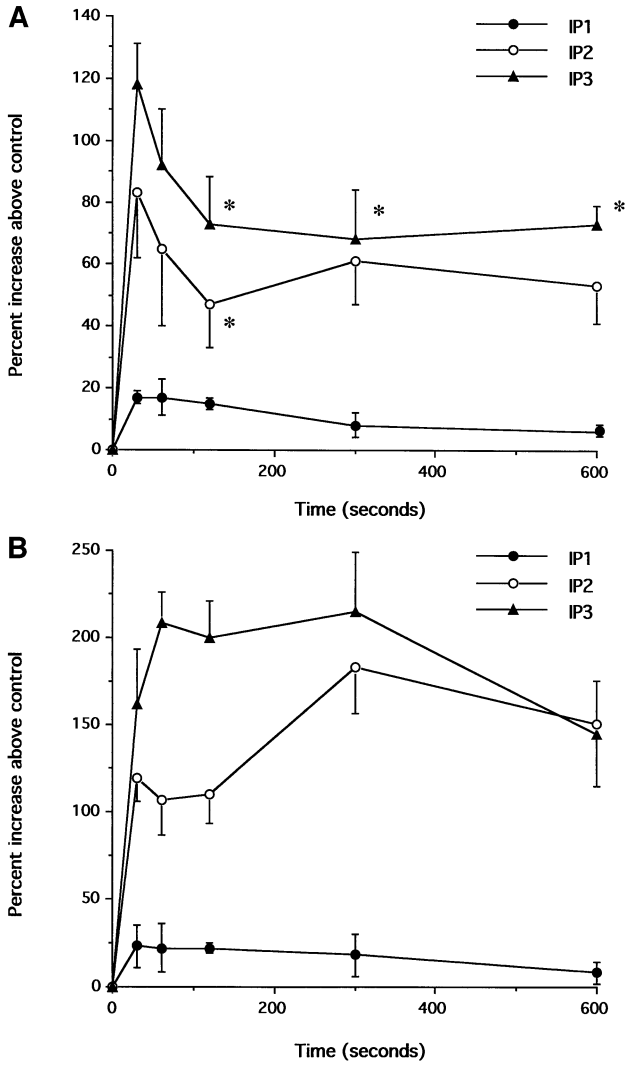

FIG. 2. Time course of TxA_2-induced increases in inositol phosphates. Mouse mesangial cells stably expressing the TxA_2 receptor were stimulated with 10 μM of U46619 for the indicated times. Panel A shows results for clone I expressing ≈250 fmol TxA_2 receptors/mg protein, and panel B shows results for the clone III expressing ≈2500 fmol TxA_2 receptors/mg of protein. Experiments were performed in duplicate and data points are the means ± SEM of 8 experiments. Basal levels of IP generation are presented in Table 2. Key: *$P < 0.05$ vs the peak inositol phosphate level by an unpaired *t*-test.

U46619. In contrast, U46619-induced increases in $[Ca^{2+}]_i$ levels were sustained in cells expressing high numbers of receptors for TxA_2 (clones III and IV). Indeed, $[Ca2+]_i$ levels remained at ≈70% of the peak levels for at least 15 min in cells expressing high numbers of TxA_2 receptors. Chelation of extracellular calcium with EDTA attenuated the prolonged increase in $[Ca^{2+}]_i$ levels in cells expressing either high or low numbers of TxA_2 receptors, suggesting that the prolonged increase in $[Ca^{2+}]_i$ levels resulted either from extracellular sources or from impaired capacitative filling of internal stores.

High levels of receptor expression may activate downstream effectors in a ligand-independent fashion [28]. To

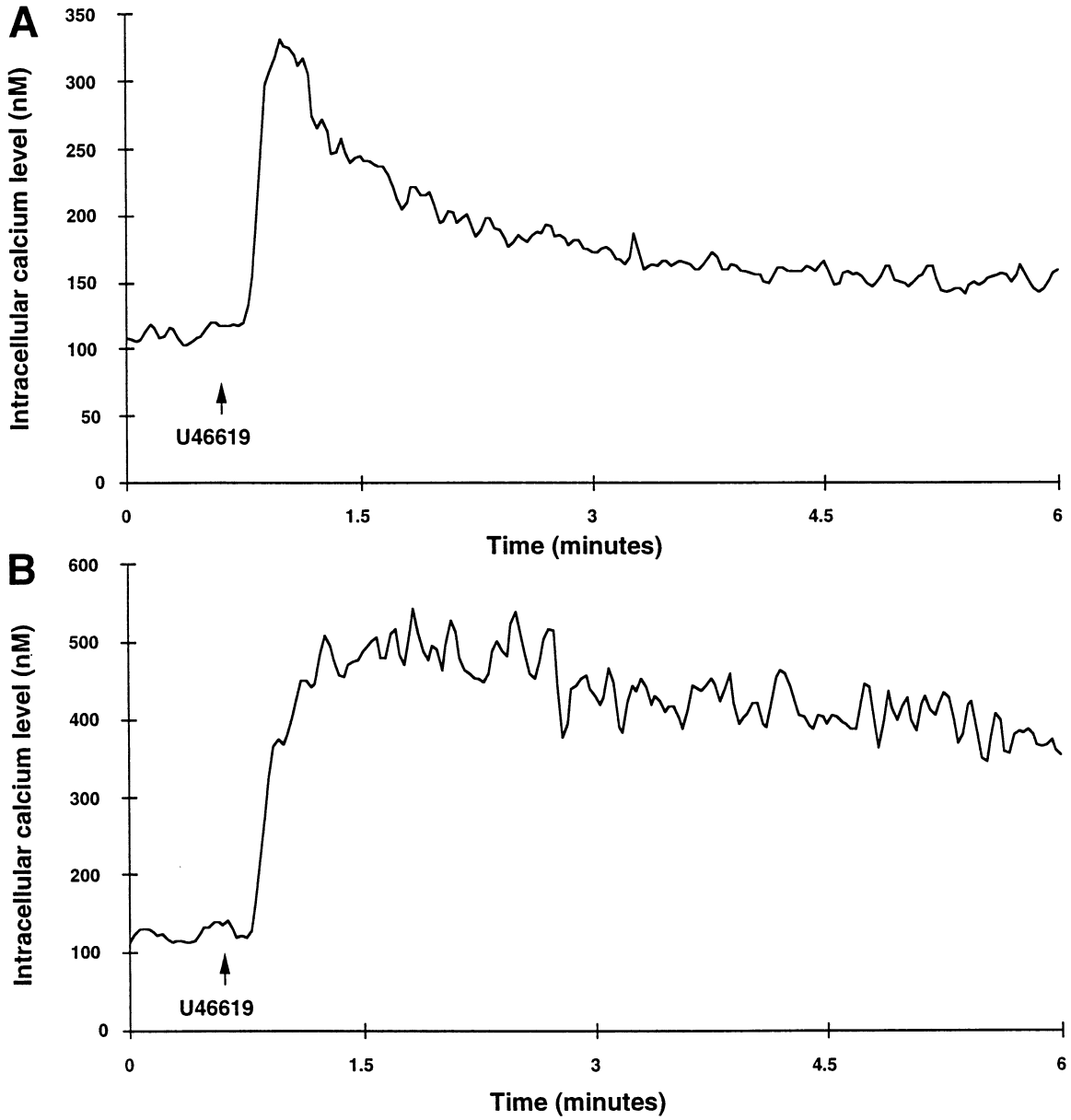

FIG. 3. Time course of TxA_2-induced increases in $[Ca^{2+}]_i$ levels. Mesangial cell clones were stimulated with 10 μM of U46619 and then perfused with 10 μM of U46619 for the remainder of the study period. Panel A shows results for clone I expressing ≈250 fmol of TxA_2 receptors/mg protein, and panel B shows results for clone III expressing ≈2500 fmol of TxA_2 receptors/mg protein. Results shown are representative tracings from 16 studies in clones expressing low numbers of TxA_2 receptors and from 15 studies in clones expressing high numbers of TxA_2 receptors.

determine if high levels of TxA_2 receptor expression resulted in ligand independent activity, we first measured basal levels of IP1, IP2, and IP3 in cells expressing low numbers of TxA_2 receptors (clones I and II) and in cells expressing high numbers of TxA_2 receptors (clones III and IV). As shown in Table 2, basal levels of IP generation were similar in cells expressing either low or high numbers of TxA_2 receptors. To further investigate ligand-independent activity of the TxA_2 receptor, we measured IP1 generation in cells expressing high numbers of TxA_2 receptors in the presence and absence of lithium chloride (20 mM). Lithium chloride was included in the incubation medium to inhibit IP breakdown, primarily by inhibiting breakdown of IP1 [29]. After a 10-min incubation period, IP1 levels were similar in the presence (24,742 ± 2,255 dpm) or absence (25,382 ± 3,735 dpm) of lithium chloride (P = NS; N = 6). These data suggest that the TxA_2 receptor does not exhibit significant ligand independent activity in our model system.

Effect of TxA_2 Receptor Number on Homologous Desensitization

To study the role of receptor number in desensitization of TxA_2 receptors, cells expressing either high or low numbers

TABLE 2. Basal levels of IP generation in mouse mesangial cell clones

	IP generation* (dpm)		
	IP1	IP2	IP3
Clone I	28,019 ± 2,656	3,826 ± 358	2,176 ± 184
Clone II	28,139 ± 2,659	4,514 ± 748	2,589 ± 549
Clone III	26,645 ± 1,746	4,177 ± 360	2,152 ± 149
Clone IV	27,611 ± 1,739	4,392 ± 660	2,361 ± 647

*Basal IP generation was measured in the absence of lithium chloride. Data points are the means ± SEM of 4 experiments.

of TxA_2 receptors were exposed to 10 μM of U46619, washed to remove bound agonist, and then rechallenged with 10 μM of U46619 as described in Materials and Methods. TxA_2 receptor responsiveness was assessed by measuring IP3 generation. As shown in Table 3, prior exposure to the TxA_2 agonist reduced subsequent U46619-induced increases in inositol phosphates by ≈50% in cells with low numbers of TxA_2 receptors (clones I and II). In contrast, pretreatment with U46619 had little effect on subsequent U46619-induced PI hydrolysis in cells with higher numbers of TxA_2 receptors (Clones III and IV). When the data were expressed as the percent response in vehicle-treated cells (Table 3), TxA_2 receptor responsiveness was reduced significantly in clone I and clone II compared with either clone III or clone IV.

Desensitization of G-protein coupled receptors is manifest not only by a reduction in maximal receptor responsiveness, but also by a rightward shift of the concentration–response curve [2]. We therefore investigated the effect of prior exposure to 10 μM of U46619 on subsequent IP3 generation induced by rechallenging cells with either 10-nM, 100-nM, 1-μM, or 10-μM concentrations of TxA_2 agonist. The resulting concentration–response curves are shown in Fig. 4 for clone II expressing low receptor numbers (panel A) and clone III expressing high receptor numbers (panel B). Pretreatment with U46619 increased the EC_{50} from 54 to 134 nM in clone II expressing low numbers of TxA_2 receptors (panel A). In contrast, pretreatment with U46619 had little effect on the EC_{50} in clone III expressing high levels of TxA_2 receptors (14 nM [vehicle] vs 16 nM [U46619; P = NS). When the same data were expressed as

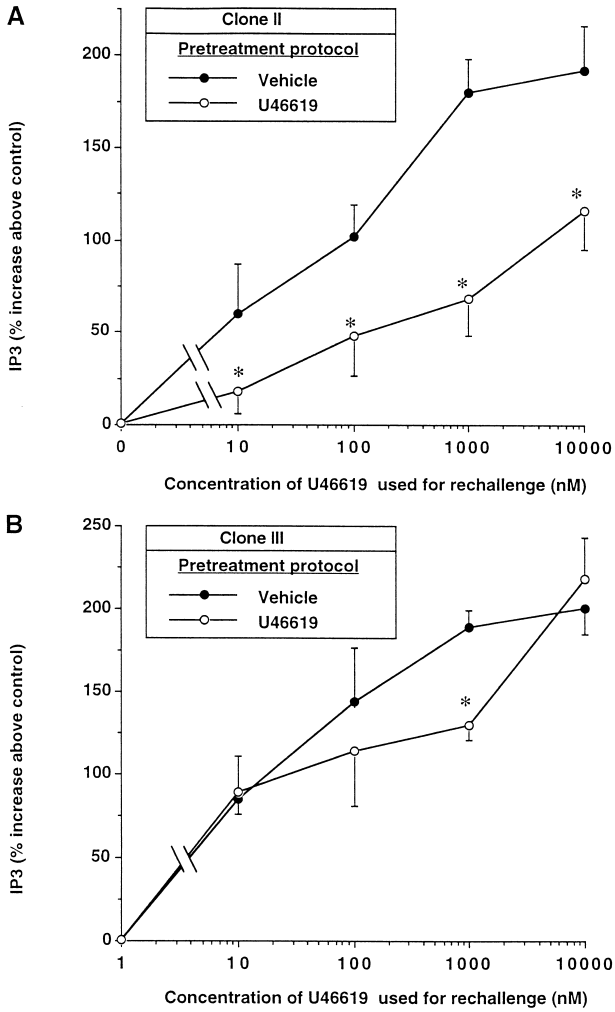

FIG. 4. Effect of prior exposure to U46619 on subsequent TxA_2-induced IP3 generation. Mesangial cell clones were incubated with 10 μM of U46619, washed to remove bound agonist, and then rechallenged with either 10 nM, 100 nM, 1 μM, or 10 μM of U46619 as described in Materials and Methods. Generation of IP3 was measured in cells rechallenged with the TxA_2 agonist. The concentration–response curve is shifted rightward to a greater extent in clone II expressing low numbers of TxA_2 receptors (panel A) compared to clone III expressing high levels of TxA_2 receptors (panel B). Experiments were performed in duplicate and data points are the means ± SEM of 12 experiments. Basal levels of IP3 generation are presented in Table 2. *$P < 0.05$ vs vehicle-treated cells by an unpaired t-test.

TABLE 3. Homologous desensitization of mouse mesangial cell clones

		IP3 generation (% increase above control)		
	Receptor number (fmol/mg protein)	Pretreated with vehicle	Pretreated with U46619	% Response in vehicle-treated cells
Clone I	264 ± 52	86 ± 12	47 ± 20*	47 ± 12†
Clone II	517 ± 29	194 ± 26	116 ± 21*	59 ± 6†
Clone III	2288 ± 257	244 ± 34	218 ± 52	88 ± 10
Clone IV	3704 ± 205	229 ± 14	258 ± 22	107 ± 7

Cells were pretreated with 10 μM of U46619 prior to washing and rechallenge with 10 μM of U46619 as described in Materials and Methods.
*$P < 0.025$ vs cells pretreated with vehicle.
†$P < 0.05$ vs either clone III or clone IV. Data points are the means ± SEM of 5–11 experiments per clone. Basal levels of IP3 generation are presented in Table 2.

TABLE 4. Effect of receptor number on the extent of homologous desensitization

Concentration of U46619 used to rechallenge clones	Percent response in vehicle-treated cells	
	Clone II	Clone III
10 nM	22 ± 13*	109 ± 18
100 nM	36 ± 16*	74 ± 8
1 μM	34 ± 6*	70 ± 8
10 μM	54 ± 5*	88 ± 10

Cells were pretreated with 10 μM of U46619 prior to washing and rechallenge with the indicated concentrations of U46619 as described in Materials and Methods. Data points are the means ± SEM of 12 experiments.

*$P < 0.05$ vs clone III.

the percent response in the vehicle-treated cells (Table 4), the extent of TxA_2 receptor desensitization was reduced significantly in clone III expressing high numbers of TxA_2 receptors compared with clone II expressing low numbers of TxA_2 receptors at the 10-nM, 100-nM, 1-μM and 10-μM concentrations of U46619.

We next determined if calcium responses were desensitized to a second application of U46619. For these experiments, cells were perfused with 10 μM of U46619 for the indicated times, washed with buffer, and then rechallenged with 10 μM of U46619. Representative studies are shown in Fig. 5. In all clones, $[Ca^{2+}]_i$ levels tended to remain above baseline even after washing, which made quantitation of the responses difficult. Despite this limitation, we

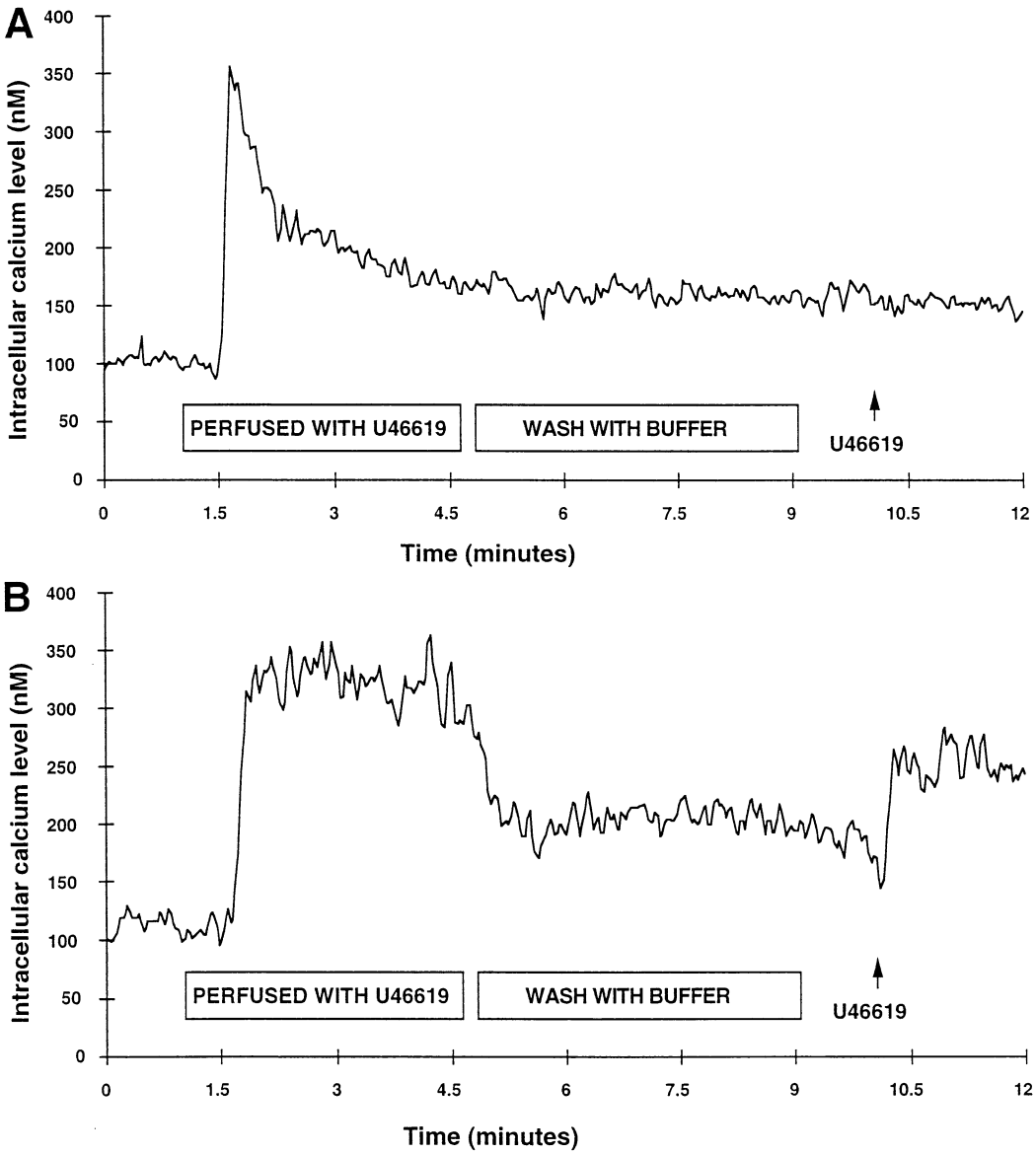

FIG. 5. Homologous desensitization of TxA_2-induced increases in $[Ca^{2+}]_i$ levels. Mesangial cell clones were stimulated with 10 μM of U46619 and then perfused with 10 μM of U46619 prior to washing and rechallenge with the TxA_2 agonist. Panel A shows results for clone II expressing ≈500 fmol TxA_2 receptors/mg of protein, and panel B shows results for clone III expressing ≈2500 fmol TxA_2 receptors/mg of protein. Results shown are representative tracings from 8 studies in clones expressing low numbers of TxA_2 receptors and from 12 studies in clones expressing high numbers of TxA_2 receptors.

TABLE 5. Heterologous desensitization of mouse mesangial cell clones

		Thrombin-induced IP3 generation (% increase above control)	
	Receptor number (fmol/mg protein)	Pretreated with vehicle	Pretreated with U46619
Clone II	517 ± 29	76 ± 19	81 ± 19
Clone IV	3704 ± 205	70 ± 12	77 ± 13

Cells were pretreated with 10 μM of U46619 prior to washing and stimulation with 1 μM of thrombin. Data points are the means ± SEM of 6 experiments. Basal levels of IP3 generation are presented in Table 2.

found that increases in $[Ca^{2+}]_i$ levels following a second application of U46619 were absent or blunted in cells expressing lower numbers of TxA_2 receptors (panel A). In contrast, rechallenge with U46619 consistently caused an increase in $[Ca^{2+}]_i$ levels in cells expressing high numbers of TxA_2 receptors (panel B).

To determine if cells expressing low and high levels of TxA_2 receptors were similar with regard to signal transduction mechanisms downstream from the receptor, cells expressing either high or low numbers of TxA_2 receptors were exposed to 10 μM of U46619 or its vehicle, washed to remove bound agonist, and then stimulated with 1 μM of thrombin. TxA_2 receptor responsiveness was assessed by measuring IP3 generation. As seen in Table 5, thrombin-induced IP3 generation was similar in clone II expressing low numbers of TxA_2 receptors and in clone IV expressing high numbers of TxA_2 receptors. Moreover, thrombin-induced IP3 generation was not affected by pretreatment with U46619. These data suggest that the clones are similar with regard to IP production downstream from the TxA_2 receptor and demonstrate the homologous nature of U46619-induced desensitization of the TxA_2 receptor.

Regulation of TxA_2 Receptors by PKC

Previous studies have suggested that PKC plays a key role in regulating TxA_2 receptor responsiveness [11, 12]. To determine if PKC contributes to homologous desensitization of the TxA_2 receptor in our model system, we incubated cells expressing low number of TxA_2 receptors (clones I and II) with U46619 or its vehicle in the presence or absence of the selective PKC inhibitor GF109203X (1 μM) [30]. After 10 min, cells were washed to remove bound agonist, and then rechallenged with 10 μM of U46619 as described in Materials and Methods. TxA_2 receptor responsiveness was assessed by measuring IP3 generation, and data were expressed as a percentage of the IP3 response in cells desensitized with vehicle to normalize for differences in U46619-induced IP3 generation between clones I and II (see Table 1). In these experiments, IP3 generation was increased in cells desensitized in the presence of GF109203X (71 ± 11% of the baseline response) compared with cells desensitized in the absence of GF109203X (26 ± 8% of the baseline response; $P < 0.01$, $N = 6$ experiments). These data suggest that PKC contributes to homologous desensitization of the TxA_2 receptor in our model system.

We next investigated the effect of receptor number on desensitization of TxA_2 receptors induced by direct activation of PKC with the phorbol ester PDBu. For these studies, cells were incubated with 1 μM of PDBu, washed, and then stimulated with U46619 as described in the Materials and Methods. Results of these experiments are shown in Table 6. In contrast to the homologous desensitization experiments, prior exposure to PDBu decreased subsequent U46619-induced PI hydrolysis to a similar extent in clones II and IV expressing either low or high numbers of TxA_2 receptors, respectively. This effect of phorbol esters on the percent increase in IP3 generation was not due to a change in basal IP3 production because basal IP3 levels were similar in the absence (4532 ± 734 dpm) and presence (3872 ± 594 dpm) of 1 μM of PDBu (P = NS, $N = 3$ experiments). Prior exposure of clone II to the inactive phorbol ester 4α-DD had no significant effect on subsequent TxA_2 receptor responsiveness (167 ± 18 [vehicle] vs 161 ± 25% increase above control [4α-DD]; P = NS, $N = 4$ experiments). Thus, receptor number did not affect desensitization of TxA_2 receptors following direct activation of PKC by phorbol esters.

DISCUSSION

Desensitization of G-protein coupled receptors is mediated, at least in part, by direct phosphorylation of receptors by general kinase systems as well as by receptor-specific kinases [2]. In the case of receptor-specific kinases, receptor phosphorylation is followed by binding and inactivation of

TABLE 6. Effect of TxA_2 receptor number on PKC-induced desensitization

		IP3 generation (% increase above control)		
	Receptor number (fmol/mg protein)	Pretreated with vehicle	Pretreated with PDBu	% Response in vehicle-treated cells
Clone II	517 ± 29	227 ± 38	114 ± 27*	51 ± 10
Clone IV	3704 ± 205	244 ± 19	107 ± 16*	45 ± 10

Cells were pretreated with 1 μM of PDBu prior to washing and stimulation with 10 μM of U46619 as described in Materials and Methods. Data points are the means ± SEM of 4 experiments per clone. Basal levels of IP3 generation are presented in Table 2.

*$P < 0.05$ vs cells pretreated with vehicle.

receptors by a second group of protein cofactors termed arrestins, which may impair receptor–G–protein coupling [2, 3]. These reactions might be regulated by: (1) the concentration or activity of the regulatory kinases or protein cofactors, (2) the concentration of receptor substrate, (3) the affinity of the kinases or cofactors for the receptor substrate, or (4) compartmentalization of the enzymatic reactions within the cell [31]. In the present study, we investigated the effect of receptor number on desensitization of G-protein coupled receptors. We found that increasing TxA_2 receptor number above a certain threshold level impaired homologous desensitization of TxA_2 receptors. Failure of cells to desensitize caused prolonged and sustained activation of the TxA_2 receptor. These data provide evidence that increasing the amount of receptor substrate attenuates homologous desensitization of G-protein coupled receptors.

There are several potential explanations for attenuated desensitization in cells expressing high levels of TxA_2 receptors. One possibility is that high numbers of TxA_2 receptors saturate the regulatory enzymes or protein cofactors. In this regard, several groups have investigated the stoichiometric requirements for receptor specific kinases and arrestins in desensitization of G-protein coupled receptors. In the β-adrenergic receptor system, overexpression of the β-adrenergic receptor kinase and β-arrestin augments desensitization of β_2-adrenergic receptors in cells that express high levels of receptor substrate [14]. In *Drosophila*, mutations of photoreceptor-specific arrestin genes results in a marked reduction in retinal arrestins [32]. Analysis of the light response in these arrestin-deficient mutants demonstrates prolonged activation of rhodopsin *in vivo* [32]. These data indicate that desensitization is modulated by the amounts of regulatory enzymes and protein cofactors. Our results suggest that the amount of receptor substrate alone may also modulate desensitization responses.

Although U46619-induced desensitization of TxA_2 receptors was attenuated by increases in receptor number, we found that the number of TxA_2 receptors did not affect desensitization following direct activation of PKC by phorbol esters. This finding suggests that the mechanisms of agonist-specific desensitization are different from the mechanisms of desensitization induced by activation of general kinase systems such as PKC. This hypothesis is supported by studies of the β-adrenergic receptor. Desensitization of β-adrenergic receptors is mediated by at least three independent processes including: (1) phosphorylation of the receptor by receptor-specific kinases, (2) receptor phosphorylation by general kinase systems such as PKC or protein kinase A, and (3) sequestration of receptors away from the cell surface [2, 3, 33]. Agonist-specific desensitization of β-adrenergic receptors is mediated to a large extent by receptor-specific kinases, particularly at high agonist concentrations [33]. Although it is not known if receptor-specific kinases contribute to TxA_2 receptor desensitization, one possible explanation for the difference between U46619-compared with PKC-induced desensitization observed in the present study is that desensitization caused by receptor-specific kinases is more sensitive to high levels of receptor substrate.

An alternative explanation for the difference between U46619- and phorbol ester-induced desensitization of the TxA_2 receptor may relate to the level of PKC activity induced by each agent. In this regard, increasing TxA_2 receptor number beyond a threshold level of ≈500 fmol/mg protein produces little additional increase in PLC activity (Table 1) and, presumably, little additional production of the PKC-activator diacylglycerol. Once this threshold level is reached, increases in the number of TxA_2 receptors may not cause additional stimulation of PKC, and, as a result, desensitization is impaired. In contrast, by pharmacologically stimulating PKC to higher levels of activity with phorbol esters, TxA_2 receptor responsiveness may be attenuated in cells expressing even high numbers of TxA_2 receptors.

In the present studies, a 10-min period was used to induce desensitization. This time period is likely sufficient to produce not only receptor phosphorylation but also sequestration of TxA_2 receptors away from the cell surface [33]. It is possible that this regulatory mechanism may become saturated in cells expressing high levels of TxA_2 receptors, resulting in impaired desensitization. While the contribution of internalization to the extent of TxA_2 receptor desensitization is not known with certainty, desensitization of β-adrenergic receptors is caused largely by direct receptor phosphorylation, with receptor internalization alone producing only a modest reduction in receptor responsiveness [33]. Indeed, inhibition of sequestration of β-adrenergic receptors has little effect on homologous desensitization [34]. If these findings are generalizable to other receptor systems, it seems unlikely that saturation of the processes involved in receptor internalization plays a significant role in the attenuated desensitization observed in cells expressing high levels of TxA_2 receptors.

Other groups [14, 35] have not found that receptor number modulates short-term regulatory mechanisms. For example, Pippig *et al.* [14] reported that the extent of desensitization of β_2-adrenergic receptors was similar in cells expressing ≈80 and ≈600 fmol/mg protein in Chinese hamster ovary cells. Using a quantitative method for measuring the extent of receptor desensitization, Lohse [35] found that the reduction in signal transduction efficiency induced by desensitization was not affected by decreasing the number of β_2-adrenergic receptors using a β-adrenergic receptor alkylating agent in A431 cell lines. In this study, desensitization decreased maximal receptor responsiveness to a greater extent in cells with lower numbers of β_2-adrenergic receptors, but changed the EC_{50} by less [35]. As a result, the signal transduction efficiency was unchanged in cells treated with the alkylating agent. The failure to demonstrate an effect of receptor number on desensitization in other receptor systems may result from a number of factors including differences in the regulatory mecha-

nisms between receptor types or a failure to increase receptor number to levels required to saturate the regulatory processes. Further studies will be required to determine if our findings are generalizable to other G-protein coupled receptors.

In summary, we found that prior exposure to U46619 reduced subsequent U46619-induced increases in IP3 and $[Ca^{2+}]_i$ levels in clones expressing low numbers of TxA_2 receptors, but had little effect on these responses in clones expressing high receptor numbers. Failure of TxA_2 receptors to desensitize caused sustained increases in $[Ca^{2+}]_i$ levels and PI hydrolysis. These findings suggest that increasing the abundance of receptor substrate attenuates the rapid phase of homologous desensitization of G-protein coupled receptors.

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