Regulation of thromboxane receptor activation in human platelets
(desensitization/uncoupling/down-regulation)

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ABSTRACT  Thromboxane A₂ (TxA₂) is a potent platelet agonist that serves as an amplifying signal after exposure of platelets to other stimulants, such as thrombin, in vitro. Exposure of platelets to the TxA₂ receptor agonists U46619 and SQ 26,655 (1.4 μM) resulted in a 60-90% decrease in subsequent TxA₂ receptor-stimulated aggregation, calcium release, and protein kinase C activation. The desensitization was rapid, with a half-time of 2-3 min. The sequence of events involved in TxA₂ receptor desensitization involves initial uncoupling of the receptor from a guanine nucleotide binding (G) protein followed by eventual receptor down-regulation. Consistent with this hypothesis were (i) a 60-70% decrease in SQ 26,655-stimulated platelet GTPase activity, (ii) a shift to the right of the dose-response curve for U46619-stimulated release of calcium [EC₀, 275 ± 51 nM (control)] vs. 475 ± 71 nM (desensitized); P < 0.01], and (iii) a delayed loss of receptor sites. In summary, exposure of platelets to TxA₂ receptor agonists results in rapid desensitization of the biochemical and functional responses to interaction with its receptor in human platelets. The kinetics of these events are consistent with the hypothesis that thisicosanoid functions in the regulation as well as amplification of platelet activation in vivo.

Thromboxane A₂ (TxA₂) is a potent agonist of platelet activation, causing shape change, secretion, and aggregation (1). TxA₂ is also an important potentiator of platelet activation by other platelet stimuli, such as ADP and epinephrine. These agents stimulate the release and subsequent metabolism to TxA₂ of arachidonic acid (2, 3). TxA₂, largely derived from platelets, is synthesized in vivo even under physiological conditions (4), implying continuous platelet activation, which would seem likely to be subject to regulatory constraints. One potential mechanism whereby platelet function might be controlled would be the decreased response of platelets to TxA₂ after exposure to agonist, or desensitization.

We have characterized the molecular events involved in TxA₂ receptor desensitization by comparing the biochemical responses to TxA₂ receptor stimulation in untreated and desensitized platelets. Our results indicate that desensitization of platelet TxA₂ receptors is mediated initially by impaired coupling between the TxA₂ receptor and its second messenger system—guanine nucleotide binding (G) protein-coupled phospholipase C activation. Uncoupling of receptor stimulation from phospholipase C activation is followed, upon extended exposure to agonist, by a decrease in platelet TxA₂ receptor density.

MATERIALS AND METHODS

Materials. 9,11-Dimethylmethano-11,12-methano-16-(4-hydroxyphenyl)-13,14-dihydro-13-aza-15-β-ω-tetranor-TxA₂ (PTA-OH) and 127I-labeled PTA-OH (127I-PTA-OH) were gifts from P. Halushka (Medical University of South Carolina); [1S-[1,2(SZ),3(1E,3S),4]]-7-[3-[hydroxy-1-ocenyl]-7-oxabicyclo(2.2.2)hept-2-yl]-5-heptenoic acid (SQ 26,655) and [1S-[1,2(SZ),3,4]]-7-(3-[2-(phenylaminocarboxyl)hydrazino]methyl)-7-oxabicyclo(2.2.1)hept-2-yl)-5-heptenoic acid (SQ 29,548) were gifts from M. Ogletree (Squibb Institute, Princeton, NJ); (1S)-hydroxy-11,9-(epoxymethano)prosta-5Z,13E-dienoic acid (U46619) was a gift from R. Gorman (Upjohn, Kalamazoo, MI). Reagents for polyacrylamide gel electrophoresis were from Bio-Rad. Other reagents were from Sigma except where indicated otherwise.

Desensitization and Preparation of Washed Platelets. Blood was drawn from healthy human volunteers (who had not taken any medication for at least 10 days) by venipuncture into EDTA (5 mM) and indomethacin (5 μg/ml) (final concentrations). The blood was centrifuged for 10 min at 160 x g, and platelet-rich plasma (PRP) was removed. For desensitization, PRP was incubated at room temperature for 30 min (or as indicated in individual experiments) with the TxA₂ receptor agonists U46619 (1.4 μM) (5), SQ 26,655 (1.4 μM) (6), or vehicle (control). The platelets were washed as described (7). All buffers contained indomethacin at 5 μg/ml.

Platelet Aggregation. Platelets (0.5 ml; ~10⁹ platelets per ml) were preincubated at 37°C for 2 min, then stimulant was added, and the extent of aggregation was monitored by light transmission in a Biodata Pap 4 aggregometer.

Calcium Measurement. Intracellular free calcium (Ca²⁺) measurements were made by monitoring the intensity of fura-2 fluorescence. PRP was incubated with 1 μM fura-2 AM (Molecular Probes) at 37°C for 30 min. The fura-2-loaded platelets were desensitized and washed as described above. Changes in fluorescence of the fura-2-loaded platelets at excitation wavelengths of 340 and 380 nm (less autofluorescence of the platelets in the absence of fura-2 loading) were followed, and addition of various agonists were recorded. The ratio of fluorescence at excitation wavelength 340 nm to fluorescence at excitation wavelength 380 nm (relative fluorescence intensity) is a measure of Ca²⁺ concentration (8).

Phosphorylation of 47-kDa Protein. Phosphorylation of platelet 47-kDa protein, a protein kinase C substrate, was assessed as described (9). After two washes in phosphate-free buffer, control and desensitized platelets were resuspended to a concentration of 2 x 10⁹ platelets per ml in the same buffer [15 mM Tris-HCl, pH 7.4/0.150 mM NaCl/5 mM dextrose/indomethacin (5 μg/ml), also containing 500 μM CaCl₂ and 200 μCi of ³²P orthophosphate per ml (carrier-free in HCl, neutralized with 1 M Tris (pH 7.4); 1 Ci/ml] after addition of various agonists were recorded. The ratio of fluorescence at excitation wavelength 340 nm to fluorescence at excitation wavelength 380 nm (relative fluorescence intensity) is a measure of Ca²⁺ concentration (8).

Abbreviations: TxA₂, thromboxane A₂; G protein, guanine nucleotide binding protein; Ca²⁺, intracellular free calcium; PRP, platelet rich plasma; PTA-OH, 9,11-dimethylmethano-11,12-methano-16-(4-hydroxyphenyl)-13,14-dihydro-13-aza-15-β-ω-tetranor-TxA₂; 127I-PTA-OH, 127I-labeled PTA-OH; ³²P, ³²P-orthophosphate; ³²P-OH, ³²P-orthophosphate labeled platelet rich plasma; ³²P-OH, ³²P-labeled platelet rich plasma; H-SQ 29,548, ³H-labeled SQ 29,548.

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molecular mass of the most heavily phosphorylated protein after stimulation was 47 kDa by comparison with concomitantly running molecular mass standards. The extent of phosphorylation in the 47-kDa protein was assessed by laser desensitometry.

**Binding Studies.** Saturation binding. 125I-labeled PTA-OH (prepared by iodination of the TXA2 receptor antagonist PTA-OH (11) or obtained from Amersham [200 Ci/mmol]) diluted 1:50 with 100 nM 125I-PTA-OH to give final concentrations of 5–60 nM; or 3H-labeled SQ 29,548 (3H-SQ 29,548) (from DuPont–New England Nuclear; 40 Ci/mmol) at final concentrations of 2.5–30 nM, in 50 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM dextrose/1% bovine serum albumin (binding buffer) was incubated with ~5 × 10² washed platelets in a final volume of 200 μl. Nonspecific binding was measured by including 10 μM 125I-PTA-OH or unlabeled SQ 29,548. Incubations were done in duplicate at 37°C for 30 min. The reaction was terminated by dilution with 4 ml of ice-cold 10 mM Tris-HCl (pH 7.4) and the reaction mixture was immediately filtered through Whatman GF/C glass fiber filters (Fisher). The filters were then washed with an additional 3 × 4 ml of 10 mM Tris-HCl buffer.

Competition binding. 3H-SQ 29,548 (0.5 nM) was incubated with ~5 × 10² platelets in the presence of various concentrations (1 nM to 10 μM) of U46619 or PTA-OH in binding buffer in a total volume of 200 μl. Incubations were done in duplicate at 37°C for 30 min and were terminated as described for saturation binding assays. After addition of 10 ml of scintillation fluid, the filters were assayed by liquid scintillation counting.

Binding analyses were performed by computerized nonlinear curve fitting using the LIGAND program, as described by Munson and Robard (12).

**GTPase Assays.** Stimulation of platelet high-affinity GTPase activity was assessed as an indication of receptor–G protein coupling (13). Platelet membranes were prepared, following desensitization, as described (14). GTPase activity was measured essentially by the method of Cassel and Selinger (15) with minor modifications. The reaction mixture, in a final volume of 50 μl, contained 5 mM MgCl₂, 1 mM p[NH]ppA, 0.25 mM ATP, 1 mM phosphocreatine, creatine phosphate kinase (50 units/ml), 1 mM dithiothreitol, 1 mM EGTA, 0.75 mM [γ-³²P]GTP (30 Ci/mmol; Amersham), 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4). High-affinity GTPase activity was defined as that inhibited by the inclusion of 1 mM GTP in the assay. Each point was measured in triplicate with and without 1 mM GTP. After incubation for 10 min at 37°C, 1 ml of ice-cold 5% charcoal (Norit A) in 20 mM phosphate (pH 7.5) was added, and the mixture was vortexed and centrifuged at 3000 rpm for 5 min in a Beckman Microfuge. Aliquots (200 μl) were assayed by liquid scintillation counting. A blank value was obtained in the absence of added platelet membranes and always represented 2–5% of total [γ-³²P]GTP added.

**Statistical Analysis.** Pairwise comparisons were performed by the Student’s t test.

**RESULTS**

**TXA2 Receptor Desensitization.** Platelets preincubated with either of the TXA2 receptor agonists (U46619 or SQ 26,655) were 60–70% less responsive to a subsequent stimulus with either agonist, as measured by the aggregation response. Occasionally, full desensitization was observed (an example is shown in Fig. 2). Thrombin (0.1 unit/ml)-stimulated aggregation was slightly decreased (by 10%) by preincubation with either TXA2 mimic (the decrease in thrombin stimulation was significantly less than TXA2 receptor agonist desensitization; P < 0.001) (Fig. 1A). The onset of desensitization was rapid—desensitization was maximal by 15 min of exposure to the TXA2 receptor agonists. The half-time of desensitization was 2–3 min (Fig. 1B).

The specificity of the induction of desensitization by interaction of the TXA2 receptor agonists with the TXA2 receptor was demonstrated by the inhibition of the induction of desensitization in the presence of the TXA2 receptor antagonist SQ 29,548 (Fig. 2).

Platelet aggregation has been associated with activation of phospholipase C, with resultant production of inositol trisphosphate and 1,2-diacylglycerol, which stimulate release of Ca²⁺ and the activation of protein kinase C, respectively. Resting Ca²⁺ concentrations were identical in control and desensitized platelets (0.68 ± 0.01 and 0.65 ± 0.03 fluorescence units, respectively). U46619 (1 μM) or SQ 26,655 (1 μM) stimulation of Ca⁴⁺ release in platelets desensitized with either TXA2 receptor agonist was attenuated to 60–70% of that obtained in control platelets (Fig. 3A). Thrombin stimulation of Ca²⁺ release was significantly greater (P < 0.05) decreased by 27% ± 6% in desensitized compared with control platelets. The decrease in agonist stimulation of Ca²⁺ release was significantly greater for the TXA2 receptor agonists than for thrombin (P < 0.001). [Control stimulation of Ca²⁺ release by 1 μM U46619 was 0.61 ± 0.1; by 1 mM SQ 26,655, it was 1.24 ± 0.15; and by thrombin (0.1 unit/ml), it was 1.6 ± 0.2 fluorescence units above basal.]

**Fig. 1.** Desensitization of the platelet aggregation response. (A) Platelets were desensitized with U46619 (open bars) or SQ 26,655 (solid bars) and subsequently stimulated with U46619 (1 μM), SQ 26,655 (1 μM), or thrombin (0.1 unit/ml). (B) Platelets were desensitized with SQ 26,655 for 5, 15, 30, or 60 min prior to stimulation of aggregation with 1 μM SQ 26,655. Results are expressed as percentage stimulation in control platelets and represent the mean ± SEM of 10 (A) and 5 (B) experiments. ***, P < 0.001; *, P < 0.05.

**Fig. 2.** Inhibition of induction of desensitization by the TXA2 receptor antagonist SQ 29,548. Platelets were incubated with either vehicle (control), SQ 29,548 (1 μM), SQ 29,548 (1 μM) + U46619 (1.4 μM), or U46619 (1.4 μM) for 30 min, washed twice, stimulated with U46619 (1 μM), and aggregation was monitored. In this experiment, U46619 stimulation was, as occurred occasionally, completely desensitized. The effects of the antagonist were replicated in four independent experiments.
Fig. 3. Desensitization of Ca\(^{2+}\) release and of 47-kDa protein phosphorylation. Control (open bars), U46619 desensitized (hatched bars), and SQ 26,655 desensitized (solid bars) platelets were stimulated with either U46619 (1 μM), SQ 26,655 (1 μM), or thrombin (0.1 unit/ml). (A) Ca\(^{2+}\) release was measured as relative fluorescence intensity over basal at excitation wavelengths of 340 and 380 nm as described in the text. (B) The 47-kDa protein phosphorylation was quantitated by laser densitometry as described in the text. The data are expressed as percentage of phosphorylation in control platelets. Results represent the mean ± SEM of nine experiments. **, \(P < 0.005\); *, \(P < 0.05\).

Phosphorylation of platelet 47-kDa protein in control and desensitized platelets was assessed as an index of protein kinase C activation (16). U46619 (1 μM), SQ 26,655 (1 μM), thrombin (0.1 unit/ml), and 12-O-tetradecanoylphorbol 13-acetate (10 nM) all stimulated \(^{32}\)P incorporation into a 47-kDa protein in control platelets. In platelets desensitized with either U46619 or SQ 26,655, stimulation of phosphorylation by the Tx\(_A_2\) receptor agonists was attenuated (to 27–37% of control) (Fig. 3B). Thrombin stimulation of 47-kDa protein phosphorylation was not significantly changed in desensitized compared to control platelets.

**Tx\(_A_2\) Receptor Coupling.** We investigated the possibility that Tx\(_A_2\) receptor desensitization resulted from impaired Tx\(_A_2\) receptor coupling. Tx\(_A_2\) receptor coupling to its associated G protein in desensitized compared with control platelets was assessed by measurement of platelet high-affinity GTPase and by comparison of the dose–response curves for U46619 stimulation of calcium release. GTPase activity was stimulated 41% ± 2% over basal by SQ 26,655 (5 μM) and 27% ± 4% over basal by thrombin (0.5 unit/ml) in control platelets (basal activity was measured in the presence of the antagonist SQ 29,548 and was 6.70 ± 0.3 pmol·mg\(^{-1}\)·min\(^{-1}\)). Desensitization resulted in no change in basal or thrombin-stimulated activity, but SQ 26,655 stimulation was decreased to 12% ± 4% over basal GTPase activity, indicative of an impaired Tx\(_A_2\) receptor–G protein coupling in desensitized platelets (Fig. 4).

**Tx\(_A_2\) Receptor Binding Properties.** We investigated the contribution of changes in number and function of Tx\(_A_2\) receptors to desensitization. Tx\(_A_2\) receptor affinity for agonist was measured by competition of U46619 for \(^{3}\)H-SQ 29,548 binding sites (and analyzed by the programs LIGAND and ALLFIT) and yielded a \(K_d\) value of 100 ± 10 nM for both control and desensitized platelets (\(n = 7\)). The EC\(_{50}\) for U46619 stimulation of Ca\(^{2+}\) release was increased in desensitized compared with control platelets (EC\(_{50}\) = 475 ± 71 nM and 275 ± 51 nM, respectively (Fig. 5)).

The affinity of the Tx\(_A_2\) receptor for the antagonist PTA-OH was assessed, also by competition for \(^{3}\)H-SQ 29,548 binding sites. There was an apparent increase in receptor affinity for the antagonist in desensitized compared with control platelets. Analysis of the binding curves revealed that PTA-OH was binding to two affinity states of the receptor. In desensitized platelets, the proportion of low-affinity sites (\(K_d = 6 ± 2.5 \mu M\)) was decreased from 50% ± 8% in control to 21% ± 5% in desensitized platelets, resulting in an overall apparent increase in affinity for PTA-OH. There was a concomitant increase in the proportion of high-affinity receptor sites (\(K_d = 130 ± 19 \mu M\); \(n = 7\)).

It has been demonstrated in other receptor systems that desensitization may be associated with a decrease in receptor density (17–19). Saturation binding of \(^{125}\)I-PTA-OH to control platelets (\(B_{max} = 295 ± 30\) fmol per 10\(^8\) platelets) and platelets desensitized with either U46619 or SQ 26,655 for 1 hr (\(B_{max} = 211 ± 25\) and 232 ± 35 fmol per 10\(^8\) platelets, respectively (\(n = 11\))) revealed a 20–30% decrease in receptor sites after desensitization. However, using \(^{3}\)H-SQ 29,548 to measure receptor number, no significant decrease was observed in desensitized (\(B_{max} = 212 ± 14\) fmol per 10\(^8\) platelets) compared to control platelets (\(B_{max} = 245 ± 8\) fmol per 10\(^8\) platelets (\(n = 13\))) at this time.

The desensitization incubation was extended to 24 hr and receptor density was assayed, using \(^{3}\)H-SQ 29,548, at various
The sequence of events thought to be involved in homologous desensitization of the β-adrenergic receptor has been extensively defined (23). In the continued presence of agonist, the β-adrenergic receptor is rapidly phosphorylated—the receptor becomes uncoupled from G, and, therefore, from the adenylate cyclase enzyme. The receptor is then sequestered from the membrane surface and ultimately down-regulated, or converted to a form that can no longer bind radioligands and is not detectable by immunofluorescence (24). We have made several observations consistent with a similar sequence of events during TxA2 receptor desensitization.

The TxA2 receptor stimulation has been shown to be mediated by a G protein—in permeabilized platelets, U46619-induced phosphatidic acid production and secretion were inhibited by the G protein inhibitor GDP[βS] (25). Stimulation of a high-affinity GTPase by TxA2 receptor agonists (U46619 and U44069) has also been demonstrated (26, 27). We assessed TxA2 receptor stimulation of GTPase activity as a measure of receptor–G protein coupling in control and desensitized platelets. After 30 min to 1 hr of desensitization, there was little change in TxA2 receptor properties—no change in affinity for agonist and no loss of [3H]-SQ 29,548 binding sites. There was, however, a 60% reduction in GTPase stimulation by SQ 26,655, consistent with a loss of TxA2 receptor–G protein coupling. Thrombin stimulation of GTPase activity was unchanged in desensitized platelets, suggesting that either the small (10–30%) decrease in aggregation and Ca2+ responsiveness to thrombin stimulation in desensitized platelets is not mediated by a change in thrombin receptor activation, or that the GTPase assay was not sensitive enough to detect a small change in thrombin-stimulated GTPase activity. Since the TxA2 receptor and the thrombin receptor appear to couple to distinct G proteins (25), it is not clear whether the locus of the modification responsible for impaired TxA2 receptor–G protein coupling is the TxA2 receptor or the G protein.

The dose–response curves in control and desensitized platelets for U46619 stimulation of Ca2+ release also suggest that uncoupling of the TxA2 receptor from phospholipase C is the first stage in its desensitization—the EC50 for U46619 was increased in desensitized platelets and may indicate an uncoupling of receptor compared to control.

After 1 hr of desensitization, there was a 20–30% decrease in TxA2 receptor number using the radioligand 125I-PTA-OH to measure binding. This receptor down-regulation was not observed when the radioligand [3H]-SQ 29,548 was used.

While it could be argued that the difference in 125I-PTA-OH and [3H]-SQ 29,548 binding in desensitized platelets may be due to residual agonist, we feel this is unlikely. If U46619 or SQ 26,655 had been retained after washing and were not efficiently displaced by 125I-PTA-OH, we would expect to see a decrease in affinity for the ligand. In fact, there was no change in affinity for 125I-PTA-OH after desensitization (Kd = 23 ± 2 nM in control and 16 ± 2 nM in desensitized platelets; P > 0.05). Heterogeneity in binding of TxA2 receptor radioligands has been a feature of platelet TxA2 receptor studies—there have been discrepancies in the sites labeled by [3H]-U46619 and 125I-PTA-OH (28). While the [3H]-U46619 and 125I-PTA-OH site difference has been attributed to different properties of agonist and antagonist binding, there may be a more fundamental difference in the nature of the sites identified or in the relative specificity of the ligands used. Another indication of heterogeneity of platelet TxA2 receptor sites is the observation that the antagonist PTA-OH recognizes two affinity states of the receptor—states that are not discriminated by the other agonists and antagonists used in this study. One interpretation of the existence of two sites for PTA-OH and the relative decrease in the proportion of low-affinity sites following desensitization is that the low- and
high-affinity sites for PTA-OH represent coupled and uncoupled forms of the receptor, respectively. This is supported by the observation that the EC50 for U46619 stimulation of Ca2+ release in control platelets is higher than the Kd for U46619 binding, suggesting that there are uncoupled or poorly coupled receptors in control platelets. After 30 min of desensitization, there is an increase in the proportion of uncoupled receptors (reflected in an increased EC50 for U46619 in desensitized platelets).

After the initial rapid uncoupling of TxA2 receptor and its associated G protein, there was, in the continued presence of agonist, a slow down-regulation of receptor, demonstrated by a 50% ± 8% loss in receptor number following 24 hr of desensitization, as measured using 3H-SQ 29,548. This long-term down-regulation of receptor number may have some clinical relevance; for example, in conditions associated with sustained augmentation of TxA2 biosynthesis, such as severe peripheral vascular disease (29).

The TxA2 receptor was subject to heterologous desensitization following platelet exposure to thrombin, suggesting that refractoriness to TxA2 stimulation may be of general importance in regulating platelet activation by stimulants other than TxA2 itself.

Given the rapid amplification that occurs upon platelet stimulation due to release of arachidonic acid and its metabolism, an understanding of the regulation of the complex pathways of platelet activation is desirable. The rapid uncoupling of the TxA2 receptor upon exposure to TxA2 agonists and subsequent down-regulation of receptor number and the heterologous desensitization of the TxA2 receptor by thrombin are consistent with the hypothesis that desensitization to TxA2 functions as an important element in the physiological control of platelet activation in vivo.

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