Monoclonal Anti-idiotypic Antibody to a Potent Thromboxane A$_2$ Receptor Antagonist and Its Interaction with Thromboxane A$_2$ Receptor*

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A monoclonal anti-idiotypic antibody that interacts with thromboxane A$_2$ receptor was generated using an anti-idiotypic approach. Antibody sequences against a potent receptor antagonist, HS-145, were generated in rabbit. The idiotypic antibodies were then selected by an affinity procedure using SQ29,548-Affi-Gel-102 matrix. The selected idiotypic antibodies were used as surrogate receptor for anti-idiotypic antibody generation. A mouse monoclonal antibody, 3D-9E-12, was generated. It was shown to displace $^{125}$I-HS-145 from affinity-purified idiotypic antibodies. It also inhibits $^{125}$I-IS-145 binding to thromboxane A$_2$ receptor in human platelet membranes in a dose-dependent manner. Furthermore, it attenuated U46,619-induced increase in $[^{35}]$uridine 5'-O-(thiotriphosphate) binding and GTPase activity in human platelet membranes. Finally, it inhibited U46,619- but not PAF-induced platelet aggregation. These results indicate that 3D-9E-12 acts as a specific antagonist in the thromboxane A$_2$ receptor.

Thromboxane A$_2$ is one of the most important arachidonic acid metabolites in human platelets (1). It acts as a potent vasoconstrictor and induces platelet aggregation (2). It is thought to be involved in various cardiovascular disorders such as atherosclerosis (3), thrombosis (4), and angiogenesis (5). Although the receptor has been purified (6) and the cDNA (7) has been cloned and sequenced, antibodies against the receptor are not presently available.

As the thromboxane receptor is only present in very minute quantities in tissues (6), the classical method of generating antibodies with purified receptor is not very practical. An alternative method needs to be sought. This approach is based on Jerne's theory of idiotypic network (8). It is believed that anti-idiotypic antibodies can be generated against idiotypic antibodies that recognize a ligand for a receptor (9). The generated anti-idiotypic antibodies that are specific for the binding site of the idiotypic antibodies may cross-react with the receptor. This approach does not require purified receptor as an antigen. Many interesting anti-receptor antibodies have been reported using this approach, including acetylcholine (10), adenosine (11), $\beta$-adrenergic (12), aldosterone (13), dopamine (14), glucocorticoid (15), insulin (16), naticinocorticoid (17), opioid (18), substance P (19), and thrytropin (20) and PAF (21) receptors.

The success of this approach depends on a reliable method to isolate a fraction of the idiotypic antibodies that their binding sites are structurally similar to that of the receptor. In our approach, we used a high affinity antagonist, HS-145 (22), as the thromboxane A$_2$ receptor ligand. Rabbit anti-HS-145 antibodies were generated. The selection of antibodies with binding sites that are structurally similar to that of the receptor was done with an affinity procedure using an SQ29,548-Affi-Gel-102 affinity matrix. SQ29,548 (23), another potent thromboxane A$_2$ antagonist, is structurally different from that of HS-145. We assumed that the affinity purification might provide an alternate method of selecting those binding populations which might mimic receptor. These idiotypic antibodies were used as surrogate receptors to generate monoclonal anti-thromboxane A$_2$ receptor antibody. Here we describe the production of such an anti-idiotypic antibody, which functions like an antigen that interacts specifically with the thromboxane A$_2$ receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**

HS-145, $^{125}$I-HS-145 (2,000 Ci/mmol), and $^{125}$I-IS-145 (2,000 Ci/mmol) were synthesized in our laboratory according to an improved procedure (24). $[^{35}]$GTP (600 Ci/mmol) and $[^{35}]$GTPyS (1,000 Ci/mmol) were purchased from Du Pont-New England Nuclear. SQ29,548 was a kind gift from Squibb. Bovine thyroglobulin, bovine serum albumin, complete and incomplete Freund adjuvants, polyethylene glycol 4000 (hydriodoms grade), 1-ethyl-3-(dimethylamino)propylcarbodiimide hydrochloride (EDC), ammonium persulfate, acrylamide, SDS, antibiotic-antimyotic mixture, gentamycin, RPMI 1640, HY medium, hybridoma enhancing medium, fetal bovine serum (Hybrimax), phorbol myristate acetate, pristane, Tween 20, casein, gelatin, activated charcoal, bovine $\gamma$-globulin, protein A, goat anti-mouse immunoglobulin G, polylysine, pineal platelet A$_2$ and agrore were purchased from Sigma. Dicyclohexylcarbodiimide was obtained from Aldrich. The EIA/RIA 5-well strips, EIA/RIA 96-well plate, and tissue culture flasks were purchased from Costar or Falcon. Horseradish peroxidase (HRP) substrate was purchased from Kirkegaard & Perry. Goat anti-mouse IgG-HRP conjugate and Goat

* The abbreviations used are: PAF, platelet activating factor; RIA, radioimmunoassay; EIA, enzyme immunoassay; U46,619, (15S)-hydroxy-11,9-epoxymethanoprosta-(5z,13z)-dienoic acid; HS-145, ([5z]-1a,2a,5S,7S,9R)-7-[(3R)-3-[(2-hydroxytetramisulfonylamido)bicyclo[2.2.1].heptyl]-hept-5-enoic acid]; IS-145, (52)-7-[(3R)-3-endo-[(4-iodobenzensulfonylamido)bicyclo[2.2.1].heptyl]-hept-5-enoic acid]; EDC, 1-ethyl-3-(dimethylamino)propylcarbodiimide hydrochloride; HRP, horseradish peroxidase; PRP, platelet-rich plasma; PPP, platelet-poor plasma; GTPyS, guanosine 5'-O-(thiotriphosphate).

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Preparation and Purification of Rabbit Polyclonal Anti-HS-145 Antibodies

**Preparation of Antigen and Immunization Protocol**—Three mg of HS-145 and 10 mg of thyroglobulin were dissolved in 0.5 and 1.5 ml of phosphate buffered saline (PBS), respectively. The two were mixed and 2 mg of EDC was added. After the mixture was stirred for 1 h at 4 °C, another 2 mg of EDC was added. The addition of EDC was repeated 4 h later. The mixture was allowed to react overnight at 4 °C with stirring.

After the coupling procedure, the protein solution was concentrated to 0.5 ml and desalted with a 20 ml Sephadex G-25 column using PBS as elution buffer. The flow-through fractions were pooled. Less than 10% of the added HS-145 was recovered from the uncoupled fractions. The protein solution was then aliquoted in 1 mg/tube and lyophilized. A New Zealand White rabbit was injected subcutaneously with 1 mg of HS-145-thyroglobulin conjugate emulsified with complete Freund adjuvant on 12 sites. Prior to immunization, blood was collected to be used as a source of control antisera. After 4 weeks it was injected again with 1 mg of the conjugate emulsified in complete Freund adjuvant through the same route. Injection was repeated every 4-5 weeks. The animal was bled 1 week after injection, and the sera were stored at -20 °C until used.

**Affinity Purification of Rabbit Polyclonal Anti-HS-145 Antibodies**—Ten mg of SQ29,548 was dissolved in 1 ml of anhydrous dimethylformamide. Ten mg of dicyclohexyl carbodiimide was added with 5 mg of N-hydroxysuccinimide. The mixture was allowed to react for 1 h. Five ml of Affi-Gel-102 was washed extensively with dimethylformamide. The reaction mixture was added to the gel with an additional 10 mg of dicyclohexyl carbodiimide. After the slurry was kept at room temperature overnight, it was drained and washed successively with 50 ml of dimethylformamide, 50 ml of acetone, and finally 50 ml of methanol. The gel was stored in methanol until used. By comparison of UV absorbance before and after coupling, it was estimated that about 8 pmol of SQ29,548 was coupled to each milliliter of gel.

One ml of the serum was diluted with 1 ml of Pierce Immunoapure binding buffer and applied to a 2 ml protein A column (from Pierce). The flow-through fractions were applied to the column twice. Afterward, the column was washed with 20 ml of the binding buffer (until there was no protein in the eluant). Six ml of elution buffer was added. One ml fractions were collected. The protein peak was pooled and dialyzed against phosphate-buffered saline (PBS). The protein was eluted above water with 1 ml of SQ29,548-Affi-Gel-102 and incubated by end-to-end rotation overnight. The gel was then packed into a column and washed extensively with PBS. The binding fraction was eluted with Pierce elution buffer. The protein peak was pooled and dialyzed against phosphate-buffered saline.

**Radioimmunoassay of HS-145**—The assay was run in 10 x 75-mm glass test tubes in duplicates. The standard assay buffer, 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1% gelatin, was used for diluting antibodies, HS-145 standards, and 125I-HS-145. The assay mixture (0.2 ml) contained 0.1 ml of HS-145 standards or analogs, 0.05 ml of diluted antisera, and 0.05 ml of 125I-HS-145 (18,000 cpm). The mixture was incubated at room temperature for 1 h, and the separation of the bound and free HS-145 was achieved by adding 1 ml of the buffer followed by 0.4 ml of 3% charcoal suspension in buffer. After mixing, the assay mixture was centrifuged at 2,000 x g for 5 min. The supernatant (bound form, 1 ml) was used for radioactivity determination.

**Production of Hybridoma**

Four to six-week-old female BALB/c mice were used for generation of monoclonal antibodies. Affinity-purified rabbit anti-HS-145 IgG (200 μg) in 100 μl of PBS was emulsified with 100 μl of complete Freund adjuvant for each mouse. The emulsion was injected intraperitoneally into the mice. After 2 weeks, each mouse was injected again with 100 μg of the antigen in 100 μl of PBS together with 100 μl of incomplete Freund adjuvant. Prior injections were done every 4 weeks. Ten days after injection, the mice were bled. The serum was separated using normal procedure. The titers of the antibodies were checked against the binding toward the affinity-purified rabbit anti-idiotypic IgG and the ability to displace 125I-HS-145 from the thromboxane A2 receptor in human platelet membranes (see below). After an appropriate titer was detected, the mice were allowed to rest. In order to prevent anaphylaxis, the mice were boosted intravenously after the titer dropped back to base level. Fusion was done 3 days after the final boost.

**Direct Binding Assay for Anti-HS-145 Antibodies**

**Enzyme-linked Immunosorbent Assay**

**EIA plates were coated by addition of 1 μg of affinity-purified rabbit IgG in 100 μl of 0.1 M sodium bicarbonate buffer (pH 9.6) into each well. After overnight incubation at 4 °C, the coating buffer was removed. To each well, 100 μl of 5% bovine serum albumin was added and incubated at room temperature for 1 h. The BSA solution was then removed, and the wells were washed three times with 300 μl of PBS. Hybridoma supernatant (100 μl) was added into each well. The incubation was done at 4 °C overnight. After incubation, the supernatant was removed and the wells were washed three times with 300 μl of PBS/well. One μg of goat anti-mouse IgG hors eradish peroxidase conjugate in 100 μl of PBS was added into each well. The plates were then incubated at room temperature for an hour before washing extensively with PBS. Color development was done with the addition of 100 μl of horseradish peroxidase substrate (3,3',5,5'-tetramethylbenzidine and hydrogen peroxide). Color developed within 30 min and was monitored at 650 nm.

**Receptor Binding Study on Human Platelet Membranes**

**Human platelet membranes (100 μg), prepared as described previously (20), were added into 200 μl of 25 mM Tris-HCl buffer, pH 7.4, containing 5 mM CaCl2, 125I-HS-145 (0.1 μM; approximately 100,000 cpm/tube) was added with various amounts of monoclonal anti-idiotypic antibody. Incubation took place for 45 min at 37 °C. The reaction was then stopped with 4 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4. Filtration was done under vacuum using a Whatman GF/C glass fiber filter. The filter was washed 3 times with 4 ml of the same Tris buffer. Radioactivity was determined by counting in 10 ml of scintillation cocktail.

**Human Platelet Aggregation Study**

Platelet aggregation was carried out as described by Mustard et al. (27). Venous blood was obtained from healthy volunteers who had not taken any medications for 2 weeks or more. The blood was collected with 0.1 volume of ACD (85 mM trisodium citrate, 121 mM dextrose, and 71 mM citric acid) and centrifuged at 180 x g at room temperature for 15 min to obtain the platelet-rich plasma (PRP), which was carefully transferred to a sterile plastic tube. The lower layer was centrifuged at 3,000 x g at room temperature for 35 min to obtain the platelet-poor plasma (PPP), which was used as control. The platelet count of the PRP was done with a Coulter counter, and the platelet count was adjusted to 2.5 x 10^9 cells/ml with the addition of normal saline. The aggregometer cuvette with 0.5 ml of PPP was inserted into the machine at 37 °C to adjust the base line. PRP (0.5 ml) was then inserted for the maximum value. Various amounts of the antibody were added to the PRP at 37 °C for 1 min before aggregation was initiated by the addition of 1 μM U46,619. The process of aggregation was recorded.
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case), only a small fraction of anti-idiotypic antibodies rec-

counting 10 ml of scintillation
The reaction was stopped with 4 ml of ice-cold washing buffer
bodies. The first approach relies on the fact that the immune
mixture.

carried out to determine background hydrolysis.
The filter was washed
The mixture was filtered through a Whatman GF/C fiber glass filter.

The reaction was terminated with the addition of 0.5 ml of charcoal suspension (5% Norit-A charcoal in 20 mM sodium phosphate, pH 7.4, and 0.2% bovine serum albumin). It was incubated at room temperature for 5 min and then centrifuged at 3,000 × g for 5 min at room temperature. The supernatant (500 ml) was taken out for counting. Control experiments without any human platelet membrane were carried out to determine background hydrolysis.

[^35]GTPγS Binding Assay
[^35]GTPγS binding was assayed as described previously (28). A typical assay contained 100 μg of human platelet membranes in 200 μl of assay buffer and various amounts of monoclonal anti-idiotypic antibody plus 1 μM U46,619 in a final volume of 200 μl. The mixture was vortexed and incubated for 30 min at 37°C. Ten μl of 4 mM [γ-[^35]P]GTP (0.3 μCi, 2,500 cpm/μmol) was added and incubated again at 37°C for 10 min. The reaction was terminated with the addition of 0.5 ml of charcoal suspension (5% Norit-A charcoal in 20 mM sodium phosphate, pH 7.4, and 0.2% bovine serum albumin). It was incubated at room temperature for 5 min and then centrifuged at 3,000 × g for 5 min at room temperature. The supernatant (500 ml) was taken out for counting. Control experiments without any human platelet membrane were carried out to determine background hydrolysis.

RESULTS AND DISCUSSION

Characterization of Rabbit Anti-HS-145 Antibodies—There are two different approaches to generate anti-idiotypic antibodies. The first approach relies on the fact that the immune system, according to Jerne’s theory (8), generates anti-idiotypic antibodies against overproducing idiotypic antibodies as a measure of control. As a result, immunizing an animal with an antigen may result in the generation of anti-idiotypic antibodies that bear the likeness of the antigen. However, for this approach to work, it is necessary to use monoclonal antibodies, since anti-idiotypic and idiotypic antibodies neutralize each other. After fusion, highly diluted cells are plated on 96-well plates to make sure that idiotypic and anti-idiotype clones will not be present within a well at the same time.

Anti-idiotypic antibodies can also be generated by immunizing mice with idiotypic antibodies. One of the drawbacks of this approach is that most of the monoclonal antibodies generated would recognize the structural regions of the idiotypic antibodies. Only a few would recognize the binding sites of the idiotypic antibodies. As the binding site of the idiotypic antibodies served as a surrogate receptor (in our case), only a small fraction of anti-idiotypic antibodies recognized the receptor. A good screening method needs to be designed to distinguish anti-idiotypic antibodies that recognize the receptor from those that do not.

Because we chose to use the latter approach, an obvious problem needed to be solved. Although the idiotypic antibodies might have high affinity toward the antigen, they might not bear structural likeness to the receptor. Although one might use affinity-purified idiotypic antibodies, one might still obtain little useful anti-idiotypic antibodies, since most of the idiotypic antibodies might not resemble the receptor. One way to overcome this obstacle was to generate monoclonal idiotypic antibodies. Monoclonal idiotypic antibodies were then characterized. Those that bound the antigen in a manner similar to that of the receptor were used as surrogate receptor. This method was not very attractive because it involved another tedious step of monoclonal antibody generation. Instead, we selected the fraction of polyclonal antibodies from rabbit that resembled the receptor by affinity methods, and used them as surrogate receptor.

Polyclonal idiotypic antibodies were generated from rabbit using bovine thyroglobulin-HS-145 conjugate as antigen. Very high titer of specific antibodies was observed after a second injection of the antigen. Serum was collected after the third boost. IgG fraction was obtained using a protein A column. The displacement curve of the purified IgG indicates high affinity antibodies (Fig. 1). IC50 was estimated to be 40 ± 23.7 pm (n = 3). In order to select for the receptor resembling fraction, an SQ29,548-Affi-Gel-102 column was used (Fig. 2).

The rationale was that those IgG binding a structurally different thromboxane A2 antagonist should resemble the receptor. A fraction of IgG was then obtained. The purified IgG fraction was characterized with the binding of 125I-HS-145 and the competitive binding with several structurally different thromboxane A2 analogs (Fig. 3). The apparent affinity of the IgG fraction was Kd = 2.2 ± 0.96 nM (n = 3) that was very close to that of the receptor. The potency order of the analogs for the displacement of specific binding of 125I-HS-145 was identical to that of the receptor (28). Hence, it was assumed that the affinity-purified IgG fraction indeed resembled the receptor.

Characterization of Monoclonal Anti-Idiotypic Antibody—There were some important criteria for the identification of a useful monoclonal anti-idiotypic antibody. The criteria included: 1) interaction with rabbit idiotype antibodies at their binding sites, 2) interaction with thromboxane A2 receptors at the molecular level, and 3) exhibition of biological activities. On the basis of these criteria, experiments were designed for the monoclonal anti-idiotypic antibodies to examine the above three criteria.
Anti-idiotypic Antibody and Thromboxane A<sub>2</sub> Receptor

FIG. 2. Affinity purification of rabbit anti-HS-145 IgG with SQ29,548-Affi Gel-102. Rabbit anti-HS-145 IgG was incubated with the affinity gel overnight at 4 °C. The gel was then washed extensively with binding buffer. Elution was done at tube 11 with elution buffer as described under "Experimental Procedures." One-ml fractions were collected. Each tube was assayed for 125I-HS-145 binding and protein concentration.

FIG. 3. Competitive binding studies on affinity-purified rabbit anti-HS-145 IgG. In a 200-μl incubation buffer, the IgG was incubated with 0.02 nM 125I-HS-145 with different amounts of competitive ligands for 1 h at room temperature. The incubation was terminated, and the bound and free 125I-HS-145 were determined as described under "Experimental Procedures." B<sub>0</sub> is the binding in the absence of unlabeled ligand. B<sub>i</sub> is the binding in the presence of unlabeled ligand. Nonspecific binding was determined using preimmunized rabbit IgG as control, which was typically less than 1%. The data are representative of three separate experiments with qualitatively similar results.

To test the interaction between the antibodies, several experiments were performed. In an enzyme-linked immunoassay with rabbit idiotypic antibodies coated on 96-well plates, the anti-idiotypic antibodies bound to the plates in a dose-dependent manner (Fig. 4). In order to rule out the possibility that this binding may be directed toward the structural portion of the idiotypic antibodies, an increasing amount of HS-145 was added to the assay system to examine if it competed with anti-idiotypic antibody. For mouse monoclonal antibody, 3D-9E-12, its binding was displaceable by HS-145 in a dose-dependent manner with an IC<sub>50</sub> of 2.2 nM (Fig. 5).

To further verify that the binding was indeed specific, radioimmunoassay was carried out. 125I-HS-145 was incubated with rabbit idiotypic antibodies for 1 h, after which different amounts of monoclonal antibody 3D-9E-12 were added. It was shown that the bound radioactivity could be displaced by the monoclonal antibody in a dose-dependent manner (Fig. 6). Thus, 3D-9E-12 was established as a true anti-idiotypic antibody.

One of the most important criteria for a useful anti-idiotypic antibody is that it interacts with the receptor. Many anti-idiotypic antibodies interact with the idiotypic antibodies but fail to interact with the receptor. Since the objective of this application was to generate antibodies that recognize the receptor, it was even more important for this verification. The
most direct method was to show that the antibody displaced specific radioactive ligand from the receptor. As shown in Fig. 7, up to 60% of the specific $^{125}$I-IS-145 binding was displaced by 100 $\mu$g of the monoclonal antibody 3D-9E-12 ($K_I = 0.98 \pm 0.452$ nM, $n = 3$). The displacement was shown to be dose-dependent, whereas mouse IgGsa at the same concentration had no effect.

In this phase of study, the objective was to verify that the monoclonal antibody not only interacted with the receptor but also elicited biological responses. It was also important to characterize the antibody as to its biological activities that might increase its usefulness for the study of this receptor.

In platelet aggregation assay, 3D-9E-12 was shown to inhibit U46,619-induced platelet aggregation (Fig. 8). However, it failed to inhibit the aggregation induced by PAF. Under very similar conditions, 50 $\mu$g of mouse IgGsa also failed to inhibit aggregation in either case. Therefore, it was clear that 3D-9E-12 was indeed specific for the receptor.

Another set of experiments was performed to test whether the monoclonal antibody interfered with the signal transduction of the receptor via G-protein. Under normal circumstances, 1 $\mu$M U46,619 induced a 50–60% increase in $[^{35}S]GTP\gamma S$ binding and about 80% increase in GTPase activity. As shown in Fig. 9, 500 $\mu$g of 3D-9E-12 inhibited half of the increase. Similar observation was made in case of GTPase activity (Fig. 10).

Based on the above observations, we might conclude that 3D-9E-12 was indeed anti-idiotypic and interacted with the receptor at the molecular level. First, it bound to affinity-purified rabbit idiotypic antibodies and displaced $^{125}$I-HS-145 from its binding site. Therefore, it was truly anti-idiotypic, acting on the combining site of the idiotypic antibodies. Second, it competed with $^{125}$I-IS-145 for the thromboxane A$_2$ receptor binding in human platelet membranes. Thus, it interacted with the receptor specifically. Moreover, it functionally inhibited U46,619-induced platelet aggregation, but failed to inhibit that induced by PAF. These results suggest that the inhibition was highly specific for thromboxane A$_2$ receptor only. Furthermore, the antibody inhibits U46,619-induced increases in $[^{35}S]GTP\gamma S$ binding and GTPase activity in human platelet membranes, indicating that it was capable of
anti-idiotypic antibody and thromboxane A2 receptor

Interfering with the signal transduction at the molecular level.

We concluded that mouse monoclonal anti-idiotypic antibody 3D-9E-12 to thromboxane A2 receptor was successfully generated. It proved that our approach of generation of anti-receptor antibody through a two-step approach was versatile. Since the receptor involved is present in a minute amount, isolation of sufficient quantity of pure receptor for antibody production is a formidable task. Anti-idiotypic approach becomes a feasible and valuable avenue to produce anti-receptor antibodies.

CONCLUSION

The successful generation of anti-thromboxane A2 receptor antibody using the anti-idiotypic approach proved the validity of Jerne's network theory. In a practical sense, it offers a very good method for the generation of anti-receptor antibodies even when pure receptor is not available. Although the affinity of the antibody generated in these studies was not very spectacular, it did show very interesting pharmacological profiles. It possessed antagonistic properties in several pharmacological studies, bearing close resemblance to its parent compound HS-145, although the antibody was about 3 orders less potent. The decrease in potency may be due to its bulkiness. Nonetheless, it provided us with a rare opportunity in studying the binding site of this antibody. From the cDNA to this antibody, it will be possible for us to elucidate the specific sequence of this antibody related to receptor binding. We believe that such an approach may help us to understand the sequence requirements for thromboxane A2 receptor binding, which in turn may lead to new drug discovery. Another possible use of this hybridoma cell line may be the generation of mutated antibodies of altered affinity. Using site-directed mutagenesis, we may be able to generate new sequences that possess even higher affinities to the receptor.

REFERENCES

FIG. 8. Inhibition of U46,619-induced platelet aggregation by 3D-9E-12. Freshly prepared human platelet-rich plasma in normal saline was incubated with different amounts of 3D-9E-12. It was then stimulated with 10 μM U46,619. The aggregation was recorded as described under "Experimental Procedures." The data are representative of three separate experiments with qualitatively similar results. 1, 10 μM U46,619; 2, 10 μM U46,619 + 50 μg of mouse IgG2; 3, 10 μM U46,619 + 10 μg of 3D-9E-12; 4, 10 μM U46,619 + 50 μg of 3D-9E-12; 5, 10 nM PAF; 6, 10 nM PAF + 50 μg of 3D-9E-12.

FIG. 9. Inhibition of U46,619-induced increase in [35S]GTP binding by 3D-9E-12. Human platelet membranes (100 μg) were incubated with various amounts of 3D-9E-12 and 1 μM U46,619 for 30 min at 37 °C. [35S]GTP was added and incubated for another 30 min as described under "Experimental Procedures." The reaction mixture was filtered, and retained radioactivity was counted. Mouse IgG2 was used as a control. The data are representative of three separate experiments with qualitatively similar results.

FIG. 10. Inhibition of U46,619-induced increase in GTPase activity by 3D-9E-12. Human platelet membranes (100 μg) were incubated with different amounts of 3D-9E-12 with 1 μM U46,619 for 30 min at 37 °C. [γ-32P]GTP was added. After incubation for 10 min, the reaction was terminated with charcoal as described under "Experimental Procedures." The supernatant was counted after centrifugation. Mouse IgG2 was used as a control. The data are representative of three separate experiments with qualitatively similar results.
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