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CHARACTERIZATION OF THE INHIBITION OF U46619-MEDIATED HUMAN PLATELET ACTIVATION BY THE TRIMETOQUINOL ISOMERS

EVIDENCE FOR ENDOPEROXIDE/THROMBOXANE A_2 RECEPTOR BLOCKADE

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Abstract—Sites of inhibition for the trimetoquinol (TMQ) isomers on 15*S*-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid (U46619)-, 12-*O*-tetradecanoylphorbol 13-acetate (TPA)- and A23187-induced human platelet activation were investigated. Experiments using washed human platelets were designed to characterize relationships among functional (aggregation, secretion) and biochemical (protein phosphorylation, metabolism of inositol phospholipids and radioligand displacement analysis) processes of platelet activation by U46619 and the specificity of inhibition by the TMQ isomers. Thromboxane A_2 receptor stimulation by U46619 in human platelets resulted in a time- and concentration-dependent breakdown of inositol phospholipids [phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol (PI)], phosphatidic acid (PA) accumulation, phosphorylation of 20 and 45 kD proteins, aggregation and serotonin secretion. The TMQ isomers stereoselectively inhibited all U46619-mediated platelet activation processes. *R*(+)-TMQ was 40- and 22-fold more potent than *S*(−)-TMQ as an inhibitor of U46619-induced platelet aggregation and serotonin secretion respectively. In addition, *R*(+)-TMQ blocked U46619-induced 20 kD protein phosphorylation, 45 kD protein phosphorylation, PIP_2, PIP and PI breakdown, and PA accumulation with a potency which was 8-, 13-, 45-, 37-, 33- and 33-fold greater than the *S*(−)-isomer respectively. In contrast to *S*(−)-TMQ, *R*(+)-TMQ produced a concentration-dependent inhibition of specific [^{3}H]U46619 binding to endoperoxide/thromboxane A_2 receptor sites in washed platelets. In other experiments, *S*(−)-TMQ was more potent than *R*(+)-TMQ as an inhibitor of TPA- and A23187-induced platelet aggregation and serotonin secretion, and of TPA-induced phosphorylation of 45 and 20 kD proteins. The inhibitory potencies of *S*(−)-TMQ against TPA- or A23187-induced responses were similar to those needed for antagonism of U46619-mediated platelet activation. In contrast, much higher concentrations of *R*(+)-TMQ were required for blockade of TPA or A23187 versus U46619-mediated responses in human platelets. Taken collectively, the data show that the TMQ isomers interfered with the endoperoxide/thromboxane A_2 receptor-mediated phospholipase C-signal cascade of inositol phospholipid hydrolysis, calcium mobilization, and protein phosphorylation leading to platelet aggregation and secretion. *R*(+)-TMQ acted as a pharmacologically selective and highly stereospecific [*R*(+)-TMQ $\gg$ *S*(−)-TMQ] antagonist of endoperoxide/thromboxane A_2 receptor sites in platelets. Additionally, *S*(−)-TMQ was more potent than *R*(+)-TMQ as an antagonist of platelet activation by inducers of protein kinase C activity and calcium mobilization. Thus, the isomers of TMQ may also interfere by a yet undefined mechanism with calcium-regulated cytosolic processes in platelets.

Prostaglandin endoperoxides and thromboxane A_2 are generated from arachidonic acid derived from membrane phospholipids and are potent inducers of human platelet aggregation and secretion reactions [1]. Since they have very short biological half-lives, stable thromboxane-mimetic PGH_2 endoperoxides, U46619 and U44069 [2], have been used to characterize the mechanism of thromboxane action in various tissues [3–5]. U46619 (Fig. 1) is a potent inducer of platelet aggregatory and secretory responses and is capable of lowering cytosolic cAMP levels [6]. In human platelets, these stable prostaglandin endoperoxides are reported to activate platelet phospholipase C [7] and to increase inositol phospholipid metabolism, phosphatidic acid formation [7–10], calcium mobilization from intracellular storage pools [11–13], and phosphorylation of the 45 kD and 20 kD proteins involved in aggregation and secretion [7, 14–17]. Thus, the phospholipase C-signal cascade appears to play a central role in human platelet activation by the prostaglandin endoperoxides U46619 and U44069 [7–10, 13].

Trimetoquinol (TMQ) is a tetrahydroisoquinoline which exists as two optical isomers and bears little

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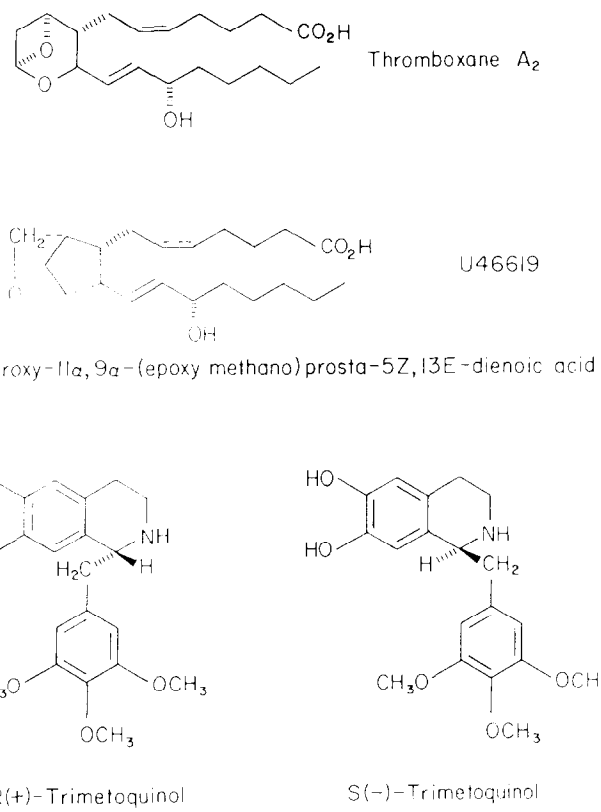

Fig. 1. Chemical structures of thromboxane A_2, U46619 and the optical isomers of trimetoquinol.

structural resemblance to the prostanoid nucleus of either thromboxane A_2 or U46619 (Fig. 1). Early reports [3, 5, 12, 18] showed that *R*(+)-TMQ is a more potent inhibitor of ADP-, collagen-, epinephrine-, arachidonic acid-, U44069-, U46619- and thromboxane A_2-induced human platelet activation than *S*(−)-TMQ. The inhibitory effect of TMQ on human platelet function is independent of prostaglandin biosynthesis or cAMP [18]. This drug was proposed to act as a thromboxane A_2 receptor antagonist since inhibition of the prostaglandin-dependent pathway of platelet activation by TMQ is stereoselective [*R*(+)-TMQ > *S*(−)-TMQ] and competitive against U46619 [3]. However, Pollock *et al.* [8, 19] suggested that antiplatelet action of TMQ isomers against thromboxane A_2-mediated platelet activation is related to the blockade of a specific transduction mechanism (e.g. inhibition of phosphatidylinositol hydrolysis or calcium influx) in platelets. In this regard, radioligand analysis with the stable thromboxane A_2 agonist, U44069, has suggested that TMQ does not act as a thromboxane A_2 receptor antagonist since displacement of [^{3}H]U44069 [20] from putative endoperoxide/thromboxane A_2 binding sites was not observed with high concentrations of *R*(+)-TMQ in human platelets. Thus, TMQ does not appear to block the action of prostaglandin endoperoxides or thromboxane A_2 at the receptor level in human platelets [20] but interferes with an event in the phospholipase C-signal cascade involving either hydrolysis of inositol phospholipids or calcium mobilization [8, 19].

In an attempt to further understand the mechanism of endoperoxide/thromboxane A_2 blockade for this unique tetrahydroisoquinoline class of antiplatelet agents, we have examined functional (aggregation, secretion) and biochemical effects of the TMQ isomers on U46619-induced receptor-linked hydrolysis of inositol phospholipids (including phosphatidylinositol 4,5-bisphosphate, PIP_2; phosphatidylinositol 4-monophosphate, PIP; and phosphatidylinositol PI), phosphatidic acid (PA) accumulation, and protein phosphorylation (20 kD and 45 kD proteins) and on the specific binding of [^{3}H]U46619 to endoperoxide/thromboxane A_2 receptor sites in human platelets. To further assess potential cytosolic sites of inhibitory action, the concentration-dependent effects of the TMQ isomers were evaluated on human platelet activation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA; aggregation, secretion, and protein phosphorylation) and A23187 (aggregation and secretion).

MATERIALS AND METHODS

Materials

Sources of chemicals used in this study were as follows: [^{14}C]serotonin (58 mCi/mmol) and carrier-free sodium [^{32}P]phosphate (50 mCi/ml) were purchased from the Amersham Corp. (Arlington Heights, IL) and New England Nuclear (Boston, MA) respectively. U46619 (15*S*-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid) and [^{3}H]U46619 (22.4 Ci/mmol) were obtained from the

UpJohn Diagnostics Co. (Kalamazoo, MI) and New England Nuclear respectively. Bovine serum albumin (fraction V), apyrase, A23187, ethyleneglycol bis(amino-ethylether)acetic acid (EGTA), Tris–HCl, Tris base, sodium dodecyl sulfate (SDS), L-phosphatidylinositol 4,5-bisphosphate, L-phosphatidylinositol 4-monophosphate, phosphatidylinositol, phosphatidic acid, prostaglandin E_1 (PGE_1), standard marker proteins for gel electrophoresis, and TPA were purchased from the Sigma Chemical Co. (St. Louis, MO). Acrylamide and bis-acrylamide were purchased from Bio-Rad Laboratories (Richmond, CA). TLC plates (silica gel H, 250 micron thickness, 20 cm × 20 cm) were obtained from Whatman Chemical Separation Inc. (Clifton, NJ). FMC PAG bond film was purchased from the FMC Corp. (Rockland, ME). Kodak XR-5 X-Omat X-ray film was purchased from the Eastman Kodak Co. (Rochester, NY). The stereoisomers of TMQ [*R*(+)-TMQ Lot No. 703010; *S*(−)-TMQ Lot No. 960030] were gifts from Dr. Yoshio Iwasawa (Tanabe Seiyaku Co. Ltd., Saitama, Japan). Formula 963 and Protosol were purchased from New England Nuclear. All other chemicals were of reagent grade.

Collection of blood and preparation of washed platelets

Blood was taken by venipuncture from healthy human volunteers who reported being free of medication for at least 10 days prior to blood collection. Whole blood was mixed with acid–citrate dextrose (ACD; 0.8% citric acid, 2.2% trisodium citrate and 2.45% dextrose) solution (6:1, v/v) [21]. Platelet-rich plasma (PRP) was then prepared by centrifugation at 120 *g* for 15 min at room temperature. PRP was centrifuged at 1100 *g* for 5 min, and the resulting platelet pellet was resuspended in a modified Tyrode's washing solution [NaCl, 137 mM; KCl, 2.7 mM; NaH_2PO_4, 0.36 mM; $MgCl_2$, 0.1 mM; $NaHCO_3$, 12 mM; dextrose, 0.56 mM; and bovine serum albumin (Fraction V, 0.35%) without calcium, pH 6.5] [22] in the presence of 20 mM EGTA. Apyrase (EC 3.6.1.5) (34 μg/ml) was added to the platelet suspension to prevent accumulation of ADP. The washing procedure was repeated three times. The final platelet pellet was resuspended in the modified Tyrode's incubation solution, without calcium and apyrase, pH 7.4. Platelets were counted by phase contrast microscopy using a Neubauer counting chamber (Spencer, Inc., Buffalo, NY) and were adjusted to 3×10^8/ml for aggregation and secretion studies.

Aggregation and serotonin secretion

Platelet aggregation studies were conducted at 37° by the turbidometric method of Born [23] as modified by Mustard *et al.* [24], in a Payton dual channel aggregometer (model 600; Buffalo, NY) with constant stirring at 1100 rpm. Washed platelets (0.5 ml) were incubated for 3 min prior to the initiation of aggregation, and this time period also served as the preincubation interval for all compounds added to modify platelet aggregation. The light transmission through modified Tyrode's solution was used to determine a maximum aggregation response, and aggregation responses were measured as percent of the maximal aggregation response to a given stimulus.

In experiments designed to examine inhibitory potencies of TMQ isomers, the minimum concentration of an inducer which produced a near maximal aggregation response (70–90% of maximum light transmittance) was chosen. Concentrations used were 1 μM U46619, 30 nM TPA and 10 μM A23187, and aggregation responses to these inducers were monitored for 2, 5 and 3 min respectively.

The release of the contents of platelet granules was measured by monitoring the secretion of radioactive serotonin from platelets prelabeled with [^{14}C]-serotonin ([^{14}C]5-HT). Platelets were incubated with [^{14}C]5-HT (0.04 μCi/ml platelet suspension) for 30 min during the third wash and were washed again to remove any residual radioactivity from the medium. Platelets were resuspended in modified Tyrode's incubation solution without calcium, pH 7.4. Aliquots of washed platelet suspension were transferred into cuvettes and caused to aggregate by proaggregatory agents in the presence or absence of antiaggregatory agents. Samples were immediately centrifuged at 10,000 *g* for 1 min in a Beckman microfuge (Beckman Instruments Inc., Palo Alto, CA). An aliquot (100 μl) of the supernatant fraction was transferred to scintillation vials, and 0.5 ml of a tissue solubilizer, Protosol, was added. After 2 hr, 10 ml of Formula 963 was added to the vial, and the radioactivity was measured on a Beckman scintillation counter (model LS 6800, Beckman Instruments Inc.) using external standardization to monitor the extent of quench. The amount of [^{14}C]5-HT released was calculated by subtracting the radioactivity contained in the control nonstimulated sample from the total radioactivity in each drug-modified sample. Data were expressed as a percentage of the total radioactivity in platelets as described previously by Mayo *et al.* [3]. The effect of inhibitors was expressed as the percent inhibition of the maximal release by each inducer. In these studies, the mean percent (± SEM) of total [^{14}C]5-HT released from platelets in the presence of 1 μM U46619, 30 nM TPA and 10 μM A23187 was 43.3 ± 4.4 (N = 7); 36.8 ± 7.2 (N = 9) and 52.7 ± 11.9% (N = 3) respectively.

Preparation and stimulation of [^{32}P]phosphate-loaded platelets

The platelet pellet from PRP was resuspended in Tris–HCl washing buffer (50 mM Tris–HCl, 0.1 mM $MgCl_2$, pH 7.0) along with 20 mM EDTA. Apyrase (34 μg/ml) was added to the platelet suspension to prevent accumulation of ADP. After the second wash, platelets were suspended in the buffer and incubated with carrier-free [^{32}P]phosphate (1 mCi/3 ml of platelet suspension) for 90 min at 37° and then were centrifuged at 1100 *g* for 5 min. The resulting platelet pellet was washed and then resuspended in the isotonic Tris–HCl resuspending buffer (20 mM Tris–HCl, 140 mM NaCl and 5.5 mM glucose), pH 7.4) at 2×10^9 platelets/ml. Aliquots (50 μl) of the platelet suspension were used to conduct the protein phosphorylation experiments with U46619 in the presence or absence of the isomers of TMQ.

The phosphorylation reaction was stopped by adding 50 μl of SDS sample buffer (4% SDS, 100 mM 2-mercaptoethanol, 10% sucrose, and 0.0025% bromphenol blue). Platelets mixed with sample buffer were solubilized by heating in a boiling water bath for 2 min.

SDS polyacrylamide gradient slab gel electrophoresis

Proteins dissolved in the sample buffer were subjected to SDS-polyacrylamide gradient slab gel electrophoresis according to the method of Laemmli [25] with some modification. A 3% acrylamide gel was used as a stacking gel and 7–15% gradient of acrylamide as a separating gel. Electrophoresis was carried out for about 12 hr at room temperature. Proteins on the gel were stained with 0.03% Coomassie brilliant blue R for about 10 hr and destained in 10% acetic acid and 40% isopropyl alcohol for about 8 hr. Gels were dried on FMC PAG Bond film and then exposed to Kodak XR-5 X-Omat X-ray film to prepare an autoradiograph for the detection of radioactive zones. The presence of darkened zones on autoradiographs was scanned with a densitometer linked to an IBM computer to quantitate the relative intensity of each band.

Molecular weight of phosphorylated proteins were determined by co-electrophoresing samples with the following standard marker proteins: lysozyme, 14,300; beta-lactoglobin, 18,400; trypsinogen, 24,000; pepsin, 35,000; egg albumin, 40,000; and bovine serum albumin, 66,000.

Incubation of [^{32}P]-loaded platelets and lipid extraction

Labeled platelet suspensions were prepared as described for the protein phosphorylation studies. Platelets (3×10^8 platelets/ml) were resuspended in the isotonic Tris–HCl resuspending buffer. Duplicate 0.5-ml samples of the platelet suspension were incubated for 5 min at 37° before adding any drug. After this initial preincubation, U46619 was added at various concentrations (0.1 to 10 μM) in the presence or absence of TMQ isomers and incubated at varying times (5 sec to 2 min). To terminate the reaction, 2 ml of chloroform/methanol/conc. HCl (C/M/H) mixture (100:200:2; by vol.) was added and mixed. The C/M/H-platelet mixture was vortexed for 15 sec every 5 min for an hour. Mixtures were partitioned into two phases by addition of 0.6 ml of chloroform and 0.6 ml of 2 M KCl solution containing 5 mM EDTA and centrifuged at 1100 *g* for 5 min. The lower chloroform phase was removed using a siliconized glass pipet. The upper phase was washed with 2 ml chloroform. The resulting lower phase was removed and added to the earlier chloroform phase. The combined chloroform extract was evaporated to dryness under a flow of nitrogen gas in a heat chamber.

Thin-layer chromatographic separation of phosphoinositides

Dried lipids were redissolved in chloroform (100 μl) and spotted on silica gel H (20 cm × 20 cm plates) impregnated with 1% potassium oxalate containing 2 mM EDTA. The first solvent system used for the silica gel was the chloroform/methanol/4 N ammonium hydroxide (45:35:10; by vol.) [16]. After the solvent front had moved up to a predetermined margin (14 cm) beyond the origin, the silica gel plate was air-dried and redeveloped in chloroform/methanol/acetone/acetic acid/water (3:1:2:1.5:0.5, by vol.). The R_f values for the first solvent system were: phosphatidylinositol 4,5-bisphosphate (0.15), phosphatidylinositol 4-monophosphate (0.38), phosphatidylinositol (0.60) and phosphatidic acid (0.63). The R_f values of the redeveloped plate in the second solvent system were: phosphatidylinositol 4,5-bisphosphate (0.21), phosphatidylinositol 4-monophosphate (0.45), phosphatidylinositol (0.82) and phosphatidic acid (0.92).

Location and measurement of labeled-phosphoinositides

The TLC plate was air-dried and then exposed to Kodak XR-5 X-Omat X-ray film to prepare an autoradiograph. Autoradiographs were used to locate respective phosphoinositides and phosphatidic acid which were scraped from the silica plate. Silica gel scrapings were placed in scintillation vials with Formula 963 for liquid scintillation counting in a Beckman liquid scintillation counter (model LS 6800), to determine [^{32}P]phosphate incorporation. Data were expressed as net cpm present in zones corresponding to each phosphorylated compound.

Binding studies

Human platelets were washed three times by suspension and centrifugation (1000 *g*, 3 min) in 50 mM Tris buffer, pH 7.0, containing 10 mM $MgCl_2$, 0.1 mg/ml apyrase and 1 μM PGE_1. The first suspension also contained 5 mM EGTA, and the final suspension consisted of 1×10^9 platelets/ml in 20 mM Tris buffer, pH 7.4, containing 10 mM $MgCl_2$, 140 mM NaCl and 5.5 mM dextrose.

Platelets (0.1 ml) were added to 0.9 ml of the Tris buffer-mixture containing 15 nM [^{3}H]U46619 (22.4 Ci/mmol) and selected compounds. Following a 10-min incubation at 37°, samples were rapidly filtered by vacuum through Whatman glass fiber GF-B disks, and washed three times with 5-ml volumes of ice-cold Tris buffer, pH 7.4. Filters were placed in a vial containing 0.5 ml water and 10 ml of Formula 963 fluid, and the amount of [^{3}H] bound was measured by liquid scintillation spectrometry.

Displacement caused by a 10 μM concentration of unlabeled U46619 was used as the measure of specific binding (20–30% of total binding). Filter binding constituted about 65% of the nonspecifically bound [^{3}H]. Neither U46619 nor *R*(+)-TMQ displaced binding of [^{3}H] from filters.

Statistics

Data from U46619 were calculated as effective concentration-50 (EC_{50}) values as determined graphically from plots of percent of maximal light transmittance (or maximal serotonin release) versus log molar concentration. Blockade of U46619-, TPA- or A23187-mediated responses by the optical isomers of TMQ were calculated as inhibitory concentration-50 (IC_{50}) values. Differences between means were

compared by Student's *t*-test using the 5% level of significance.

RESULTS

U46619-mediated human platelet activation

Aggregation and secretion studies. Since TMQ competitively inhibits U46619-induced platelet aggregation and secretion in platelet-rich plasma and contraction of rat aorta [3, 5], the TMQ isomers were tested against U46619-induced activation in washed human platelets. Preliminary studies with U46619 in a calcium-deficient medium demonstrated that this thromboxane A_2 agonist was able to induce shape change, aggregation and secretion in washed human platelets in a concentration-dependent manner (Fig. 2A). The simultaneous measurement of [^{14}C]serotonin release from platelet dense granules also showed a concentration-dependent proportional increase of secreted [^{14}C]serotonin by U46619. The minimal concentration of U46619 required to induce maximal aggregation and serotonin secretion in these platelet preparations was determined to be about 1 μM, and the calculated EC_{50} values (mean ± SEM; N = 4) for U46619-induced aggregation and serotonin secretion were 163 ± 3 and 226 ± 23 nM respectively.

Superimposed aggregation tracings of U46619-induced aggregation in the presence and absence of TMQ isomers are shown in Fig. 2B. The TMQ isomers were concentration-dependent, stereoselective inhibitors of aggregation and secretion induced by U46619. The IC_{50} (mean ± SEM; N = 4) values against U46619-induced aggregation were 0.99 ± 0.02 and 39.6 ± 4.3 μM for *R*(+)-TMQ and *S*(−)-TMQ, respectively, yielding a calculated potency ratio (95% confidence limits) of 40 (34.9–47.3). Similarly, the IC_{50} values (mean ± SEM; N = 4) of *R*(+)-TMQ and *S*(−)-TMQ against serotonin secretion induced by U46619 were 0.24 ± 0.01 and 5.30 ± 0.67 μM, respectively, with a potency ratio of 22 (18.5–25.9, 95% confidence limits).

Protein phosphorylation studies. Exposure of [^{32}P]-phosphate-loaded platelet suspensions to U46619 increased the incorporation of [^{32}P]phosphate into mainly two phosphoproteins designated as 20 kD and 45 kD proteins. The phosphorylation of these two proteins by U46619 was increased in both a concentration- and time-dependent manner, and the times for a half-maximal increase in radioactive phosphate incorporation into the 20 kD and 45 kD proteins were 10 and 8 sec respectively (Fig. 3). In this regard, the time course of the protein phosphorylation reactions coincided with the shape change response caused by U46619 (1 μM). Moreover, the concentrations of U46619 required for half-maximal protein phosphorylation were 0.1 and 0.16 μM for the 20 kD and 45 kD phosphoproteins, respectively (Fig. 3), similar to the concentrations required for U46619-mediated aggregation and secretion.

The comparative inhibitory potencies of TMQ isomers against U46619-induced protein phosphorylation are summarized in Fig. 4. The phosphorylation of both 20 kD and 45 kD proteins stimulated by U46619 was inhibited by both TMQ isomers in a concentration-dependent manner. *R*(+)-TMQ reduced U46619-induced phosphorylation of these proteins at significantly lower concentrations than *S*(−)-TMQ. Calculated IC_{50} values of *R*(+)- and

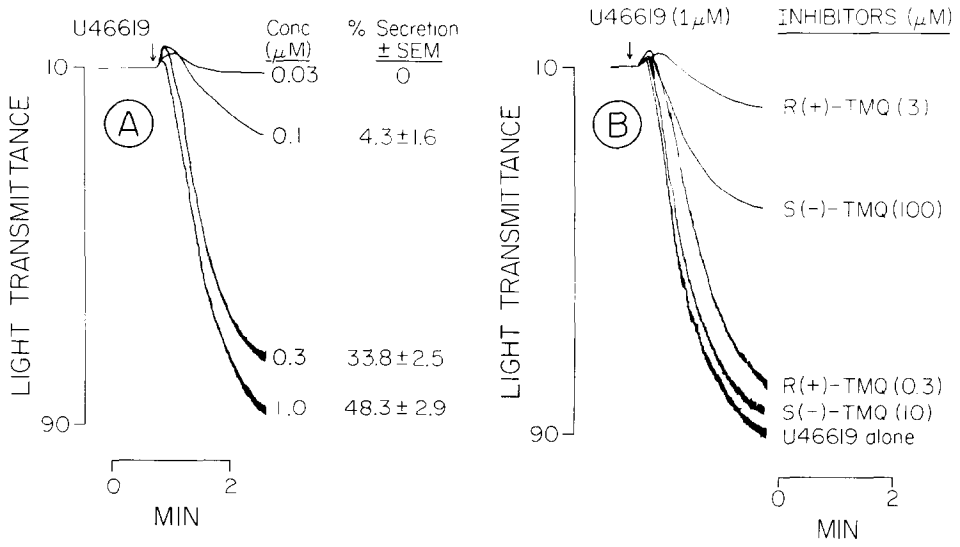

Fig. 2. (A) Concentration-dependent effect of U46619 on human platelet aggregation and [^{14}C]serotonin secretion. Various concentrations of U46619 were added to platelet suspensions, and aggregatory responses were recorded for 2 min. Aggregation tracings are the representative of four experiments. Data on [^{14}C]serotonin secretion are expressed as a percent of total [^{14}C]serotonin. Values are the means ± SEM of four experiments. (B) Concentration-dependent inhibitory effects of trimetoquinol (TMQ) isomers on U46619-induced aggregation in washed human platelets. Diluent or drugs (concentration is shown beside each tracing) were added to platelets 1 min before the addition of U46619 (1 μM). Superimposed tracings are representative of four to six experiments.

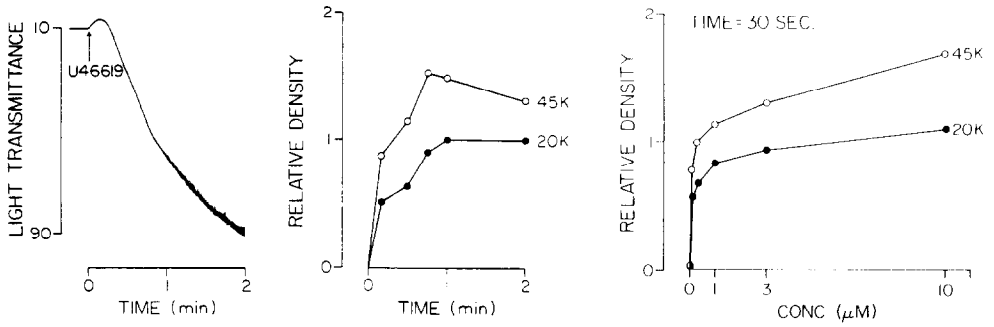

Fig. 3. Concentration- and time-dependent effects of U46619 on 20 kD and 45 kD protein phosphorylation and on aggregation in human platelets. Platelets, which were labeled with [^{32}P]phosphate, were stimulated by various concentrations of U46619 (0.03 to 10 μM) at 37° for 30 sec (concentration-dependent effect) or by 1 μM U46619 at 37° for various times (time-dependent effect). Aggregation stimulated by 1 μM U46619 at 37° for 2 min is included. Aggregation and protein phosphorylation were measured as described in Materials and Methods. Data are representative of three experiments.

S(−)-TMQ for the 20 kD protein were 5.4 versus 42.8 μM and those for the 45 kD protein were 3.9 versus 50.3 μM respectively (Table 1).

Inositol phospholipid metabolism studies. Addition of U46619 caused a time-dependent degradation of [^{32}P]phosphate-labeled inositol phospholipids (PIP_2, PIP, and PI) and accumulation of phosphatidic acid in washed human platelets (Fig. 5). A rapid loss of about 50–60% of the [^{32}P]phosphate radioactivity from PIP_2 was consistently observed within 15 sec of U46619 stimulation. This rapid time-dependent decrease of the radioactivity in PIP_2 was followed by a progressive recovery in the incorporation of [^{32}P]phosphate into PIP_2. The time-dependent changes by U46619 in [^{32}P]PIP or [^{32}P]PI were very similar to that of PIP_2, showing a 35–40% decrease within 15 sec of agonist stimulation. Like PIP_2, the amount of labeled [^{32}P] in PIP and PI also increased after 15 sec and almost reached the initial values (time = 0) after 120 sec (Fig. 5). Changes of radiolabeled phosphoinositides induced by U46619 were accompanied by a simultaneous increase in appearance of [^{32}P]phosphatidic acid, an indicator of the metabolism of phosphoinositides.

U46619 produced a concentration-dependent decrease in radioactivity of the various phosphoinositides (Fig. 5). [^{32}P]Phosphoinositides (PIP_2, PIP and PI) were decreased sharply and significantly by the addition of U46619 (0.03 to 1.0 μM) and reached a near maximal effect with 1 μM U46619. These

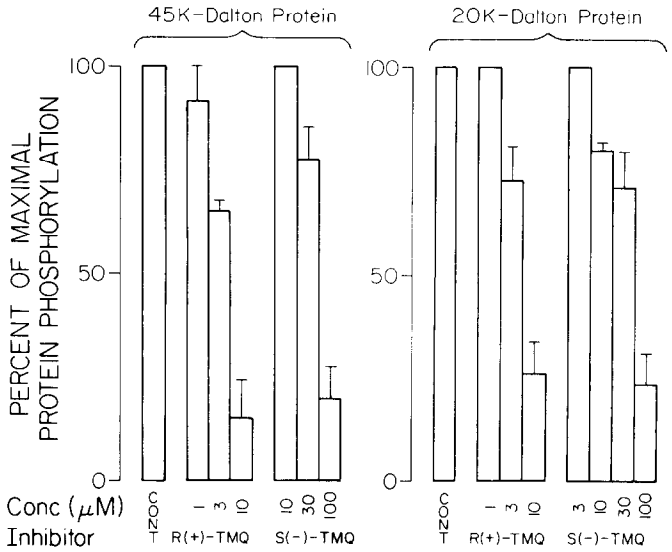

Fig. 4. Concentration-dependent inhibitory effects of trimetoquinol (TMQ) isomers against U46619 (1 μM)-induced 20 kD and 45 kD protein phosphorylation in washed human platelets. Inhibitors were added to [^{32}P]phosphate prelabeled-platelet suspension 1 min before the addition of 1 μM U46619. Platelets were then incubated for 30 sec. Protein phosphorylation was assayed as described in Materials and Methods, and data are expressed as percent inhibition of phosphorylation caused by 1 μM U46619 alone. Values are expressed as the mean ± SEM of three experiments.

Table 1. Comparative inhibitory potencies of the trimetoquinol (TMQ) isomers against U46619-mediated human platelet activation

Parameter	IC_{50}* (μM)		
	R(+)-TMQ	*S*(−)-TMQ	I.A.R.†
Functional responses			
Aggregation	0.99 ± 0.02	39.6 ± 4.3	40
Secretion	0.24 ± 0.01	5.3 ± 0.7	22
Protein phosphorylation			
20 kD protein	5.40 ± 1.2	42.8 ± 4.7	8
45 kD protein	3.90 ± 1.0	50.3 ± 6.8	13
Phosphoinositide turnover‡			
PIP_2	0.32 ± 0.08	14.4 ± 1.5	45
PIP	0.53 ± 0.23	19.9 ± 4.4	37
PI	0.51 ± 0.14	17.0 ± 3.9	33
PA	0.32 ± 0.04	10.7 ± 0.1	33

* Concentration required to block responses by 50%. Values represent the mean ± SEM of N = 3–4 experiments.

† I.A.R. (isomeric activity ratio) = IC_{50} for *S*(−)-TMQ/IC_{50} for *R*(+)-TMQ.

‡ PIP_2 = phosphatidylinositol 4,5-bisphosphate; PIP = phosphatidylinositol 4-monophosphate; PI = phosphatidylinositol; and PA = phosphatidic acid.

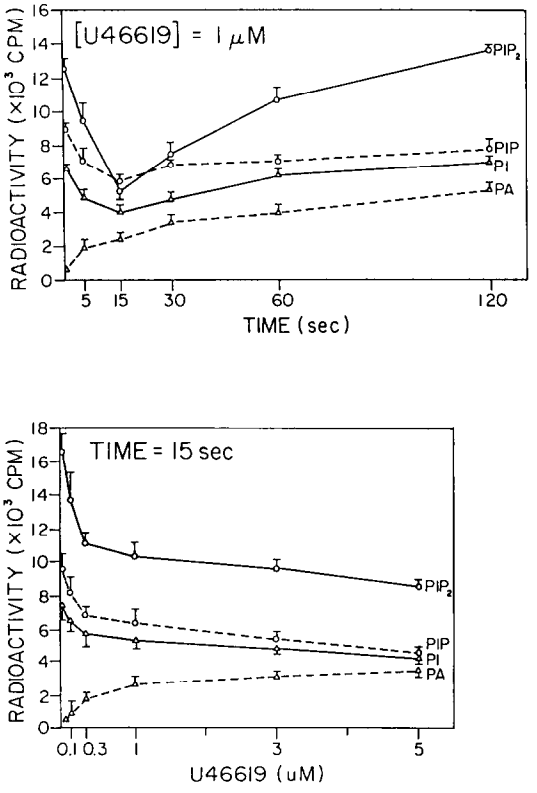

Fig. 5. Time- and concentration-dependent changes of U46619-induced [^{32}P]phosphate-labeling of inositol phospholipids and phosphatidic acid in human platelets. Aliquots (500 μl) of [^{32}P]phosphate-labeled platelets were incubated with 1 μM U46619 for various time periods (time-dependent effects) or with various concentrations of U46619 (0.03 to 5 μM) for 15 sec at room temperature (concentration-dependent effects). Reactions were stopped by adding a chloroform/methanol/conc. HCl (100:200:2) mixture. Phospholipids were extracted and analyzed as described in Materials and Methods. Values represent the mean ± SEM of three to four experiments.

concentration-dependent decreases in [^{32}P]phosphoinositides by U46619 were also accompanied with a concurrent increase in PA.

A near maximal change in each phosphoinositide and PA was seen with 1 μM U46619, and this concentration was chosen for the subsequent studies with the TMQ isomers. Both isomers of TMQ blocked the PIP_2, PIP and PI metabolism with a nearly identical stereoselectivity and isomeric activity ratio as they inhibited U46619-mediated human platelet aggregation and secretion (Fig. 6 and Table 1). TMQ inhibition of phosphoinositide metabolism was associated with a concurrent and nearly identical concentration-dependent decrease in the production of PA (Fig. 6). As summarized in Table 1, the isomeric activity ratios and individual IC_{50} values for each isomer against PIP_2, PIP and PI breakdown, and PA formation induced by U46619 were identical ($P > 0.05$).

Radioligand displacement studies. Using [^{3}H]-U46619 as a radioligand of endoperoxide/thromboxane A_2 receptor sites in platelets [26], we found that only *R*(+)-TMQ gave a concentration-dependent displacement ($IC_{50} = 0.59$ μM) of specific [^{3}H]U46619 binding to washed human platelets (Fig. 7). In contrast, *S*(−)-TMQ gave a significant displacement ($P < 0.05$) of [^{3}H]U46619 binding only at 10 μM. The endoperoxide/thromboxane A_2 receptor antagonist, *trans*-13-azaprostanoic acid also inhibited the specific binding of [^{3}H]U46619 to platelets (a 97% displacement of [^{3}H]U46619 was observed at 50 μM; data not presented).

TPA- and A23187-mediated human platelet activation

Aggregation, secretion and protein phosphorylation studies. Both TMQ isomers blocked the effects of these two inducers on platelet aggregation and serotonin secretion in a concentration-dependent manner and gave a rank order of inhibitory potency of *S*(−)-TMQ > *R*(+)-TMQ (Table 2). The secretory responses to A23187 and TPA were

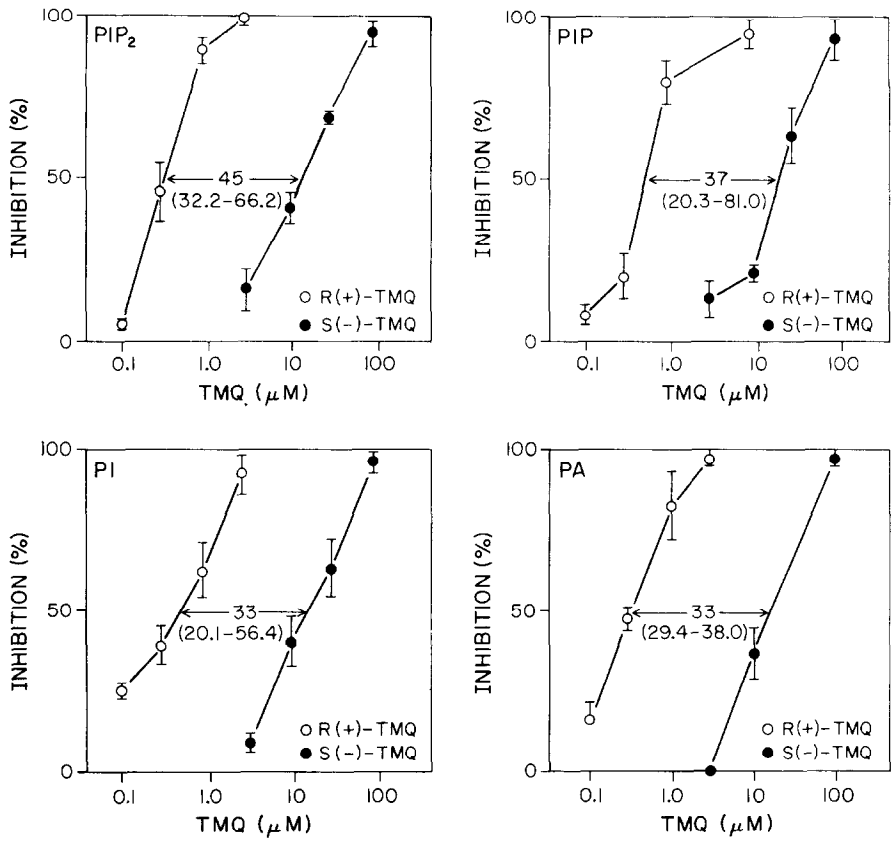

Fig. 6. Inhibitory effects of various concentrations of trimetoquinol (TMQ) isomers on U46619-induced changes in [^{32}P]phosphate-labeled inositol phospholipids and phosphatidic acid in human platelets. Various concentrations of TMQ isomers were preincubated with [^{32}P]phosphate-prelabeled platelets for 1 min before addition of 1 μM U46619. Incubations were performed at room temperature for 15 sec. Extraction and analysis of phospholipids were conducted as described in Materials and Methods. Data are expressed as the percent inhibition of the respective inositol phospholipids (PIP_2, PIP, PI) and phosphatidic acid (PA) seen with 1 μM U46619 alone. Data in parentheses are the 95% confidence limits. Values represent the mean ± SEM of duplicate determinations in three experiments.

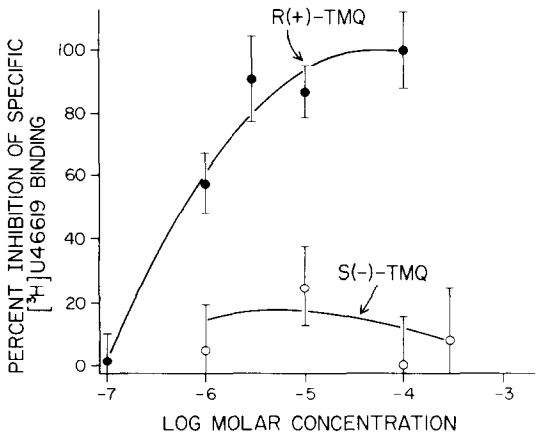

Fig. 7. Concentration-dependent inhibition of specific [^{3}H]U46619 binding to intact human platelets by the trimetoquinol (TMQ) isomers. Data are expressed as the mean percent inhibition ± SEM of N = 3–15 experiments with 4–5 replicates per experiment. Specific binding of [^{3}H]U46619 (dpm/10^8 platelets = 4089 ± 116, mean ± SE, N = 3) was 20–30% of total ^{3}H bound.

blocked by TMQ in a highly stereoselective manner [$S(-) \gg R(+)$] (Table 2). The IC_{50} values for $S(-)$-TMQ against TPA and A23187 were nearly identical to that obtained against U46619-induced platelet activation (Table 1). In contrast, the IC_{50} values for $R(+)$-TMQ against TPA- and A23187-mediated platelet activation were from 60- to 400-fold greater than needed for blockade of U46619-mediated responses. Other studies showed that the TMQ isomers inhibited TPA-induced phosphorylation of 45 and 20 kD proteins with the same stereoselectivity as needed to block aggregation and secretion responses to this inducer (Table 2).

DISCUSSION

Breakdown of membrane phosphoinositides by various stimuli is closely coupled to receptor occupation, and initiates changes in subsequent cytosolic biochemical events [27]. Rittenhouse [7] first reported that U46619 addition to human platelets activates phospholipase C and inositol phospholipid hydrolysis by a receptor coupled event and pathway

Table 2. Comparative inhibitory potencies of trimetoquinol (TMQ) isomers against A23187- and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced human platelet aggregation and secretion, and TPA-induced phosphorylation of 45 kD and 20 kD proteins*

Parameter	IC_{50}† (μM)		
	S(−)-TMQ	*R*(+)-TMQ	I.A.R.‡
A23187			
Aggregation	46.0 ± 2.6	227 ± 56.7	4.9
Secretion	28.8 ± 15.6	233 ± 62.3	8.1
TPA			
Aggregation	43.4 ± 2.1	63.2 ± 5.1	1.5
Secretion	17.8 ± 1.4	400 ± 21.7	22.4
45 kD	83.5 ± 17.2	276 ± 42.4	3.3
20 kD	149 ± 25.3	>300§	2.0

* Various concentrations of each TMQ isomer (1–500 μM) were added to washed platelets 1 min before addition of either 10 μM A23187 or 30 nM TPA. Changes in aggregation or serotonin secretion responses were measured after 3 min as described in Materials and Methods.

† Inhibitory concentration-50 (IC_{50}) values were determined from individual graphs. Values are the mean ± SEM of N = 3–6 experiments.

‡ I.A.R. (isomeric activity ratio) = IC_{50} for *R*(+)-TMQ/IC_{50} for *S*(−)-TMQ.

§ Concentrations of 300 μM did not give an inhibition greater than 35%.

independent of stimulation of phospholipase A_2 or diacylglycerol lipase. In this regard, our experiments demonstrated that the thromboxane A_2-agonist U46619 [2] is able to activate human platelets leading to aggregation and secretory reactions that are related to degradation of inositol phospholipids (PIP_2, PIP and PI), PA accumulation, and an increase in [^{32}P]phosphate incorporation into 20 kD and 45 kD proteins. Increased PA formation as a metabolite of inositol phospholipid degradation induced by U46619 and U44069 in platelets has been reported by others [7–10]. Our studies also show that the time course of phosphoinositide metabolism coincided closely with platelet shape change and protein phosphorylation responses to U46619. Further, PIP_2 was the major labeled inositol phospholipid to be degraded by this stimulus of platelet activation. Taken collectively, the mechanism of platelet activation by the thromboxane A_2-mimetic, U46619, like other inducers [10, 15–17], may involve a receptor-mediated activation of phosphoinositide-specific phospholipase C, triggering a series of biochemical events culminating in aggregation and secretion.

Previous reports indicated that the TMQ isomers interfere with an early event in thromboxane A_2-mediated platelet activation, possibly at putative thromboxane A_2 receptors [3, 5] or through an inhibition of inositol phospholipid metabolism [8, 19]. The present experiments were designed primarily to characterize potential biochemical sites of inhibition for the TMQ isomers against the U46619-induced pathway of platelet activation. Our studies demonstrate that inositol phospholipid degradation and PA accumulation by U46619 were blocked by TMQ isomers in a concentration-dependent manner and with the same stereoselectivity [*R*(+)-TMQ > *S*(−)-TMQ] as against U46619-mediated protein phosphorylation, aggregation and secretion. Moreover, the isomeric activity ratio of TMQ isomers as inhibitors of inositol phospholipid metabolism and accumulation of PA ranged from 33- to 45-fold and closely paralleled with the ratios determined for blockade of U46619-induced aggregation and secretion (40 and 22-fold, respectively; Table 1). This evidence of the antagonism by the TMQ isomers against functional and biochemical changes by U46619 strongly indicated that these compounds may act at the putative endoperoxide/thromboxane A_2 receptor level to block the action of thromboxane A_2 or by antagonism between the receptor and endogenous phospholipase C-linked hydrolysis of inositol phospholipids in platelets.

To provide evidence for or against an interaction of the TMQ isomers at endoperoxide/thromboxane A_2 receptor sites, their comparative abilities to inhibit specific [^{3}H]U46619 binding in platelets were examined (Fig. 7). *R*(+)-TMQ, unlike *S*(−)-TMQ, was a concentration-dependent inhibitor of [^{3}H]-U46619 binding in intact platelets. Further, the stereoselectivity [*R*(+)-TMQ ≫ *S*(−)-TMQ] and potency for *R*(+)-TMQ against [^{3}H]U46619 binding were identical to that required for the blockade of U46619-mediated inositol phospholipid degradation. Characterization of [^{3}H]U46619 or [^{3}H]U44069 as a useful radioligand for endoperoxide/thromboxane A_2 receptor sites in human platelets has been confirmed by others [20, 26]. Thus, we believe that the TMQ isomers, and in particular *R*(+)-TMQ, are acting as antagonists at endoperoxide/thromboxane A_2 receptor sites in human platelets.

Our results on the inhibition of [^{3}H]U46619 binding by *R*(+)-TMQ differ from an earlier report by Armstrong *et al.* [20]. They reported that *R*(+)-TMQ, at 28 μM, does not displace [^{3}H]U44069 from specific binding sites in platelets. These differences are not easily explainable since U46619 and U44069 are thromboxane A_2-mimetics [2–5, 8], and the TMQ isomers are known to be potent antagonists of both U46619- and U44069-mediated aggregation in human platelets [3, 12, 19, 20]. Several methodological differences exist between the studies which may account for these two opposite findings. They are: (1) incubation times (4 min vs 10 min); (2) incubation temperature (37° vs room temperature), and (3) radioligand employed (U44069 vs U46619). In this regard, our experiments used a longer time and higher temperature of incubation which may decrease the equilibrium time required for interaction of the TMQ isomers with endoperoxide/thromboxane A_2 receptor sites. Nevertheless, it is clear from our studies that *R*(+)-TMQ is an endoperoxide/thromboxane A_2 receptor antagonist.

Phosphorylation of two endogenous proteins (20 kD and 45 kD) has been linked to the secretory and aggregatory responses of various inducers of platelet function [13, 16, 28–31] and may occur secondary to inositol phospholipid degradation and formation of PA, diacylglycerol, or inositol phosphates [10, 16, 28]. Like other inducers, U46619 produced time- and concentration-dependent changes

in the phosphorylation of both the 45 and 20 kD proteins (Fig. 4). In this regard, the 20 kD protein has been identified as a light chain of myosin and is phosphorylated by the action of a calcium, calmodulin-dependent kinase, myosin light chain kinase (MLCK) [32], whereas the 45 kD protein is reported to be phosphorylated by a calcium, phospholipid-dependent protein kinase C [33, 34]. In the present studies, the TMQ isomers blocked both inositol phospholipid degradation and phosphorylation of the 20 kD and 45 kD proteins by U46619. Further, the U46619-induced phosphorylation of 20 kD and 45 kD proteins was inhibited by the TMQ isomers with the same stereoselectivity [$R(+)$-TMQ > $S(-)$-TMQ] as these compounds inhibited aggregatory and secretory responses to U46619 (Table 1). However, the isomeric-activity ratio for the inhibition of U46619-induced protein phosphorylation by the TMQ isomers was considerably smaller (8- and 13-fold) than the antagonism of inositol phospholipid degradation or of functional responses (22- to 45-fold). This result suggested that the TMQ isomers may possess another inhibitory site of action in platelets in addition to the blockade of endoperoxide/thromboxane A_2 receptor sites.

Our studies with the phorbol ester, TPA, and the calcium ionophore, A23187, were designed to determine whether the TMQ isomers acted as a cytosolic and prostaglandin-independent site of action in platelets. TPA is known to stimulate protein kinase C activity [35, 36] which mediates platelet activation by a mechanism dependent upon 45 and 20 kD proteins, and by a mechanism independent of inositol phospholipid degradation and diacylglycerol production. The TMQ isomers blocked TPA-induced aggregation, secretion, and protein phosphorylation with a stereoselectivity opposite to their antagonism of U46619-mediated responses in platelets. In contrast to TPA, shape change, aggregatory and secretory responses to A23187 have been related to calcium mobilization from intracellular or extracellular sources [11, 37, 38] in human platelets. Since our work was done in the absence of extracellular calcium, we suggest that the action of this inducer was mediated through an intracellular site in platelets. Our results of the rank order of inhibitory potency for TMQ isomers [$S(-)$-TMQ > $R(+)$-TMQ] against both of these inducers (Table 2) were similar to antiaggregatory effects reported for blockade of bacterial phospholipase C [39, 40] and thrombin [39] mediated responses in human platelets, and are opposite to that for antagonism of the endoperoxide/thromboxane A_2-mediated or prostaglandin-dependent pathway. Taken collectively, these data suggest that the TMQ isomers may act on calcium-dependent cytosolic sites in platelets as an additional mechanism of inhibitory action.

U46619- or A23187-mediated platelet aggregation and calcium mobilization are blocked by agents that elevate cAMP [11, 18, 41], and the antiaggregatory actions of the TMQ isomers against these inducers may be, in part, related to an elevation in platelet cAMP levels. However, results by Shtacher *et al.* [18] and our laboratory [42] indicate that TMQ does not stimulate platelet adenylate cyclase activity, inhibit cAMP phosphodiesterase activity or elevate platelet cAMP levels in concentrations up to 200 μM. Based upon the experimentally determined inhibitory potencies against U46619 and A23187 obtained in our studies (Tables 1 and 2) and others [4, 13, 18], it is unlikely that $R(+)$- or $S(-)$-TMQ acts by increasing platelet cAMP levels. If, as proposed by others [18, 43], TMQ interferes with redistribution of intraplatelet calcium in bound and ionized states, then this agent may possess a novel mechanism of inhibitory action.

Based upon differential inhibitory effects against stimuli of the prostaglandin-dependent and -inhibitory pathways, the TMQ isomers appear to possess at least two inhibitory sites of action in human platelets. The results also point out the value of using stereoisomers rather than racemates of drugs to assess specificity of pharmacologic actions in this system. Clearly, $R(+)$-TMQ, unlike $S(-)$-TMQ, is a selective antagonist at endoperoxide/thromboxane A_2 receptor sites, and is considerably less potent as an inhibitor against stimuli of prostaglandin-independent pathways of platelet activation. Further studies will be needed to determine the exact site(s) of inhibitory action of TMQ isomers against platelet activation by TPA, A23187, bacterial phospholipase C or thrombin. In this regard, the development of an antiaggregatory agent of the tetrahydroisoquinoline class which interferes with a variety of stimuli may have potential value for the treatment of thromboembolic disorders.

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