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Stereoselective inhibition of prostaglandin-induced calcium release from platelet membranes by trimetoquinol

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An elevation of free intracellular calcium levels and binding to calmodulin serves to link membrane-initiated events with cellular effects in biological systems [1]. Studies with platelets have examined relationships between prostaglandin endoperoxides, thromboxane A_2 (TXA_2), cyclic AMP, and calcium in the modulation of platelet activation [2–7]. Prostaglandin-mediated aggregation involves an interaction of inducers at a surface membrane to trigger the release of arachidonic acid (AA) from membrane phospholipids. Released AA is converted to the bioactive metabolites, prostaglandin endoperoxides (PGG_2 and PGH_2) and TXA_2, by the dense tubular system [8, 9]. TXA_2 is thought to increase the mobilization of calcium from the dense tubular system and the activation of platelet contractile proteins, whereas cyclic AMP promotes uptake of calcium into the dense tubular system or facilitates extrusion of calcium outside the cell [6, 7].

Trimetoquinol (TMQ) inhibits platelet activation induced by collagen, epinephrine, ADP, stable PGH_2 analogs (U46619 and U44069), AA and TXA_2 [10, 11], as well as by low dose thrombin and phospholipase C, from *Clostridium perfringens* [12]. TMQ exists as two stereoisomers, of which the $R(+)$-isomer is more potent as an inhibitor of platelet aggregation mediated by the prostaglandin-dependent pathway [10, 11]. We have proposed that the TMQ isomers act as antagonists of endoperoxide/TXA_2 action at the receptor level [11]. Other workers have indicated that TMQ does not interfere with prostaglandin biosynthesis [13, 14]. Thus, TMQ represents a unique chemical entity which is an antagonist of TXA_2 action and may be a novel agent for the treatment of thromboembolic disorders.

It may be suggested that TMQ modulates platelet function by interference with an event in the prostaglandin-dependent pathway that is common to the action of endoperoxides and TXA_2. TXA_2 has been envisioned as a calcium ionophore [15] and is capable of increasing the release of calcium from isolated membranes of the dense tubular system preloaded with radioactive calcium [16]. Previous reports have suggested that TMQ does not modify platelet adenylate cyclase activity or cyclic AMP levels [10]; thus, this agent would not be expected to alter uptake of calcium into the dense tubular system. Our experiments were initiated to determine whether the stereoisomers of TMQ block the release of calcium induced by prostaglandins in isolated platelet membranes in a concentration range which is similar to their blockade of aggregation.

In this report we have evaluated (a) the comparative actions of the stable PGH_2 analogs (U46619 and U44069) on the time-course release of calcium, and (b) the stereodependent ability of the TMQ isomers to block U46619-induced calcium release from isolated membranes of the platelet dense tubular system. U46619 has been characterized recently as a TXA_2 mimetic [17] in many biological tissues and is used as an experimental tool to assess the interaction of the TMQ isomers with TXA_2 sensitive sites for calcium release in this particulate fraction.

Methods

Chemicals. $^{45}Ca^{2+}$ (specific activity 38.9 μCi/mg), as the chloride salt, was obtained from the New England Nuclear Corp. (Boston, MA) and diluted for use. A23187, a cationic ionophore, and the epoxymethanoPGH_2 analogs [U46619: 15*S*-hydroxy-9α, 11α-(epoxymethano)prosta-5Z-13E-dienoic acid; and U44069: 15*S*-hydroxy-11α, 9α-(epoxymethano)-prosta-5Z-13E-dienoic acid] were received from the Eli Lilly Co. (Indianapolis, IN) and the UpJohn Laboratories (Kalamazoo, MI) respectively. The TMQ Isomers were a gift from the Tanabe Seiyaku Co., Ltd. (Saitama, Japan), and racemic TMQ [(+/−)-1-(3′,4′,5′-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] was synthesized in our laboratory [18]. U44069 and U46619 were prepared in ethanolic solutions and diluted in 0.05 M potassium phosphate buffer, pH 7.4. All compounds were freshly prepared in the buffer for use in the experiments.

Isolation of platelet membranes. Membranes were isolated by using the method of Robblee, *et al.* [19]. Human platelet-rich plasma was obtained from the American Red Cross or local blood banks and was centrifuged at 150 *g* for 3 min to obtain a pellet. The 150 *g* supernatant fraction was recentrifuged at 1800 *g* for 20 min, and the pellet was resuspended in citrated saline and the procedure repeated. The pellet was resuspended in a solution containing 30 mM KCl, 5 mM $MgCl_2$, 50 mM potassium oxalate, and 20 mM Tris–HCl, pH 7.0. This suspension was sonicated using a Sonic 300 dismembrator (Artek Systems Corp., Farmingdale, NY) at intermittant 30-sec periods for a total time of

1.5 min. Disrupted platelets were centrifuged at 14,000 *g* for 10 min, and the recovered supernatant fraction was recentrifuged at 40,000 *g* for 1 hr. The 40,000 *g* pellet was resuspended in the homogenizing solution, and the protein content was adjusted to 2.0 mg/ml. Protein was analyzed by the method of Lowry et al. [20], using bovine serum albumin as a standard.

Calcium uptake studies. Effects of compounds were tested as described previously [19] using a reaction mixture of 5 mg protein, 2 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, and 8 μM $CaCl_2$ (sp. act. 0.13 μCi/ml) in a final vol. of 25 ml of 20 mM Tris–HCl buffer, pH 7.0. Reactions were initiated by the addition of the protein suspension (final concentration = 0.2 mg/ml), and the vessels were oscillated at 200 rpm at room temperature for the times indicated. Aliquots were removed at various intervals, and the samples were filtered under negative pressure on filters (Millipore type HA, 0.45 μm) using a Millipore Filtration Manifold (No. 3025, Millipore Corp., Bedford, MA). Platelet membranes trapped on the filters were washed four times with 10 ml of ice-cold 0.9% saline. Filters were air-dried and dissolved into 10 ml of an emulsion-type scintillation solution (Thrift-Solv, Kew Scientific, Columbus, OH), and the radioactivity was measured by liquid scintillation spectrometry using a Beckman model LS-8100 counter. Efficiencies of not less than 92% were obtained. In the absence of protein, the filters retained less than 0.005% of the total radioactivity present in the reaction mixtures.

Calcium release studies. Steady-state levels of calcium accumulation in these membranes were seen between 60 and 80 min. At that time, aliquots (2 ml) of the mixtures were removed, transferred to plastic vials, and incubated in the presence or absence of compounds in a Dubnoff Incubator (Precision Instrument Co., Chicago, IL) maintained at ambient temperature and oscillating at 120 rpm. Inducers (U46619, U44069 or A23187) or inhibitors (TMQ) were added to the mixtures in a vol of 0.02 ml and then incubated for up to 10 min. Maximal release of calcium occurred within 4 min, and aliquots were removed at 3 min in these studies unless otherwise indicated. The contents of the vial were processed and analyzed for radioactivity as outlined above.

Platelet aggregation studies. Aggregation was monitored in the presence of various concentrations of inducers by nephelometry in a Chronolog aggregometer (model 330, Havertown, PA). The collection of human blood, preparation of platelet-rich plasma, and measurement of responses to agents were done as described previously [11]. In these experiments, each TMQ isomer was incubated with platelet-rich plasma for 3 min before the addition of the inducer. Data are expressed as the percentage of the maximal aggregation response (maximum light transmittance) for each inducer.

Statistical analysis. Comparisons between means were made using Students' *t*-test or by testing for overlap of 95% confidence intervals [21].

Results

The time-course of the uptake of calcium into these platelet membranes is given in Fig. 1. This subcellular fraction rapidly accumulated calcium during an initial 60-min period and reached a steady-state level (24.5 nmoles/mg protein) shortly thereafter. In other experiments, TMQ (1–100 μM) did not modify either the steady-state level or rate of accumulation of calcium in these preparations (data not presented).

U46619 (10 μM) was added to these preparations at conditions of steady-state accumulation and found to promote calcium release throughout a 4-min period. Calcium loss from these membranes was determined to be 0.42 nmole/mg protein (S.E.M. = 0.03; N = 3) and 1.2 nmoles/mg protein (S.E.M. = 0.1; N = 3) at 0.2 and 3 min respectively. This maximal release of calcium accounted for about 5% of the total radioactivity taken up into the membranes. Measurement of calcium loss from these membranes in the presence of U46619 and A23187 is shown in Fig. 2. Significant ($P < 0.05$) increases in calcium loss were seen with U46619 (1, 2 and 5 μM) and A23187 (2 μM). At 50 μM, U44069 was found to increase calcium loss from the preparation (2.3% of the total radioactivity; $P < 0.05$), but not at lower concentrations.

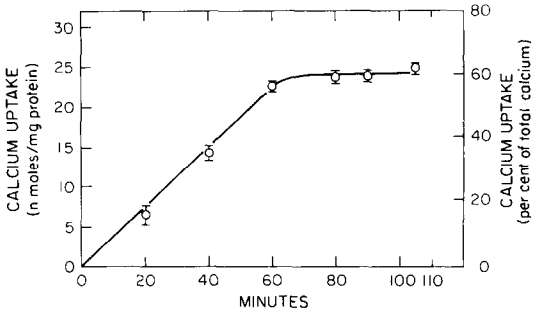

Fig. 1. Time-course of $^{45}Ca^{2+}$ accumulation in the 40,000 *g* platelet membrane fraction. Membranes were recovered by filtration, and $^{45}Ca^{2+}$ was assayed by liquid scintillation spectrometry. Calcium uptake is expressed as nmoles calcium accumulated/mg platelet protein and as a percentage of the total calcium added to the incubation mixture. No corrections were made for endogenous calcium present. Each value is the mean ± S.E.M. (N = 4).

The comparative effects of U46619 and U44069 as inducers of platelet aggregation and calcium loss from isolated membranes are given in Fig. 3. U46619 was approximately 5- and 40-fold more effective than U44069 in producing aggregation and calcium release respectively. The concentration-dependent responses to U46619 in these systems were nearly identical. In contrast, much higher concentrations of U44069 were required to promote calcium release than for platelet aggregation.

Experiments were designed to examine the effects of the TMQ isomers on U46619-induced calcium release in platelet membranes (Fig. 4). Both isomers of TMQ inhibited the release of calcium by U46619 in a

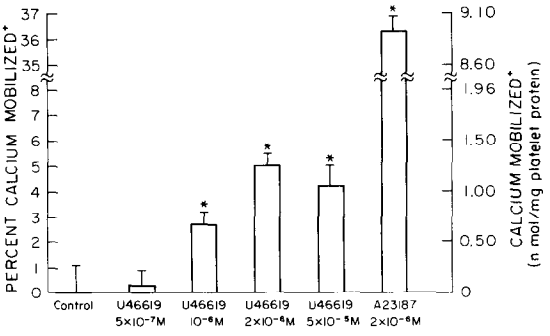

Fig. 2. Mobilization of calcium from a human platelet subcellular membrane fraction after incubation with U46619 or A23187. U46619 or A23187 was added after incubation of membranes for 60 min. $^{45}Ca^{2+}$ release was measured 3 min after the addition of the inducing agent. The amount of calcium mobilized is expressed as nmoles calcium removed/mg protein and as a percentage of the total calcium bound to the membrane fraction. Each value is the mean ± S.E.M. (N = 4). Key: (*) value is significantly different from control incubations ($P < 0.05$); and (†) no corrections were made for endogenous calcium present.

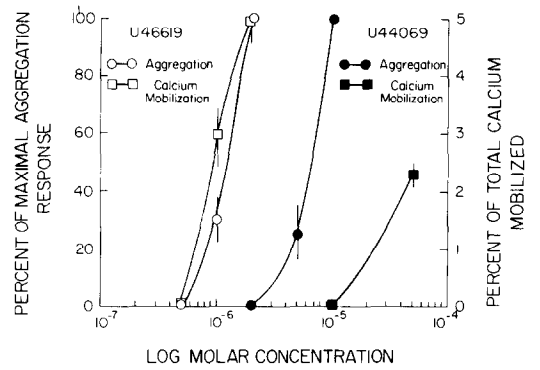

Fig. 3. Comparison of the stimulation of human platelet aggregation (in platelet-rich plasma) and calcium mobilization (from isolated platelet subcellular membranes) by the stable epoxymethano PGH_2 analogues, U46619 and U44069. Results are expressed as a percentage of the maximal aggregation response and as a percentage of the total bound calcium that was released after stimulation by U46619 or U44069. Each value represents the mean ± the range of N = 3–5. No corrections were made for endogenous calcium present.

concentration-dependent manner. The IC_{50} (concentration required for a 50% inhibition of calcium loss) values for *R*(+)- and *S*(−)-TMQ were 0.19 and 2.4 μM respectively. Thus, the blockade of U46619-induced calcium release by the TMQ isomers was stereodependent, with the *R*(+)-isomer being 12.1-fold more potent than *S*(−)-TMQ.

Discussion

The platelet granular secretion reaction associated with the secondary wave of aggregation of ADP or epinephrine, and with the prostaglandin-dependent pathway of activation by physiological inducers, is proposed to be mediated by the action of TXA_2 via calcium mobilization [6–9, 15, 16]. The stereodependent blockade of the actions of

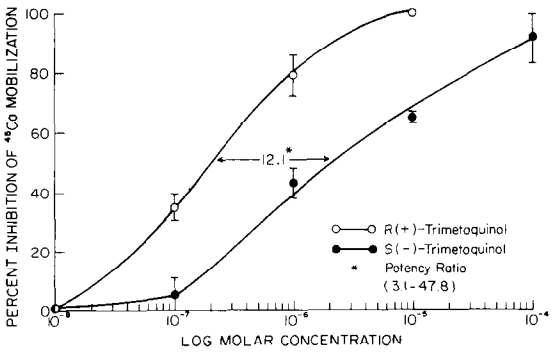

Fig. 4. Inhibition of U46619 (2 μM)-induced calcium mobilization from the platelet subcellular membrane fraction by the stereoisomers of trimetoquinol. Results are expressed as percent inhibition of calcium removal from the membrane fraction. Zero percent inhibition corresponds to 1.25 nmoles calcium mobilized/mg protein. The isomers of trimetoquinol did not modify $^{45}Ca^{2+}$ accumulation or induce mobilization in these membrane preparations. No corrections were made for endogenous calcium present. Each value is the mean ± S.E.M. of N = 4. The calculated potency ratio for the trimetoquinol isomers together with the 95% confidence limits was 12.1 (3.1–47.8).

collagen, ADP, epinephrine, AA, U46619, U44069 and TXA_2 by the TMQ isomers was suggestive of inhibition at a site common to, or subsequent to the action of the bioactive metabolites, PGG_2, PGH_2 and TXA_2 [11, 13, 14, 22]. In a previous report, we showed that the isomers of TMQ blocked aggregation and serotonin secretion by U46619 in identical stereodependent fashion and that the inhibition by TMQ was competitive in nature [11]. Using isolated platelet membranes, which are known to sequester calcium and to be responsive to the action of AA and prostaglandin metabolites [6–9, 16], we have evaluated the possibility that the TMQ isomers may act to block calcium release induced by U46619, a selective TXA_2 agonist [17].

Our experiments have shown that the stable PGH_2 analogs, U46619 and U44069, gave a concentration-dependent release of calcium from these membranes when the levels were at a steady state. The maximum release induced by U46619 in these preparations was about 5% (range = 3.3 to 8.3%; S.E.M. = 0.53; N = 9) of the total radioactive calcium taken into the membranes, and the time-course of calcium release was rapid, reaching a maximum within 4 min. Gerrard *et al.* [16] demonstrated that AA, PGH_2 and PGH_2 were able to cause calcium release from platelet membranes; however, the changes in the loss of calcium were generally of a greater magnitude or also observed during the initial calcium uptake phase. In our system, we were able to show calcium release by U46619 only under steady-state conditions. In addition, our data suggest that U46619 may release only a portion of the total calcium stores in these isolated platelet membranes. This proposal is supported in part by the observation that A23187 was capable of mobilizing calcium to a level which was about 7-fold greater than that for U46619 (Fig. 2). Our data showing a 5% calcium loss by U46619 in these membranes also supported the earlier finding of Owen and LeBreton [23]. Using intact platelets, they found that U46619 produced a mobilization of approximately 4% of the total bound calcium from intracellular sites [23].

If the mobilization of calcium from the dense tubular system is the physiologically relevant site of TXA_2 action, as suggested [6, 7, 15, 16], then concentration-dependent changes in calcium release by inducers and inhibitors of TXA_2 should be similar to their known effects on platelet function. We have shown that the qualitative effects of U46619 and U44069 on platelet aggregation and calcium release from membranes are identical. Whereas the potencies of U46619 in these two systems were the same, U44069 was considerably less active in the release of calcium than as an aggregating agent (Fig. 3). Since the concentration-dependent effects of these stable PGH_2 analogs, and specifically U46619, were similar in these pharmacological systems, calcium release from these membranes may be a direct consequence of the action of these agents on intact platelets.

Evidence for possible roles for TXA_2 and calcium mobilization comes from the use of U46619 and the stereoisomers of TMQ. If such a relationship exists, the isomers of TMQ should block the release of calcium by U46619 in accordance with their known differential effects on platelet aggregation and secretion [11]. Similar to the inhibitory actions of the TMQ isomers on aggregation produced by U46619, our data have shown that these isomers are also stereodependent inhibitors of U46619-induced calcium release from platelet membranes (Fig. 4). However, the difference of 12.1 between the effects of the TMQ isomers on calcium release was considerably lower than the value of 82.9 reported for their inhibition of U46619-induced aggregation in platelet-rich plasma [11]. These quantitative differences in the activities of the TMQ isomers on U46619-induced responses may represent potential multiple sites of action for the inducer or inhibitor in these preparations. In a recent report [11], we suggested that

U46619 may produce aggregation of intact platelets by more than one mechanism [11]. In addition, we have shown that the TMQ isomers have stereoselective inhibitory effects toward thrombin- and phospholipase C-induced aggregation [12]. In this latter study, *S*(−)-TMQ was more potent than *R*(+)-TMQ as an inhibitor of these prostaglandin-independent pathways of platelet activation [12]. Therefore, we suggest that the quantitative differences in the activity ratio of the TMQ isomers in these preparations may represent a summation of inhibitory effects by one or both isomers at more than one pharmacological site in intact platelets.

In summary, the rank order potencies of the stable PGH_2 analogs and TMQ isomers on calcium changes in the isolated platelet membranes were similar to their functional effects. These data suggest that the TMQ isomers inhibit platelet function by the prostaglandin-dependent pathway through an inhibition of calcium release from TXA_2-sensitive stores in membranes of the dense tubular system. Further experiments are required to delineate the sites of interaction of TXA_2 and TMQ. The continued use of U46619 and TMQ stereoisomers will be valuable as experimental probes in intact platelets.

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