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INHIBITION OF PLATELET AGGREGATION BY PAPAVERINE-LIKE DRUGS: EVIDENCE FOR A NOVEL MECHANISM OF ACTION

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Abstract—Trimethoquinol [6,7-dihydroxy-1-(3',4',5'-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline] (TMQ) was chosen as a model compound for studying inhibition of platelet aggregation *in vitro*, because of its β-adrenoceptor agonist properties and structural resemblance to the anti-aggregatory agent, papaverine. TMQ inhibited collagen-induced aggregation of human platelet-rich plasma (I_{50}, 2 μM), the second wave of aggregation induced by 2.5 μM ADP (I_{50}, 0.9 μM), and the second wave of aggregation induced by 45 μM epinephrine (I_{50}, 2.5 μM). Collagen-induced aggregation of human washed platelets was inhibited by TMQ (I_{50}, 1 μM). TMQ was a better inhibitor than aspirin and papaverine and had an inhibitory activity similar to indomethacin in all of the systems studied. TMQ retained inhibitory activity in the presence of both β-adrenoceptor antagonist: propranolol (50 μM), and α-adrenoceptor antagonist: phentolamine (2.5 μM). Platelet adenylate cyclase was not activated and neither cAMP nor cGMP-phosphodiesterase activities were inhibited by TMQ. PGF_2, biosynthesis by aggregating platelets during the coagulation of blood obtained from rats pretreated with aspirin (10 mg/kg, *p.o.*) or indomethacin (1 mg/kg, *p.o.*) was inhibited. However, similar pretreatment with TMQ (100 mg/kg, *p.o.*) and papaverine hydrochloride (100 mg/kg *p.o.*) had no effect. TMQ acted synergistically with the aggregation inhibitors: papaverine, aspirin, and PGE_1. The *in vitro* inhibitory action of papaverine, aspirin, and TMQ was enhanced by increasing calcium concentration. These data indicate that the platelet anti-aggregation activity of TMQ, in contrast to its myocardial stimulating and bronchodilating mechanism, is independent of adrenergic activation. Cyclic AMP accumulation or prostaglandin biosynthesis also seem not to be involved in TMQ action. Therefore, it appears that TMQ may have a novel anti-aggregatory mechanism of action.

INTRODUCTION

Investigations on the nature of the response of platelets to catecholamines have indicated the presence of both α- and β-adrenoceptors [1–4]. Activation of the platelet β-adrenoceptor by isoproterenol was shown to inhibit platelet aggregation induced by collagen, whereas β-adrenoceptor blockade by propranolol reversed this inhibition [2]. Furthermore, isoproterenol was found to stimulate the activity of adenylate cyclase in platelet lysates [3] and its inhibitory effect on platelet aggregation was increased by the addition of drugs that inhibit platelet cyclic nucleotide phosphodiesterase [2]. Since agents that increase platelet cyclic AMP levels may inhibit platelet aggregation [5], it was desirable to investigate potential dual-acting compounds namely, agents that have both β-adrenoceptor stimulating and cAMP phosphodiesterase inhibiting activities. Racemic trimethoquinol (TMQ) was chosen as a model compound since it combined a potent β-adrenoceptor stimulating activity [6,7], with a structural similarity to papaverine which is an inhibitor of both platelet aggregation [8] and platelet cyclic nucleotide phosphodiesterase activity [9].

We found TMQ to have potent platelet anti-aggregatory activity in human platelet-rich plasma and in washed platelets. This activity was unrelated to adrenergic mechanisms, stimulation of adenylate cyclase, cAMP accumulation, or inhibition of prostaglandin biosynthesis. Our studies might reflect a novel way of interfering with the platelet release reaction and platelet aggregation, involving calcium ions.

MATERIALS AND METHODS

Platelet aggregation. Venous blood was collected from human volunteers, who had not taken aspirin for at least 7 days prior, in siliconized 20-ml Vacutainer tubes (Becton & Dickinson & Co., Rutherford, N.J.) fitted with 20-gauge needles using 3.8% sodium citrate as the anticoagulant (9 vol blood to 1 vol sodium citrate). Platelet-rich plasma (PRP) was separated from the red blood cells by centrifugation for 15 min at 180 *g* at room temperature. Platelet-poor plasma (PPP) was prepared by centrifuging PRP for 2 min at 1000 *g*.

Washed platelets were prepared by centrifuging PRP for 15 min at 1110 *g*. PPP was removed and platelets were resuspended in saline containing 0.02% EDTA (disodium). The suspension was centrifuged for 10 min at 950 *g*, and the washing procedure was repeated. Platelets were finally resuspended in Tyrode suspending medium containing 0.35% bovine plasma albumin [10].

Techniques established by Born and Gross [11] were used to study platelet aggregation *in vitro* employing a Payton Dual Channel Aggregation Module (Payton Associates, Inc., Buffalo, N.Y.). One ml of PRP was added to a siliconized cuvette, containing a siliconized stirring bar, and placed in a den-

sitometer maintained at 37 and stirred at 1000 rpm. Various concentrations of test compounds were added in 50 μl of saline and preincubated with PRP for 5 min. Aggregation was initiated by the addition of (2.5 μM) ADP, (45 μM) epinephrine HCl or human mammary gland collagen (kindly donated by Dr. Harvey Weiss, Roosevelt Hospital, N.Y.) sufficient to give about 60 per cent of the maximum aggregation response, as defined by transmission through PPP.

Cyclic nucleotide phosphodiesterase activity. Washed platelets were prepared as described above and resuspended in 10 mM Tris buffer, containing 1 mM $MgCl_2$, pH 7.4. The platelet suspension was sonicated for 30 sec, frozen, thawed, and centrifuged for 1 hr at 105,000 g. The supernatant was radioassayed for both cAMP and cGMP phosphodiesterase activities, as previously described [12]. The cyclic nucleotide concentration was 10 μM. Aqueous solutions of the drugs were added directly to the reaction tubes to yield a final volume of 250 μl. Enzyme activity, calculated on the basis of the disappearance of labelled cyclic nucleotide, was expressed as picomoles of substrate hydrolyzed/mg protein/min. Drug inhibitory activity is expressed in terms of the concentration required to inhibit 50 per cent of the enzyme activity (I_{50}).

Adenylate cyclase activity. Washed human platelets were prepared as described above. Platelets were resuspended in 0.14 M sodium chloride, containing 0.15 M EDTA (disodium), and centrifuged at 120 g for 10 min to remove any remaining leucocytes. The supernatant was centrifuged at 2250 g for 15 min and the resultant pellet was resuspended in 1.0 ml of hypotonic Tris buffer (2 mM), pH 7.4. The lysed platelet preparation was centrifuged at 15,000 g for 10 min and the resultant pellet was resuspended in the assay incubation mixture, containing 80 mM Tris, 3 mM $MgCl_2$, 10 mM theophylline, and 50 μM ATP, pH 7.4. Drugs were added to give a final volume of 0.5 ml and the reaction was stopped after 10 min by placing the mixture in boiling water for 3 min. The reaction mixture was centrifuged at 2000 g for 10 min and aliquots of the supernatant were assayed for cAMP by the radio-immunoassay method of Steiner, *et al.* [13]

Prostaglandin formation. Male rats, 5 per group, weighing approximately 200 g were given various doses (up to 100 mg/kg) of the test compounds by oral intubation and sacrificed after one hr. Blood was collected and serum prepared. The serum samples were extracted with ethyl acetate. Aliquots of the extract were evaporated under nitrogen and assayed for $PGF_{2\alpha}$ content by radioimmunoassay employing antibodies raised to $PGF_{2\alpha}$ in rabbits.

Materials. The substances used in this study were: trimethoquinol [6,7-dihydroxy-1-(3′,4′,5′-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline], (synthesized by Dr S. Teitel, Hoffmann–La Roche Inc., Nutley, N.J.); papaverine hydrochloride (Mallinckrodt Chemical Works, St. Louis, Mo.); epinephrine hydrochloride (Parke Davis & Co., Detroit Michigan); isoproterenol hydrochloride (Winthrop Laboratories Inc., New York, N.Y.); adenosine-5′-diphosphate, sodium salt (Sigma Chemical Co., St. Louis, Mo.); propranolol, 1-isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride (Ayerst Laboratories Inc., New York, New York); phentolamine, 2-(*m*-hydroxy-*N*-*p*-tolylanilinomethyl)-2-imidazoline (Ciba Pharmaceutical Co., Summit, N.J.); practolol, 4′-(2-hydroxy-3-(isopropylamino)propoxy) acetanilide (Ayerst Laboratories Inc., New York, N.Y.); prostaglandin E_1 (P–L Biochemical Inc., Milwaukee, Wisconsin); theophylline (Hoffmann–La Roche Inc., Nutley, N.J.); aspirin and indomethacin (Merck & Co., Inc., Rahway, N.J.).

RESULTS

Preincubation of human citrated PRP for 5 min with 10 μM TMQ resulted in the complete inhibition of second wave platelet aggregation induced by 45 μM epinephrine (Fig. 1). In a concentration-dependent manner, TMQ also inhibited the second wave of platelet aggregation induced by 2.5 μM ADP (Fig. 2). Single phase aggregation induced by human collagen was inhibited by preincubating with different concentrations of TMQ, as shown in Fig. 3. An inhibitory response was apparent at a TMQ concentration of 1 μM and the maximum effect occurred at 10 μM. Known platelet aggregation inhibitors were also studied and I_{50} values were obtained from a plot of inhibitor concentration vs per cent inhibition (Table 1). In the inhibition of collagen-induced aggregation, TMQ [I_{50} of 2 μM) was a better inhibitor than aspirin (I_{50} of 30 μM) or papaverine (I_{50} of 20 μM) and had an inhibitory activity similar to indomethacin (I_{50} of 1 μM). TMQ had a similar potency in inhibiting the second wave platelet aggregation induced by epinephrine (Table 1). The second wave of ADP-induced aggregation appeared to be sensitive to inhibition by TMQ and indomethacin. The I_{50} values for TMQ and indomethacin were 0.9 and 0.1 μM, respectively (Table 1). TMQ was also an effective inhibitor (I_{50} of 1 μM) of the monophasic collagen-induced platelet aggregation of washed human platelets, resuspended in Tyrode's solution, containing 0.35 per cent albumin.

In various smooth muscle preparations, TMQ has been shown to be an effective β-adrenoceptor agonist [6], an effect which was blocked by the β-adrenoceptor antagonist, pronethalol [7]. Inhibition of platelet aggregation by TMQ, however, was not affected by adequate β-blocking concentrations of either propranolol or practolol (Table 2).

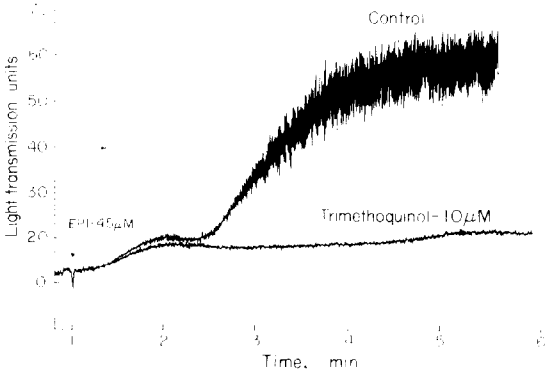

Fig. 1. Trimethoquinol inhibition of epinephrine-induced platelet aggregation in human citrated platelet rich plasma.

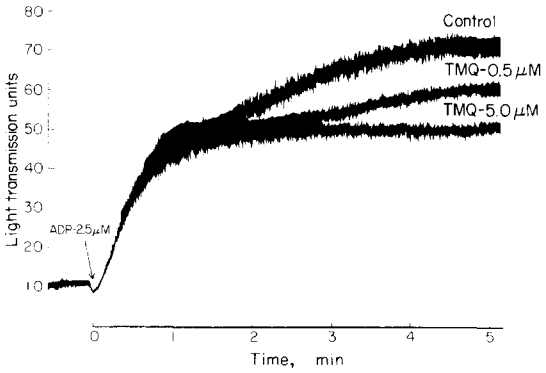

Fig. 2. Trimethoquinol inhibition of ADP-induced platelet aggregation in human citrated platelet-rich plasma.

In contrast to the findings of others [2, 3], isoproterenol (50 μM) did not inhibit collagen-induced platelet aggregation and did not influence the inhibitory action of TMQ on aggregation (Table 2). Similarly, the inhibitory activity of TMQ was retained in the presence of α-adrenoceptor blocking concentrations of phentolamine, which completely inhibited the aggregatory response of epinephrine (Table 3).

Papaverine is a potent inhibitor of cyclic nucleotide phosphodiesterase in many tissues [14], including human platelets [9], and thereby causes an elevation of intracellular cAMP. Since the elevation of intracellular cAMP is thought to be associated with inhibition of platelet aggregation [5] TMQ or papaverine may inhibit platelet aggregation by inhibiting platelet phosphodiesterase activity. The inhibition of cyclic nucleotide phosphodiesterase probably does not explain the mechanism of action of TMQ. Papaverine was a potent inhibitor of both cAMP and cGMP phosphodiesterase activities in a platelet supernatant preparation, whereas TMQ had only minimal inhibitory activities (Table 4). TMQ also had no effect on platelet adenylate cyclase. Neither TMQ nor isoproterenol had any effect in a broken cell preparation of human platelets in which the previously described activators of platelet adenylate cyclase, PGE_1 and NaF [15, 16], caused 3-fold increases in activity (Table 5). Confirmation of a lack of a TMQ effect on platelet cAMP accumulation was also obtained in intact washed human platelets. TMQ at concentrations below 100 μM had minimal effect on the incorporation of [^{14}C]adenine into [^{14}C]cAMP in the platelet [17]*.

The anti-aggregating activity of aspirin and other non-steroidal anti-inflammatory agents is believed to be due to inhibition of platelet prostaglandin biosynthesis [18]. Inhibition of platelet prostaglandin formation is readily detected in rats treated with aspirin or indomethacin, by allowing blood to coagulate and assaying for serum $PGF_{2\alpha}$ by radioimmunoassay. Aspirin significantly reduced $PGF_{2\alpha}$ formation at a dose of 10 mg/kg, *p.o.* and indomethacin was even more active at 1 mg/kg, *p.o.* However, neither TMQ nor papaverine had inhibitory activity on $PGF_{2\alpha}$ formation when administered at doses as high as 100 mg/kg, *p.o.* (Table 6).

TMQ platelet anti-aggregatory activity was synergistic with those of other aggregation inhibitors. The combined anti-aggregatory activities of TMQ together with either aspirin, papaverine, or PGE_1, were greater than the sum of their individual effects (Fig. 4). Calcium is an obligatory component of platelet aggregation [19]. In resuspended, washed human platelets, challenged with collagen, aggregation occurred at Ca^{2+} concentrations of 4.5 to 8.5 meq/l. Outside of this concentration range there was either an absence or a diminished aggregation response to collagen. The platelet anti-aggregatory activities of

* R. J. Haslam, personal communication

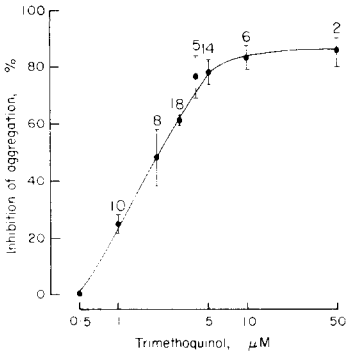

Fig. 3. Concentration response curve for trimethoquinol inhibition of collagen-induced platelet aggregation in human citrated platelet-rich plasma. The number indicated at each point on the curve represents the number of determinations at that concentration.

Table 1. Activity of platelet aggregation inhibitors in human citrated platelet-rich plasma

	I_{50} (μM)		
	Collagen	ADP	Epinephrine
Aspirin	30	30	17
Papaverine	20	5	70
Indomethacin	1	0.1	1.7
Trimethoquinol	2	0.9	2.5

Drugs were preincubated for 5 min in PRP before addition of 10 μl collagen suspension, 2.5 μM ADP or 45 μM epinephrine. I_{50} values, against single phase collagen-induced aggregation and against second wave aggregation induced by ADP or epinephrine, were obtained by inspection of duplicate plots of per cent inhibition vs at least 10 different concentrations of the drug.

Table 2. Effect of β-adrenergic agents on trimethoquinol inhibition of collagen-induced platelet aggregation

Additions	Aggregation amplitude Transmission units ± S.E.M.	
	Without TMQ	With TMQ
None	59.3 ± 0.5	14.0 ± 0.9
Propranolol (50 μM)	54.0 ± 0.6	11.0 ± 0.6
Practolol (50 μM)	59.0 ± 0.6	11.0 ± 0.4
Isoproterenol (50 μM)	58.0 ± 0.7	11.0 ± 1.1

Human PRP was incubated with propranolol, practolol or isoproteranol for 10 min with or without TMQ (5 μM) which was added 5 min before aggregation was induced by addition of collagen. Each point represents the mean of 4 incubations.

Table 3. Effect of phentolamine on trimethoquinol inhibition of platelet aggregation

Aggregating agent	Addition	Aggregation amplitude Transmission units ± S.E.M. Without TMQ	With TMQ
Collagen	None	59.5 ± 0.5	8.8 ± 0.5
	Phentolamine	46.0 ± 0.7	10.3 ± 0.3
Epinephrine	None	59.0 ± 0.4	11.8 ± 0.3
	Phentolamine	10.8 ± 0.5	9.8 ± 0.3

Human PRP was incubated with phentolamine (2.5 μM) with or without TMQ (5 μM) which was added 5 min before aggregation was induced by addition of collagen (5 μl) or epinephrine (45 μM). Each point represents the mean of 4 incubations.

aspirin, papaverine and TMQ were enhanced by increasing $[Ca^{2+}]$ within the range of 4.5–8.5 meq/l (Fig. 5). TMQ, however, was more sensitive to $[Ca^{2+}]$ than were the other anti-aggregatory agents studied. The inhibitory action of TMQ was enhanced by a Ca^{2+} concentration (6 meq/l), that was ineffective in stimulating the activities of papaverine and aspirin. At higher concentrations of Ca^{2+}, the increment in inhibiting potency due to Ca^{2+} was greater in the case of TMQ than in those cases with papaverine or aspirin (Fig. 5).

DISCUSSION

TMQ was a better inhibitor than aspirin or papaverine and had inhibitory activity similar to indomethacin against epinephrine, ADP or collagen-induced aggregation of human platelet-rich plasma. Papaverine was chosen as a reference drug for comparison with TMQ because, apart from its obvious structural resemblance to TMQ, it is an inhibitor of cyclic AMP phosphodiesterase activity [14, 9]. Inhibition of cAMP phosphodiesterase may explain the platelet anti-aggregatory activity of papaverine [9], since the elevation of intracellular cAMP is associated with the inhibition of platelet aggregation [5]. We tend to eliminate inhibition of phosphodiesterase as a mechanism of action of TMQ because TMQ was not inhibitory in a platelet supernatant system, in which the inhibitory action of papaverine was readily detected.

TMQ is a definite β-adrenoceptor agonist in some tissues because of its characteristic effects such as myocardial stimulation, bronchodilatation [6, 7], acti-

Table 4. Effect of trimethoquinol on cyclic nucleotide phosphodiesterase activity in human platelets

	I_{50} (μM) cAMP	cGMP
Papaverine	3	21
Trimethoquinol	240	340

I_{50} values were obtained by inspection of triplicate plots of per cent inhibition vs at least 3 concentrations of the drug.

Table 5. Effect of trimethoquinol on adenylate cyclase activity in human platelets

	Pmoles cAMP/mg protein/10 min ± S.E.M.
Control	640 ± 86
PGE_1 (1 μM)	1530 ± 246*
NaF (7 mM)	1690 ± 229*
Isoproterenol (1 μM)	820 ± 114
Trimethoquinol (100 μM)	820 ± 147

Each figure represents the mean of 6 incubations.
* $P < 0.01$

vation of adenylate cyclase [20] and elevation of tissue cAMP levels [21], all of which are specifically blocked by β-adrenoceptor antagonists [6, 7]. The human platelet has been reported to have a β-adrenoceptor [2 4] and an adenylate cyclase that is activated by PGE_1 and NaF [15, 16]. The powerful inhibitory activity of TMQ on platelet aggregation could thus be attributed to the activation of the platelet β-adrenoceptor and consequent elevation of intracellular cAMP. However, careful analysis of the TMQ effect on platelet adenylate cyclase activity and platelet cAMP accumulation failed to show any response, nor was the inhibitory action of TMQ on platelet aggregation antagonized by β-adrenoceptor antagonists such as propranolol or practolol. These experiments would tend to eliminate an involvement of the β-adrenoceptor or cAMP accumulation in the mechanism of action of TMQ in platelets. Our experiments might also raise a question about the validity of the occurrence of a β-adrenoceptor in the human platelet. In agreement with Clayton and Cross [1], and Vargaftig and Chignard [22] we found no anti-aggregatory effect with isoproterenol in our platelet system. This lack of effect is consistent with the failure of isoproterenol to activate platelet adenylate cyclase as found in our studies and also reported by others [15, 16].

Aspirin is considered as the standard platelet anti-aggregating drug and some inconclusive clinical trials of aspirin as an anti-thrombotic agent have been conducted [23–27]. Inhibition of platelet prostaglandin

Table 6. Effect of pretreatment of rats with platelet anti-aggregating agents on blood prostaglandin formation

Agent	Dose mg/kg *p.o.*	No. of rats	Serum $PGF_{2\alpha}$ ng/ml ± S.E.M.
Placebo		17	17.2 ± 1.4
Aspirin	10	4	6.2 ± 1.5*
Indomethacin	1	3	4.3 ± 2.1*
Trimethoquinol	100	4	18.2 ± 0.8
Papaverine	100	4	15.2 ± 0.9

Male rats weighing approx 200 g were given various doses (up to 100 mg/kg) of the test compounds orally and sacrificed after 1 hr, at which time blood was collected and serum prepared. The serum samples are extracted with ethyl acetate. Aliquots of the extract were evaporated under nitrogen and assayed for prostaglandin-like activity by radio-immunoassay employing antibodies raised to $PGF_{2\alpha}$ in rabbits.
* $P < 0.01$

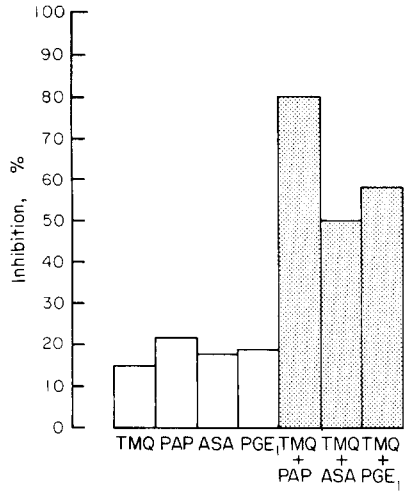

Fig. 4. Inhibition of collagen-induced platelet aggregation in human platelet-rich plasma with combinations of TMQ, papaverine, aspirin and PGE_1. Concentrations of agents added individually or in combination were: TMQ, 0.5 μM; papaverine, 25 μM; aspirin, 12.5 μM and PGE_1, 0.014 μM.

formation has been widely accepted as platelet anti-aggregatory mechanism of aspirin and indomethacin [18] however, inhibition of platelet prostaglandin biosynthesis does not explain the mechanism of action of TMQ. When tested under *in vivo* conditions, that detect the inhibitory effect of aspirin and indomethacin on platelet prostaglandin biosynthesis, TMQ had no inhibitory activity. The bioavailability of TMQ in these experiments was assured because TMQ is known to be orally absorbed in the rat with a plasma half-life of 190 min and maximum tissue levels are reached 1 hr after drug administration [28–29]. We administered 100 mg/kg of TMQ by both oral intubation and i.p. injection and measured the effect of platelet prostaglandin biosynthesis 1 hr after drug administration. This dose of TMQ, which was in excess of the inhibitory doses of aspirin (10 mg/kg) or indomethacin (1 mg/kg), was totally inactive.

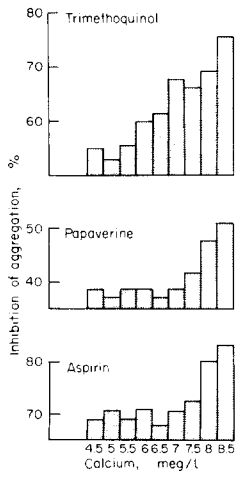

Fig. 5. Effect of calcium on drug inhibition of collagen-induced platelet aggregation in washed platelets. Human washed platelets were incubated for 5 min with trimethoquinol (1 μM), papaverine (5 μM) or aspirin (15 μM) and different concentrations of Ca^{2+} before aggregation was initiated by the addition of collagen.

Experiments with washed human platelets indicated that the platelet anti-aggregatory effects of TMQ, papaverine, and aspirin were enhanced by increasing Ca^{2+} concentration in the incubation medium. TMQ, however, appeared to be more sensitive to the influence of increased Ca^{2+} concentrations than the other anti-aggregatory agents studied. An explanation of this finding is not apparent from these experiments. It is quite possible that Ca^{2+} is displacing the drug molecule from albumin binding sites in the incubation medium thus making more free drug molecules available for inhibitory action on the platelet. Ca^{2+} has been shown to be an essential intracellular mediator of the coupling of external stimuli to internal secretion or contraction in many biological systems [30]. It is likely that an increase in intraplatelet Ca^{2+} arising from the release of intracellularly bound calcium and/or influx of Ca^{2+} from the external medium is a vital link in the initiation of platelet contractile protein interaction and the secretion of platelet storage granules [19]. ADP-induced aggregation is associated with an influx of [44] $CaCl_2$; ADP also induces a redistribution of platelet calcium from the bound state towards the ionized state [31]. TMQ could be interfering with such a redistribution of calcium.

In conclusion, our results indicate that TMQ is a potent inhibitor of platelet aggregation *in vitro*. Its inhibitory action is unrelated to adrenergic mechanisms, cAMP accumulation or prostaglandin biosynthesis. Contrary to previous findings the β-adrenoceptor agonist, isoproterenol, is devoid of platelet anti-aggregatory activity. TMQ may have a novel mechanism of action, different from that of other platelet aggregation inhibitors currently being considered clinically as potential anti-thrombotic compounds.

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