응용약물학회지, The Journal of Applied Pharmacology 3, 143-148(1995)

Further Investigation of the Action Mechanism of GS 389: a Thromboxane A_2 Antagonistic Action

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(Received June 3, 1995; accepted June 17, 1995)

Abstract—Recently, we reported that GS 389 has vasodilating action without cardiac inotropic action (Chang *et al.*, Can. *J. Physiol. Pharmacol.* **72**, 327-334, 1994). However the mechanism of action of GS 389 has not been thoroughly evaluated. In the present study, we performed functional study of GS 389 in rat trachealis, thoracic aorta, pig coronary artery by isometric tension and in human platelets by aggregation experiments. We also tested if GS 389 influences on Ca^{2+} movement and inositol phosphate metabolism. In rat trachealis, GS 389 concentration-dependently relaxed carbachol (0.1 μM)- and high K^+ (65.4 mM)-induced contraction with pIC_{50} of 4.43± 0.19 and 4.11± 0.12, respectively. In Ca^{2+}-free media, GS 389 inhibited carbachol-induced phasic contraction. In rat thoracic aorta, GS 389 inhibited ^{45}Ca uptake due to norepinephrine and high K^+, indicating that GS 389 has direct inhibitory action of Ca^{2+} movement. Furthermore, GS 389 competitively inhibited U46619-induced contraction in rat thoracic aorta and pig coronary artery with K_i values of 5.23± 0.12 and 5.56± 0.14, respectively, and inhibited U 46619-induced phosphatidylinositide (PI) turnover in rat aorta. GS 389 also concentration-dependently inhibited the human platelet aggregation against U 46619 with pIC_{50} 5.66± 0.02. These results indicate that GS 389 has thromboxane A_2 antagonistic action in vascular and platelets as well as direct action on Ca^{2+} movement, which may account, at least in part, for relaxing action of rat trachealis.

Keywords □ tetrahydroisoquinoline, thromboxane A_2, smooth muscle, platelet

TXA_2 is one of the most naturally occurring activators of platelet aggregation and vasoconstriction leading to a variety of cardiovascular disorders. Tetrahydroisoquinoline (THI) compounds (Fig. 1) have various pharmacological actions in the cardiovascular system (Dong *et al.*, 1992). Trimetoquinol, a nonprostanoid compound, and its analogs are competitive antagonists for endoperoxide/thromboxane A_2-mediated responses in human platelet and rat aorta (Mayo *et al.*, 1981). Early functional studies indicated that thromboxane (TP)-mediated responses in platelets and vascular tissues differ. Radioligand binding studies have revealed a similarity in binding specificity of structurally diverse TP receptor agonists and antagonists in platelets and vascular tissues (Hanasaki *et al.*, 1988). Binding characteristics of prostanoid compounds in platelets and vascular smooth muscles have examined, but only a few nonprostanoid TP-interacting compounds were included in those studies. THI alkaloids belong to a group of naturally occurring pharmacologically active compounds. Higenamine, THI derivative, an active ingredient of Aconiti tuber, used as anodyne and cardiotonic agent (Kosuge *et al.*, 1971; Chang *et al.*, 1986). Trimetoquinol, a synthetic THI analog, was identified as a potent bronchodilating agent, which possess β-adrenergic activity (Iwasawa and Kiyomoto, 1967) as well as platelet antiaggregatory activity (Shin *et al.*, 1991, 1992, 1993). Our laboratory has been working for several years to find pharmacologically active THI compounds, in particular, having cardiovascular activity (Park *et al.*, 1984, Chang *et al.*, 1991, 1992,1993a, 1994). In an attempt to develop more potent and more selective cardioactive drugs within the THI series, Chang *et al* (1991, 1993a) examined the effect of some THI by modifying the structure of higenamine. Recently, Chang *et al* (1994) reported that GS 389 has some different characteristic from higenamine and papaverine. In the present study, further investigation of the mechanism

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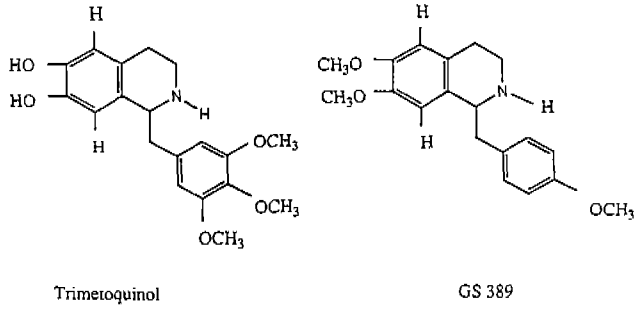

Trimetoquinol GS 389

Fig. 1. Structural similarities between trimetoquinol and GS 389

of action of GS 389 has been performed by focusing on Ca^{2+} movement and thromboxane A_2 antagonism.

Materials and Methods

General

Rats (Sprague Dawley of either sex, weighing 250~300 g) were killed by stunning and bleeding. The tracheas were excised, cleaned of adhering adipose tissue and connective tissue and cut into small pieces (3 mm long) making so called tracheal rings. The contractile force of the muscle was measured isometrically as described previously (Chang *et al.*, 1992). In brief, the rings were set up with specially designed tungsten wire for the isometric recording of tension changes in Krebs solution of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5. KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11 and EDTA 0.03. The tissues were then transferred into the water-jacketed 10-m*l* tissue baths containing Krebs solution at 37℃ and bubbled with a gaseous mixture containing 95% O_2 and 5% CO_2. Once the smooth muscle fibers were correctly oriented in the vertical plane, the tissues were connected via capillary glass rod to isometric force-displacement transducers (Grass FT 03C). Changes in isometric tension were recorded on an ink-writing curvilinear polygraph (Grass, model 7D). Before the commencement of each experiment the tissues were equilibrated for at least 60 min. Following equilibration, the tissues were treated with indomethacin (10 μM) in order to inhibit the generation of cyclooxygenase products. Indomethacin remained in the tissue bath during the whole experiment.

Effect of GS 389 against tone induced by carbachol and high K^+ solution

In these experiments the tracheas were challenged with carbachol (0.1 μM) and high K^+ (65.4 mM). When contraction reached a plateau, GS 389 was added to the bath from 100 nM to 0.1 mM with 0.5 log unit concentration interval. Cumulative log concentration-response curves for the relaxant drugs were obtained. To assess the inhibitory effect of GS 389 the tissues were exposed to GS 389 for 10 min before adding agonists and cumulative concentration-response curves were obtained by a stepwise increase in concentration of carbachol (0.1~100 μM).

Assessment of inhibitory action of GS 389 on Ca^{2+}-induced contraction in Ca^{2+}-free media, and on intracellular Ca^{2+} release

To see the effect of GS 389 directly on Ca^{2+}-induced contraction, Ca^{2+} (0.1~10 mM) was added cumulatively to the bath containing an external Ca^{2+}-free media (compositions are all the same as Krebs except 2 mM EGTA instead of $CaCl_2$), thus log concentration-response curves for Ca^{2+} were made in the presence or absence of the test compounds. After recording the magnitude of contraction by carbachol (10 μM), the tissues were washed until a stable resting tension was reached and the Krebs solution was exchanged for a high K^+, Ca^{2+}-free solution. During this period the bathing medium was changed for a high K^+, Ca^{2+}-free solution every 20 min, and after 60 min, a high K^+, Ca^{2+}-containing solution was substituted. After the contractile effect plateaued, the high K^+, Ca^{2+}-free solution was returned to the baths (point a). Soon after or 10 min later, carbachol (10 μM) was added (control). GS 389 was added prior to changing the bath solution at the point a and very soon, carbachol was added. The results were measured as the magnitude of contraction induced by carbachol at each step. The high K^+, Ca^{2+}-free solution contained (mM): KCl 159.6, $MgCl_2$ 2.1, $NaHCO_3$ 6, EGTA 2 and glucose 11; the high K^+, Ca^{2+}-containing solution contained $CaCl_2$ 2 instead of EGTA 2.

^{45}Ca uptake experiment

Rat aortas were exposed to ^{45}Ca-labeled Krebs solution for 90 min, and exposed to either control or an experimental solution which was also labeled with ^{45}Ca at the same specific activity. Tissues were pretreated for 5 min with GS 389 (0.3 mM) before exposing them to either NE (1 μM, or 0.1 mM) or high K^+ (120 mM). At the end of incubation period, the tissues were bathed in ice-cold Ca^{2+}-free Krebs with 2 mM EGTA for 40 min in order to remove extracellular Ca^{2+}. The tissues were blotted, weighed, and incubated overnight in 3 m*l* of 5 mM EDTA at room temperature. Seven ml of scintillation cocktail containing Triton X-100 were then added and the vials analyzed for ^{45}Ca in a liquid scintillation counter.

Human platelet aggregation studies

Human blood was taken by venipuncture from volunteers who reported being free of aspirin-containing mediation for at least 14 days. Whole blood was mixed with 3.8% trisodium citrate. Platelet-rich-plasma(PRP) was then prepared by centrifugation at 200 g for 10 min at room temparature and used within 2 hr of isolation. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 4000 g for 10 min. Platelet aggregation was monitored at 37℃ by a Chronolog aggregometer (model 560 VS; Havertown, PA, USA) with constant stirring at 1100 rpm. PRP (0.5 m*l*) was incubated for 5 min at 37℃ prior to the initiation of aggregation. Light transmission through PRP preparation was used to determine a maximum response to antiaggregatory drugs. U 46619 (2 μM) was used to induced platelet aggregation. Effect of GS 389 and trimetoquinol was compared.

Inositol phosphate studies

The accumulation of [^{3}H]inositol phosphates in aortic preparations was determined according to Chang *et al.*, (1993b). Briefly, aortic rings (5 mm) were equilibrated for 90 min in Krebs solution, and then incubated in a solution containing 24 mCi/m*l* of myo-[^{3}H] inositol for 4 hr at 37℃. After washing out, tissues were treated with U 46619 (10 μM), in the presence or absence of GS 389 (10 μM), and the incubation was allowed to continue for 60 min in the presence of LiCl (10 mM). The reaction was terminated by freezing with clamps precooled in liquid nitrogen. The tissues were then weighed and homogenized in 1 m*l* of 10 % TCA, centrifuged and the supernatants extracted with water saturated ether (2 m*l*×5). The supernatants were loaded into anion exchange columns (650 mg; Bio-Rad AG 1×8, formate form). The columns were eluted sequentially with 50 mM ammonium formate-5 mM sodium tetraborate and 0.1 M formic acid containing 0.2 M ammonium formate.

Statistical analysis

The results are expressed as means ± SEM for n separate experiments. The concentration of agents which produced 50% of the maximal relaxation (IC_{50}) was then estimated from the log-concentration response curves. Statistical evaluation was made using a one-way analysis of variances and Student's t-test, P values smaller than 0.05 being considered significant. Drug concentrations were presented as negative log molar concentration.

Table I. Effects of GS 389 on pIC_{50} values and maximum contractile responses produced by carbachol and KCl in isolated rat trachealis

Agonist	Maximumcontraction (g)	pIC_{50}
Carbachol (0.1 μM)	2.82± 0.19[a] (8)[b]	4.43± 0.19
KCl (65.4 mM)	1.02± 0.08 (12)	4.11± 0.12

[a]Mean± S.E. [b]Number of experiments

Results

Inhibitory action of GS 389 against muscle tone induced by different agonists

GS 389 inhibited muscle contraction induced by carbachol and KCl in a concentration-dependent manner in rat trachea. The inhibitory potency of GS 389 (pIC_{50}, M) on carbachol (0.1 μM) and high K^+ (65.4 mM) was 4.43± 0.19 and 4.11± 0.12, respectively (Table I). To test if GS 389 blocks muscarinic receptor in rat trachealis competitively, different concentrations of GS 389 were pretreated prior to applying carbachol. As shown in Fig. 2, GS 389 shifted the concentration-response curves dose dependently to the right and lowered the maximum contraction of carbachol.

Effects of GS 389 on intracellular Ca^{2+} release

GS 389 dose-dependently inhibited Ca^{2+}-induced contraction in rat trachea (data not shown). If this mechanism of GS 389 involves in inhibition of Ca^{2+} release mechanism from intracellularly stored Ca^{2+}, we exploited Ca^{2+} depletion and refilling procedures by

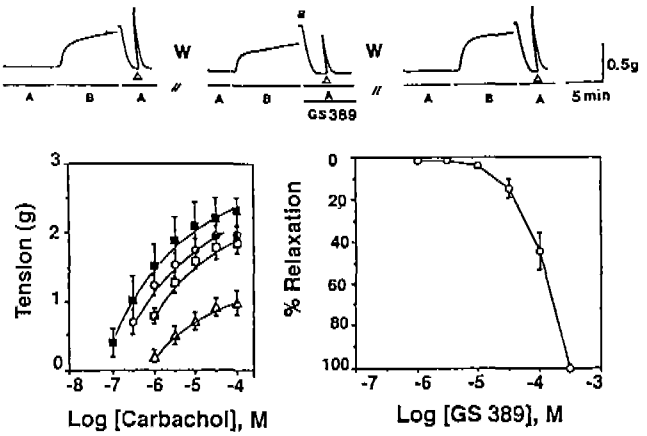

Fig. 2. Inhibition of carbachol-induced initial contraction by GS 389 (0.1 mM) and complete restoration of contraction after washing out of the agent in isolated rat trachealis. Normal Krebs solution was exchanged for a high K^+, Ca^{2+}-free solution (A). After 60 min, a high K^+, Ca^{2+}-containing solution was substituted (B). After contraction plateaued, the high K^+, Ca^{2+}-free solution was returned to the bath and catbachol (0.2 mM) was added at (△−△). Effects of GS 389 (■−■, 1 μM; ○−○, 3 μM; □−□, 5 μM; △−△, 10 μM) on the contraction-response curves of carbachol (lower left) and relaxation effect of GS 389 on carbachol (0.1 μM)-induced contraction in isolated rat trachealis (lower right).

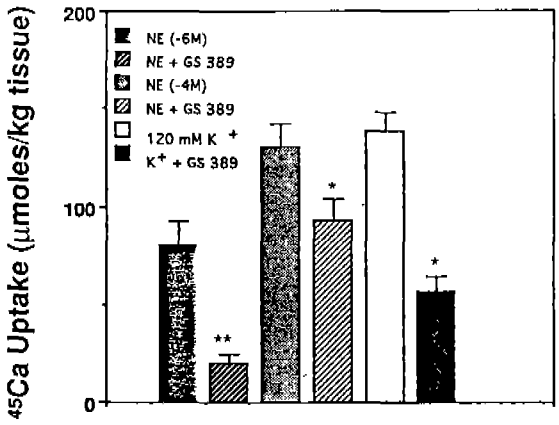

Fig 3. Inhibitory effect of GS 389 (0.3 mM) on ^{45}Ca uptake due to norepinephrine (NE, 1 μM, 0.1 mM) and high KCl (120 mM) in rat thoracic aorta. Data represents mean$\pm$3 experiments. **P<0.001, *P<0.05.

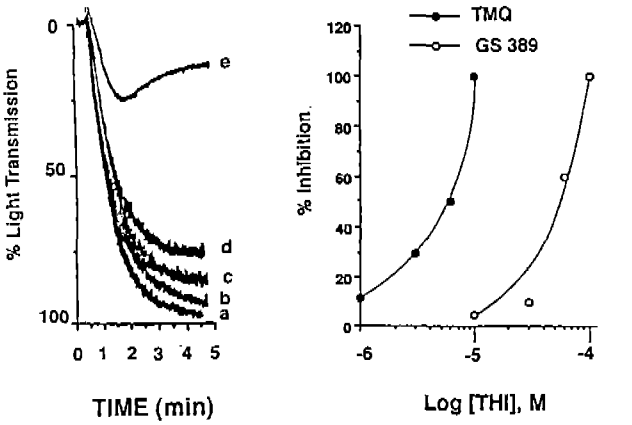

Fig. 4. Antiaggregatory action of GS 389 [0 (a), 0.2 (b), 0.4 (c), 0.8 (d) and 1 mM (e)] on U 46619-induced aggregation (left) and concentration response curves of trimetoquinol (TMQ) and GS 389 on U 46619 (2 μM)-induced aggregation in human plasma rich platelet.

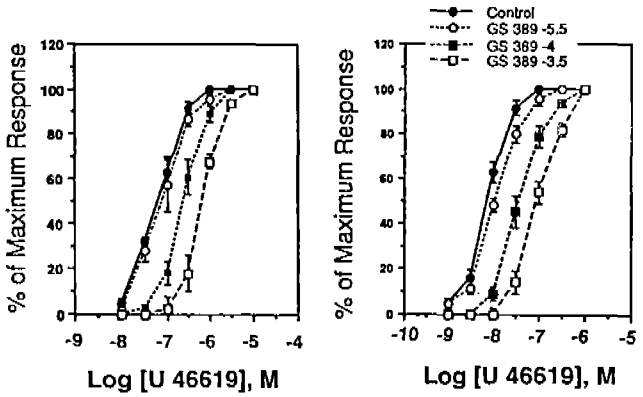

Fig. 5. Competitive inhibitory effects of GS 389 on U 46619-induced contractions of rat thoracic (left) and swine coronary artery (right).

changing the medium with high K^+-Ca^{2+}-free or high K^+-Ca^{2+} containing solutions as described by Chang *et al.*, (1993a). As shown in fig 2 upper panel, 0.1 mM GS 389 inhibited phasic contraction induced by carbachol (0.2 mM). After washing out the same procedure was applied without GS 389, in which case the phasic contraction upon carbachol stimulation was restored. High concentration of GS 389 (1 mM) completely abolished carbachol-induced phasic contraction (data not shown).

Effects of GS 389 on ^{45}Ca uptake

Since GS 389 blocked the Ca^{2+} release by carbachol in Ca^{2+}-free media and KCl-induced contraction in rat trachealis, one may speculate that its mechanism of action is at least involved in Ca^{2+}-antagonizing action. To test this possibility, we carried out Ca^{2+} uptake experiment using radio-isotope in rat thoracic aorta. The effect of GS 389 (0.3 mM) on ^{45}Ca uptake by different contractile agents are shown in Fig. 3. GS 389 significantly reduced ^{45}Ca uptake about 78% (P<0.001) and 36% (P<0.05) due to 1 mM and 0.1 mM NE, respectively. This concentration of GS 389 also caused a significant (P<0.03) inhibition of tissue ^{45}Ca uptake induced by KCl. GS 389 inhibited ^{45}Ca uptake more efficiently to KCl than NE. GS 389 by itself did not affect tissue ^{45}Ca uptake significantly.

Antiaggregatory action of GS 389 and TMQ on U 46619-induced aggregation in human platelets

We tested if GS 389 also antagonizes platelet aggregation by U46619 due to Ca^{2+} antagonistic action. Fig. 4 shows antiaggregatory action of GS 389 on U 46619-induced aggregation in human platelets. GS 389 concentration dependently (0.2~1 mM) inhibited the aggregation induced by U 46619. TMQ also concentration-dependently inhibited U 46619-mediated aggregation. At 10 mM TMQ, while 100 mM GS 389, completely (100%) inhibited U 46619-induced platelet aggregation. To our surprise, we found that GS 389 antagonized U 46619-induced aggregation in human platelet competitively with pIC_{50} value of 5.66 M. Even though the potency is weaker than TMQ, the inhibitory pattern was quite similar between the two test compounds.

Thromboxane A_2 antagonistic action of GS 389 in vascular smooth muscles.

Since TXA_2 receptors on the vascular smooth muscle are almost the same as in platelets (See introduction), we used vascular smooth muscles to further characterize this competitive inhibitory action of GS 389 to U 46619, where TXA_2 receptors are abundant. Pretrea-

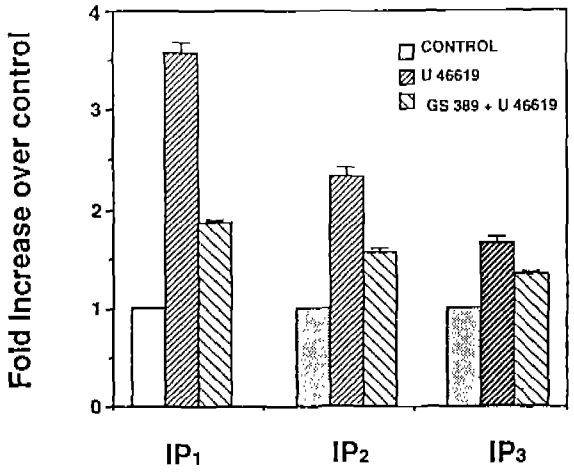

Fig. 6. Inhibitory action of GS 389 (10 μM) on U 46619 (10 μM)-induced phosphatidylinositide hydrolysis in rat throacic aorta. Data represent mean± SEM of 3 experiments. **P<0.001, *P<0.05 (U 46619 vs. GS 389+U 46619).

tment of GS 389 (0.03, 0.1, 0.3 mM) shifted the concentration response curve to U 46619 to the right without affecting the magnitude of maximum contraction, indicating competitive inhibition (Fig. 5). The inhibitory potency on rat aorta and pig coronary artery was 5.23 ± 0.12 and 5.56± 0.14, respectively.

Inhibitory action of GS 389 on U 46619-mediated PI turnover in rat aorta

To further investigate the mechanism of this TXA_2 antagonistic action of GS 389, effects of the compound on the PI turnover was performed in rat thoracic aorta. U 46619 is known to increase PI turnover in vascualr smooth muscle. As shown in Fig. 6, GS 389 inhibited PI turnover due to U 46619. For example, 10 μM U 46619 caused about 3.6 fold increase in inositol monophosphate (IP_1) over the control, which was significantly (P<0.001) inhibited by pretreatement of GS 389 (10 μM).

Discussion

The present results demonstrate that GS 389 had bronchodilating, vasodilating and antiaggregatory action. To elucidate the mechanism of these actions, we investigated effects of GS 389 on carbachol-induced initial phasic contraction in Ca^{2+}-free media in isolated rat trachealis. Pretreatment of GS 389 inhibited the carbachol-induced initial phasic contraction, indicating that GS 389 can enter into the cytoplasm and is able to block release of stored Ca^{2+} from the sarcoplasmic reticulum. This finding suggests that GS 389 may have direct effect on Ca^{2+} movement. To see this possibility, we performed the effects of GS 389 on ^{45}Ca uptake in rat aorta due to NE and KCl. As expected, GS 389 (0.3 mM, 5 min) significantly reduced the ^{45}Ca uptake in both NE and KCl-stimulated Ca^{2+} uptake. However, these results can not explain why GS 389 has no negative inotropic action in isolated cardiac muscle. Therefore, there may have some other mechanism of action besides influencing calcium movement, or cardiac muscle may regulate Ca^{2+} handling differently from those of vascular smooth muscle (Chang *et al.*, 1994). Since GS 389 showed Ca^{2+} antagonizing action in the present study, we expected this mechanism can be extended to anti-platelet aggregatory action. We, therefore, used human platelets and induced aggregation by U 46619, TXA_2 mimetic. To our surprise, we found that GS 389 competitively antagonized TXA_2-mediated aggregation activity in human platelets. We also found, as expected, that GS 389 competitively shifted the concentration-response curves for U 46619-induced contraction in rat aorta and pig coronary artery with pA_2 value of 5.23± 0.12 and 5.56± 0.14, respectively. The fact that trimetoquinol, a nonprastanoid compound, which is quite similar to GS 389 in structure (see Fig. 1), and its analogs are competitive antagonist for endoperoxide/thromboxane A_2-mediated responses in human platelet and rat aorta (Mayo *et al.*, 1981) indicates not only THI binding sites may be related with TXA_2 receptor in vascuar and platelets but also confirms that a similarity in binding specificity of structurally diverse TP receptor agonists and antagonists exists in platelets and vascular tissues (Hanasaki *et al.*, 1988). When compared the IC_{50} values between the two tissues on U 46619-induced response, rat thoracic aorta was 2.7 times less potent than that of human platelets. As comparison, effects of TMQ was analysed in U 46619-induced aggregation in human platelets. TMQ was almost 10 times more potent than GS 389. These result definetely indicates that GS 389 has TXA_2-antagonistic action. To understand more about the inhibitory action of GS 389, we tested if GS 389 has inhibitory action of PI turnover, which is regarded as important step in signal transduction (Rapoport, 1986; Legan, 1989; Chang *et al.*, 1994). In rat aorta, U 46619 increased IP_1 levels 2.5 fold over the control by U 46619, which was significantly inhibited by GS 389. Almost the same extent was inhibited by GS 389 in human platelet (data not shown).

The fact that TXA_2 is one of the most naturally occurring activators of platelet aggregation and vasoconstriction leading to a variety of cardiovascular disorders attracts us to investigate the structure activity relatio-

nships of THIs in conjuction with TXA_2 receptors.

In summary, GS 389 inhibited carbachol and high K^+-induced contraction in a concentration-dependent manner in rat trachealis. Furthermore, in Ca^{2+}-free media, GS 389 inhibited Ca^{2+}-induced contraction and carbachol-induced phasic contraction. GS 389 inhibited Ca^{2+} uptake due to NE and high K^+ in rat aorta. In human platelets U46619-induced aggregatory responses were inhibited by GS 389. PI turnover stimulated by U 46619 was inhibited by pretreatment of GS 389. These findings indicate that GS 389 not only interferes with Ca^{2+} influx and release of intracellularly stored Ca^{2+} but inhibits U 46619-induced PI turnover in rat aorta, which may be, at least in part, responsible for bronchodilator and vasodilating action, and anti-platelet aggregatory action. Recent literatures about THI analogs on thromboxane A_2 antagonistic action (Shin *et al.*, 1991, 1992, 1993) further support our conclusion that GS 389 has TXA_2 antagonistic action in vascular and platelets.

Acknowledgment

This work was supported by a Grant from Korea Science and Engineering Foundation (931-0700-039-2), Korea.

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