Korean J. of Pharmacology
Vol. 30, No. 1, pp. 101-109, 1994

Mechanisms Underlying the Inhibitory Effect of GS 283 in Various Smooth Muscles

Si Hwan Kim, Young Soo Lee, Won Seog Chong and Ki Churl Chang*

Department of Pharmacology, College of Medicine, Gyeongsang National University, Chinju 660-280, Korea

ABSTRACT

Pharmacological characterization of GS 283, a tetrahydroisoquinoline derivative has been elucidated using rat thoracic aorta, guinea pig tenea coli and rabbit mesentery artery in vitro. GS 283 showed calcium antagonistic action in vascular smooth muscle, since high K^+-induced contraction was concentration dependently inhibited. GS 283 also inhibited the contraction induced by α_1 receptor activation. Vasodilating action of GS 283 was not modified by the propranolol, indicating that GS 283 has no β receptor stimulatory action. Simultaneous measurement of intracellular calcium change and muscle tension indicated that the inhibitory effect of GS 283 was accompanied by the increase in tissue fluorescence. This increment was not due to fura 2 fluorescence but to endogenous pyridine nucleotide, suggesting that GS 283 has an effect to inhibit mitochondrial function. GS 283 had an inhibitory action on cyclic AMP and GMP-dependent phosphodiesterases from rat brain with Ki values of 2.5 and 6.7 mM. From these findings we concluded that GS 283 has multiple action such as the inhibition of cyclic nucleotide-dependent phosphodiesterases, blocking of calcium channel as well as inhibition of mitochondrial function which are responsible for vasodilatation.

Key Words: Calcium channel, Smooth muscle, Phosphodiesterase inhibitor

INTRODUCTION

Cardiovascular diseases such as hypertension, atherosclerosis, cardiac arrhythmic and stroke are the leading causes of death in western countries as well as in Korea. Recently, Chang *et al.* (1993) reported that GS 283 inhibited the contractile responses of rat and guinea pig trachea by a mechanism related to inhibition of Ca^{2+} channel. Since, many of isoquinoline or THI compounds possesses the inhibition of cyclic nucleotide PDEs (kukovetz and Poch, 1970; Van Inwegen *et al.*, 1989), Ca^{2+} blocking action (Iguchi *et al.*, 1992), and other actions (D'Ocon *et al.*, 1989, 1991; Avorra *et al.*, 1992), it remains to determined the exact mechanism of action of GS 283. Because inhibitory effect of GS 283 on Ca^{2+} channel may be a reflection of direct inhibitory action or secondary one due to inhibition of cyclic nucleotide-dependent PDE or both. Therefore, attempts have been made to further characterize the pharmacological properties of GS 283 as well as if this compound has similar pharmacological actions in other tissues as shown in trachealis.

*Auther for correspondence

MATERIAL AND METHODS

Materials

Phenylephrine(PE), triton X-100, verapamil, and 5'-nucleotidase (Ophiaphagus hannah snake

venom), 2-deoxy glucose, dithioerythritol (DTE), pyruvic acid were purchased from Sigma Chemical Company (St. Louis, MO, USA), adenosine, guanosine, [2, 8-^{3}H] cyclic AMP, [2, 8-^{3}H] cyclic GMP, were from Amersham (U.K), anion exchange (AG 1 x 8) was from Bio-rad. Fura 2 AM was from Dojindo Laboratories (Kumamoto, Japan). GS 283 was synthesized by Organic Chemistry Laboratory of Gyeongsang National University. All other chemicals and reagents used were of the highest quality available.

Muscle preparations

Healthy male Sprague-Dawley rats (250~300 g) were killed and bleeding. Their thoracic aortas were removed, cleaned of adhering fat and connective tissue. The rings (2.5 mm wide) were prepared according to Chang *et al.* (1994). Each ring was mounted using in a 10 ml water jacketed muscle chamber containing 37℃ modified Krebs-Ringer bicarbonate solution which was gassed with 95%O_2~5%CO_2 and had the following composition (mM) : NaCl (136.9), KCl (5.4), $MgCl_2$ (1.0), $NaHCO_3$ (23.8), $CaCl_2$ (1.5), EDTA (0.03). The rings were equilibrated at 1 g resting tension for more than 90 min, with washing at 20 min intervals, prior to drug addition. The Ca^{2+}-free solution was the same as above except EGTA 1 instead of $CaCl_2$, 1.5.

Measurement of vasorelaxation

For measuring vasorelaxation, contractions were obtained by adding PE, 0.3 μM Krebs-Ringer bicarbonate solutions or by changing the bath fluid with 60 mM potassium (K^+) which was made by substituting equimolar concentration of sodium from the Krebs-Ringer bicarbonate solutions. After reaching plateau of contraction, test substances were added cumulatively with 0.5 log unit intervals in concentrations. Isometric tensions were recorded on Grass physiograph (model 7E) via force transducer FT-03.

Measurement of Ca^{2+}-induced contraction

In Ca^{2+}-induced contraction experiments, bathing fluid was replaced by a Ca^{2+}-free salt solution for 1 hr. After this period, PE (10 μM) or K^+ (60 mM) were added. Ca^{2+} was added 15 min later cumulatively to the bath in concentrations from 0.1 to 2.5 mM. GS 283 was added to the bath 5 min prior to PE or K^+ in the Ca^{2+}-free solution. The contractile responses are expressed in percent of PE or K^+-induced contractions obtained in normal Krebs-Ringer bicarbonate solutions.

Effects on intracellular Ca^{2+}, $[Ca^{2+}]_i$, and muscle tension

$[Ca^{2+}]_i$ was measured according to the methods described by Ozaki *et al.* (1987) and Sato *et al.* (1988) using fura 2 (Grynkiewicz *et al.*, 1985). Muscle strips were exposed to the acetoxymethyl ester of fura 2 (5 μM) in the presence of 0.02% cremophore EL for 3 hr at room temperature. The muscle strip was then transferred to the muscle bath integrated in the fluorimeter (CAF-100) and illuminated alternately (48 Hz) with two excitation wave lengths (340 nm and 380 nm). Fluorescence at 500 nm was measured induced by these two wavelengths was calculated and used as an indicator of $[Ca^{2+}]_i$. Absolute $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura 2 for Ca^{2+} may change in smooth muscle cells (Konishi *et al.*, 1988; Karaki, 1989). Ratio of fluorescence in the resting muscle and that in the depolarized muscle with elevated external K^+ (60 mM) were considered as 0 and 100%, respectively.

Effects on oxidative phosphorylation and glycolytic metabolism

Contractile response of smooth muscle is dependent on both oxidative phosphorylation and glycolytic metabolism. Guinea pig tenea coli was prepared in normal physiological solution. After mounting the muscle as described above following treatment was performed. Incubation medium (normal Krebs' solution) was changed to glucose-free solution and then pyruvate was added to contract the muscle (glycolytic metabolism inhibition), or without change the medium the gas was changed to N_2 rather than 95% O_2-5% CO_2 and after tension completely declined, high glucose (40 mM) was added to the medium to induce contraction (oxidative phosphorylation inhibition). GS 283 was cumulatively added to both type of treatment.

Preparation of phosphodiesterase

Rats were anesthesized with pentobarbital sodium (35 mg/kg, I.M.) and the brain was immediately taken out, rinsed in ice-cold 50 mM Tris • Cl buffer (pH 7.5) containing 3.75 mM 2-mercaptoethanol (extraction buffer), and homogenized the tissues in three volumes of the same solution using a motor driven glass-glass homogenizer. The homogenates were centrifuged at 105,000 x g for 60 min and the supernatants thus obtained (crude extract) were used as the source of phosphodiesterases (Chang *et al.*, 1994).

Assay for phosphodiesterase activity

The assay method for phosphodiesterase was described previously by Chang *et al.* (1992). In brief, the standard reaction mixture contained, in a final volume of 0.1 ml, Tris. Cl buffer (pH 7.5), 5 mmol; EDTA, 0.25 mmol; $MgCl_2$, 2 mmol; 5'-nucleotidase (Ophiaphagus hannah snake venom), 10~30 μg; [2, 8-^{3}H] cyclic AMP, or [2, 8-^{3}H] cyclic GMP, 0.1 mmol, containing about 1×10^5 cpm, and the indicated amounts of crude supernatant (see above). The reaction was initiated by the addition of the supernatant and was carried out at 37℃ for 10 min. The reaction was terminated by heating the reaction mixture at 95℃ for 2 min. The unreacted nucleotides were separated from the dephosphorylated products using anion exchange (AG 1×8) chromatography.

Data analysis

The experimental results are expressed as the mean ± SEM. The concentration of agents which produced 50% of the maximal relaxation (IC_{50}) was estimated from the log concentration effective curves in each tissue. The statistical significance changes were analyzed using Student's t-test, and P values <0.05 were considered to be significant.

RESULTS

Effects of GS 283 on high K^+- and NE-induced contraction

To see if GS 283 has vasodilatory action, aortic ring were precontracted with KCl (60 mM) and PE (0.3 μM), α_1-agonist. After reaching maximum contraction, GS 283 was added cumulatively (1 ~ 100 μM) to the bath. As shown in Figure 1, GS 283 dose-dependently relaxed both high K^+ and PE-induced contraction. The inhibitory pattern of GS 283 on PE-induced contraction was relatively rapid compared to that of high K^+-induced contraction. GS 283 showed greater potency in inhibiting high K^+-induced contraction than PE-induced contraction. The 50% inhibitory concentration of GS 283 (IC_{50}) was compared in Table 1.

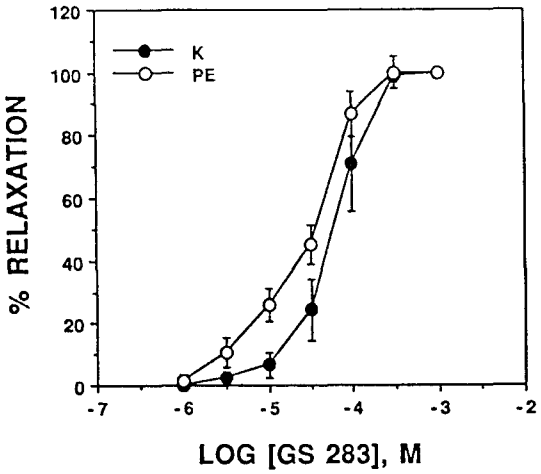

Fig. 1. Concentration response curves of GS 283 on PE (o) and KCl(●)-induced contraction in rat aorta. Each point represents mean ± SEM of 12 to 13 preparations.

Table 1. Comparison of pD_2 values of GS 283 and verapamil on PE-and high K^+-induced contraction rat thoracic aorta

Compounds	pD_2 values(M)	
	PE	High K^+
GS 283	4.75±0.03[a](12)[b]	4.54+0.05 (13)*
Verapamil	4.84±0.06 (8)	6.60±0.03 (8)**

a: mean±SEM
b: number of preparations
*significantly ($p<0.05$) different from PE vs. high K^+
**significantly ($p<0.01$) different from PE vs. high K^+

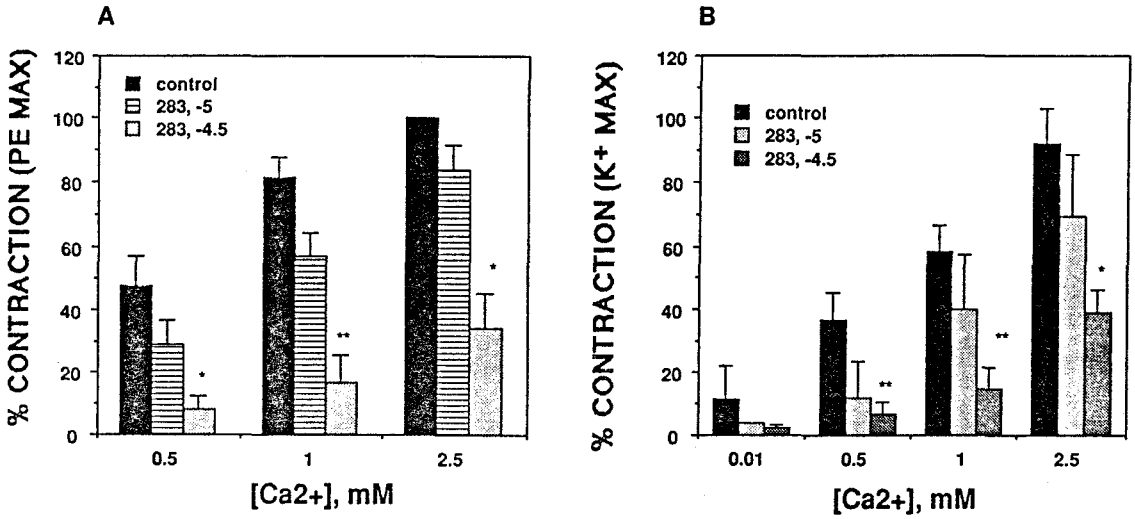

Fig. 2. Inhibitory effects of GS 283 on Ca^{2+}-induced contraction in Ca^{2+}-free media. In the presence of PE (A) or KCl (B), external Ca^{2+} (0.5~2.5 mM) caused increment of contraction, while GS 283 concentration-dependently inhibited the Ca^{2+}-induced contraction. Each column represents mean ± SEM of at least 9 preparations.

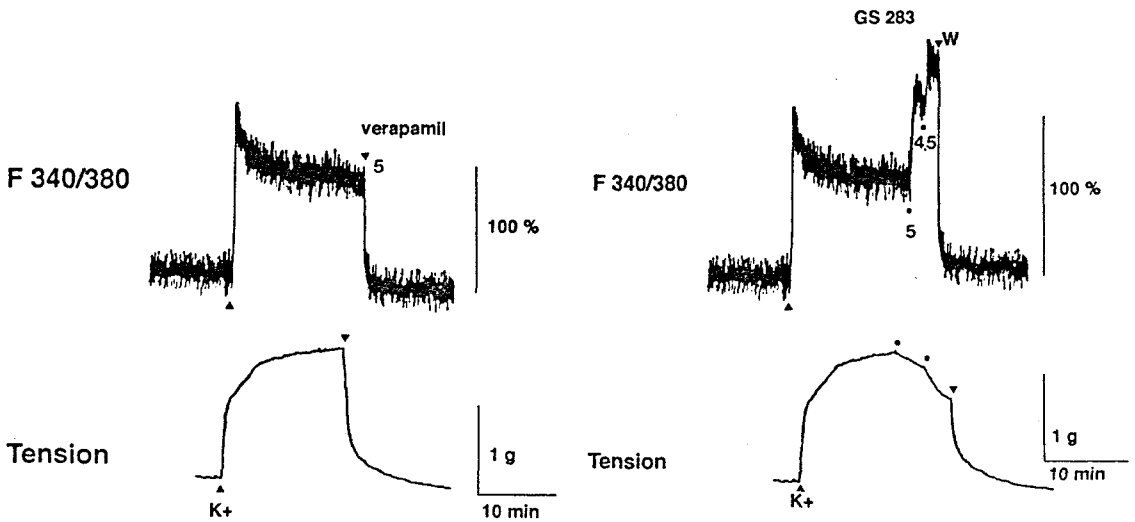

Fig. 3. Effects of verapamil and GS 283 on intracellular Ca^{2+} change and muscle tension inrat aorta stimulated by KCl, Verapamil (10 μM) completely relaxed as well as F 340/380, in contrst, GS 283 (10, 30 μM) relaxed tension but incresed F 340/380 ratio.

Calcium antagonistic action of GS 283 in aorta

To see if GS 283 has Ca^{2+} blocking action in aorta, Ca^{2+} was added cumulatively in Ca^{2+}-free solution in the presence of PE or high K^+. In Ca^{2+} free media. PE induced phasic contraction, while K^+ did not show any rapid phasic contraction, in which addition of Ca^{2+} (0.01~2.5 mM) caused

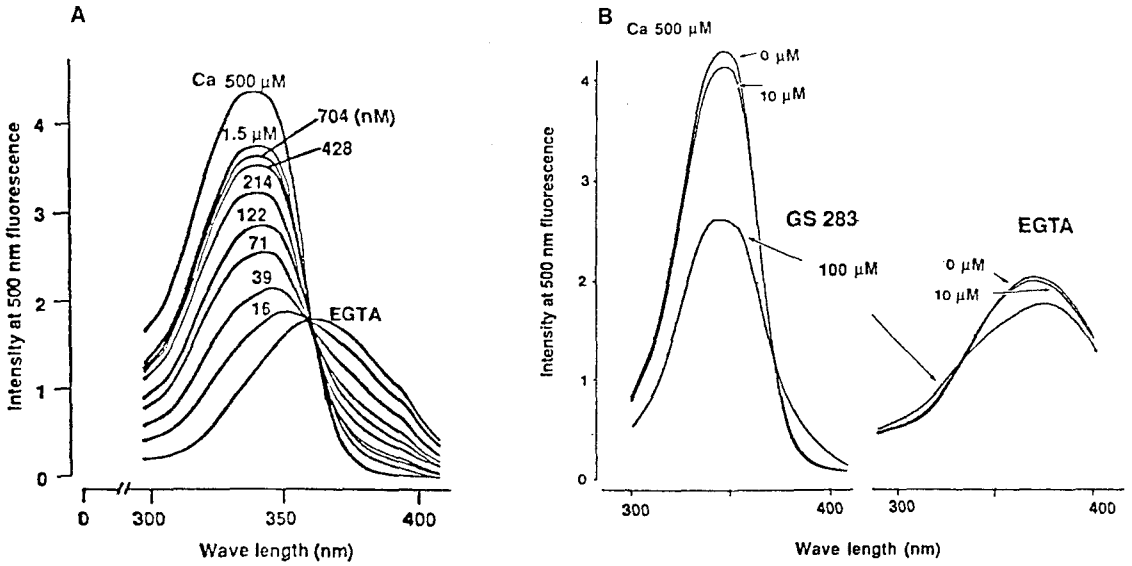

Fig. 4. Fluorospectral characteristics of fura 2-Ca^{2+} complex and effect of GS 283. Scanning excitation wave length was 300~ 400 nm and emission was 500 nm. Absoption peak was appeared at 340 and lowest at 380 nm. Concentrations of GS 283 used (10~ 30 μM) in muscle tension study, but not higher concentrations, have not interfere with the characteristics.

contraction concentration-dependently. As shown in Figure 2A and B, Ca^{2+}-dependent contraction was inhibited by the presence of GS 283. For example, 1 mM Ca^{2+} caused 60 ± 8.8 in control aorta (high K^+), whereas in the presence of GS 283 10 μM and 30 μM the same concentration of Ca^{2+} caused 40±18 and 17±7.5% over the control. On the other hand, in PE pretreated aorta, 1 mM Ca^{2+} caused 83±5.8% in control aorta, while in the presence of 30 μM GS 283 it was 14±7.1%, which was statistically significant ($p<0.01$).

Effect of GS 283 on changes in cytoplasmic Ca^{2+} concentration

Since GS 283 was known to inhibit the release of Ca^{2+} from internal store site, to see the effect of GS 283 on intracellular Ca^{2+} change, fura 2 technique was applied. Verapamil, typical Ca^{2+} blocker, decreased F340/380 ratio and muscle tension. In contrast, GS 283 increased rather decreased the fluorescence signal even though tension is decreased (Fig. 3). This may imply that GS 283 has interfere with fura 2-Ca^{2+} signal by itself or by affecting pyridine nucleotide metabolism.

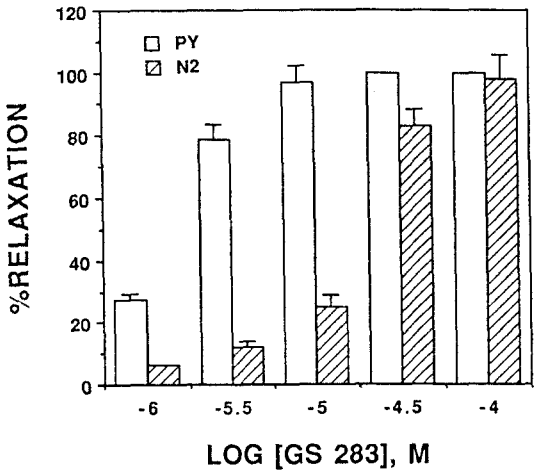

Fig. 5. Effect of GS 283 on inhibition of oxidative phosphorylation (N_2) and glycolytic metabolism (PY) in guinea pig tenea coli. Each point represents mean ± SEM of 3 experiments.

Therefore, to know if this interference was due to GS 283 by itself, fura 2-Ca^{2+} fluorescence was ana-

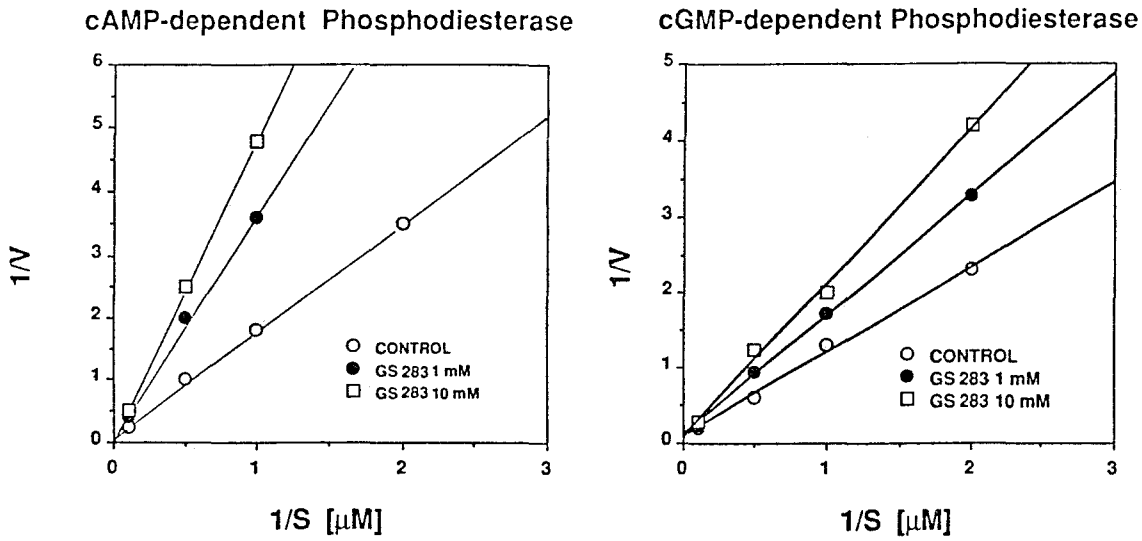

Fig. 6. Cyclic nucleotide-dependent phosphodiesterase inhibitory action of GS 283 from rat brain homogenates. Note that inhibitory concentration was relatevely higher than those used in muscle relaxation.

lyzed from 300 to 400 nm (Fig. 4B) Fura-2 characteristics are depicted in Figure 4A. The vertical line represents fluorescence intensity at 500 nm and horizontal line indicates scanning wave length. Up to 10 μM GS 283 there was no change in the fluorescence with control, but at high concentration (100 μM), the fluorescence curve was depressed (Fig. 4B). Therefore it may inhibit glycolytic metabolism reaction or oxidative phosphorylation reaction.

Effect of metabolic inhibition on GS 283 induced relaxation

To see these possibilities, guinea pig tenea coli strips were tested. As shown in Figure 5, although GS 283 had inhibited both glycolytic metabolism and oxidative phosphorylation-induced contractions, it had stronger inhibitory activity in oxidative phosphorylation reaction rather than glycolytic metabolism.

Cyclic nucleotide inhibitory action of GS 283

As shown in Figure 6, GS 283 concentration dependently inhibited the cyclic nucleotide phosphodiesterase from the rat brain. However, the concentration that inhibited was too higher than effective inhibitory concentration of vascular or non vascular smooth muscle with corresponding Ki values were 2.5 and 6.7 mM for cAMP-and cGMP-dependent phodphodiesterases, respectively.

DISCUSSION

Since GS 283 had been reported to possess Ca^{2+} antagonistic action in rat and guinea pig tracheal smooth muscle, in the present study, pharmacological characterization of GS 283 has been addressed using other smooth muscles such as aorta. If any compounds has Ca^{2+} channel blocking action, one would think that this chemical may has direct blocking action on the calcium channel or it may be the result of secondary effect of activation of some regulatory mechanism affecting calcium channel activity. In rat thoracic aorta, GS 283 concentration dependently relaxed α_1 agonist-induced contraction as well as depolarization-induced contraction, indicating that there is a possibility of possessing direct and/or indirect Ca^{2+} antagonistic action of GS 283 on vascular smooth muscle. Because PE utilized Ca^{2+}

from internal stores as well as external sites, while high K^+ only utilizes external Ca^{2+}, if GS 283 has stronger on high K^+ than PE-induced contraction, main mechanism of GS 283 would be direct Ca^{2+} channel blocking action, but the result is that when compared IC_{50} values of GS 283 on two different agonist-induced contraction, GS 283 had stronger inhibitory action on PE- than high K^+ induced contraction. This finding imply that action of GS 283, besides direct Ca^{2+} antagonistic action, decrease Ca^{2+} sensitivity or have other mechanism(s) to regulate Ca^{2+} influx. The selectivity shown by GS 283 on PE-induced contraction in normal Krebs' solution was checked in the experiments carried out in a nominally Ca^{2+} -free medium. Under these conditions, PE-induced phasic contraction was abolished by the presence of GS 283 concentration dependently, indicating that GS 283 inhibit Ca^{2+} release from the internal store site by α-adrenoceptor activation. GS 283 can enter into the cytoplasm, where it inhibited Ca^{2+} release by agonist-stimulation already reported in trachealis (Chang *et al.*, 1993). To understand better the mechanism of action of GS 283 on calcium inhibitory and/or lowering calcium sensitivity, fura-2 experiment was performed. GS 283 increased rather decreased the fluorescence signal even though tension is decreased (Fig. 3), while verapamil, typical calcium antagonist, caused relaxation and decrease fura 2 signal. Smooth muscle tissue has pyridine nucleotides that fluoresce in increasing portion to $[Ca^{2+}]_{cyt}$ (Ozaki *et al.*, 1987, 1988) and interfere with fura 2-Ca^{2+} signals (Sato *et al.*, 1988). This fluorescence, at both 340 nm and 380 nm, increased during muscle contraction by approximately 10% of the total fura-2 fluorescence. In contrast to this, the fluorescence due to fura 2-Ca^{2+} changed in the opposite directions as $[Ca^{2+}]_{cyt}$ changed (Grynkiewicz *et al.*, 1985). This may imply that GS 283 has interfere with fura 2-Ca^{2+} signal by itself or by affecting endogenous pyridine nucleotide metabolism.

Therefore, to know if this interference was due to GS 283 by itself, fura 2-Ca^{2+} fluorescence was analyzed from 300 to 400 nm. Up to 10 μM GS 283 there was no change in the fluorescence with control, but at high concentration (100 μM), the fluorescence curve was depressed (Fig. 4). Therefore there is a possibility that GS 283 may inhibit glycolytic metabolism or oxidative phosphorylation reaction. When smooth muscles are depolarized by an excess K^+ or stimulated by receptor agonists, force is generated with an increase in $[Ca^{2+}]_{cyt}$. The rate of oxygen consumption of smooth muscle increases with the isometric force development. The rate of lactate production also correlates with the isometric force. These results suggest that the contractile response of smooth muscle is dependent on both oxidative phosphorylation and glycolytic metabolism.

To know if GS 283 has an effect to inhibit mitochondrial function, guinea pig tenea coli was isolated. Oxidative phosphorylation inhibition and glycolytic metabolism inhibition was addressed by employing that N_2 gas rather than mixed gas (95% O_2-5% CO_2) and deoxy glucose rather than glucose. When tenea coli was activated with high K^+, rapid contraction of the muscle tension followed by sustained and steady contraction. From this condition, bathing solution was changed with glucose free solution with 2-deoxy glucose or N_2 gas was replaced. In glucose free solution, pyruvic acid restore the contraction even it was lower than the original contraction (data not shown). In this preparation, GS 283 was added cumulatively. GS 283 concentration dependently inhibited the sustained contraction. Likewise, GS 283 also inhibited the high glucose induced contraction in N_2 replaced medium. The inhibitory potency of the GS 283 on glycolytic reaction was less than that of oxidative phosphorylation. These results suggest that GS 283 has weak metabolic inhibitory action. Since there is a report that in intestinal smooth muscle when oxidative phosphorylation was inhibited by replacement of O_2 with N_2, the resting pyridine nucleotide (reduced form) fluorescence increased even though muscle tension decreased (Ozaki *et al.*, 1988). No changes were observed in reduced pyridine nucleotide when glycolysis was inhibited by substitution of external glucose with oxaloacetate, pyruvate, or β-hydroxybutylate. These result suggest that GS 283 may inhibit oxidative phosphorylation so that it caused relaxation of muscle tension but increased fluorescence. Since changes of reduced pyridine nucleotide and oxidized flavoprotein were not checked in the present experiment, further study is needed to clarify the inhibitory action of GS 283 on mito-

chondrial function. However, there are many reports that the redox state of pyridine nucleotide has been utilized to measure fluorometrically in variety of isolated tissues or cells (Katz *et al.*, 1987; koretsky *et al.*, 1987) including smooth muscles (Connor *et al.*, 1976; Linke and Betz, 1977). Oxidized flavoproteins are also fluorescent substances which are located specifically in the inner membrane of mitochondria. Fluorometric measurement of flavoprotein have also been applied to isolated tissues (Paddle, 1985; Hassinen and Hiltunen, 1975; Hiltunene *et al.*, 1978). From these findings the mechanism of action of GS 283 involves at least indirect regulation of Ca^{2+} movement as well as direct inhibitory action of calcium channels. To characterize the indirect regulation of Ca^{2+} movement by GS 283, involvement of cyclic nucleotide has been carried out. As pointed out in the earlier section, THI classes possess β-agonistic action and PDE enzyme inhibitory action (Kukovetz and Poch, 1970; Van Inwegen *et al.*, 1989). To examine the PDE inhibitory action of GS 283, rat brain tissue was utilized to get the PDE enzyme. Since brain tissue is easy to get large quantities of PDE enzymes than vascular smooth muscle. In this preparations, enzymes are not purified, because the purpose of the examination was to see if GS 283 has inhibitory action on the PDE enzymes. The crude preparation was used as enzyme source. GS 283 had inhibitory action on both type of cyclic nucleotide-dependent PDE. Their relative Ki values were 2.5 and 6. 7 mM for cAMP- and cGMP-dependent PDE enzymes, respectively. D'Ocon *et al.* (1989, 1991) and Avorra *et al.* (1992) reported that relaxant effect of glaucine, a THI analog, has an α_1-adrenoceptor antagonist, calcium entry blocker and as a selective inhibitor of the rolipram-sensitive cAMP-PDE, type IV PDE. There is increasing evidence for selective forms of PDE being involved in controlling different biological processes and thus selective PDE inhibition can lead to a variety of discrete pharmacological responses (Weishaar *et al.*, 1985; Beave, 1988). In this regards, further study is needed if GS 283 has which type of PDE inhibitory action and if this agent can be possibly used in clinical applications such as congestive heart failure, hypertension and asthma through toxicological experiment. In conclusion, it can be made that GS 283 has direct Ca^{2+} channel blocking action, weak metabolic inhibitory action, and PDE inhibitory action which may contribute, in part, to calcium antagonistic action.

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=국문초록=

GS 283의 평활근 억제 작용기전

경상대학교 의과대학 약리학교실

김시환 · 이영수 · 정원석 · 장기철

Tetrahydroisoquinoline 유도체인 GS 283의 약리학적 특성을 흰쥐 흉부대동맥, 기니픽 결장띠 및 토끼 장간막 동맥 및 흰쥐 뇌를 사용하여 조사하였다. 혈관 평활근에서 GS 283은 고 K^+에 의한 수축을 농도 의존적으로 억제하여 Ca^{2+} 길항작용을 보였다. 또한 α_1- 수용체 자극에 의한 수축도 억제하였다. GS 283의 혈관이완 작용은 propranolol 영향을 받지 않으므로 β- 수용체 자극작용에 의한 것이 아니었다. 세포내 칼슘이온과 근장력 변화를 동시에 측정하였을 때 GS 283의 억제효과는 조직내 형광의 증가를 수반했다. 이 증가는 fura 2 형광에 의한것이 아니라 내인성 pyridine nucleotide에 의한 것이며 이는 GS 283이 미토콘드리아 기능을 억제하는 효과가 있음을 시사했다. 흰쥐 뇌의 cAMP와 cGMP 의존성 phosphodiesterase에 대한 GS 283의 K_i값은 2.5와 6.7 mM이었다. 이상의 결과에서 GS 283의 약리 작용은 Ca^{2+} 길항작용, α_1- 수용체억제 작용 및 cyclic nucleotide 의존성 phosphodiesterase 억제 등 다양한 작용이 있으며 평활근 수축 억제에 대한 GS 283 작용에는 Ca^{2+} 길항이 가장 중요한 요인이 될 것으로 생각된다.