Korean J. of Pharmacology
Vol. 32, No. 3, pp. 373~380, 1996

Calcium Channel Blocking and Phosphodiesterase Inhibitory Action of GS386, a Dihydroisoquinoline Derivative, in Isolated Rat Trachea

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ABSTRACT

Recently we reported that GS 386, 1-(4'-methoxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline, inhibited amplitude of the Ca^{2+} current by reducing the probability of Ca^{2+} channel opening without changing channel kinetics in isolated rabbit atrial myocyte. In the present study, further investigation of the mechanism of action of GS 386 was performed using isolated rat trachealis. GS 386 concentration-dependently relaxed rat trachealis contracted by carbachol (0.3 μM) and high K^+ (65.4 mM) with IC_{50} 5.24 and 5.67 μM, respectively. Verapamil inhibited more effectively the high K^+-contracted tissues than those with carbachol in the rat trachealis muscle. In Ca^{2+}-free media, Ca^{2+}-induced contraction was inhibited by GS 386. Furthermore, high concentration of GS 386 (100 μM) but not verapamil, attenuated a phasic contraction induced not only by carbachol but also caffeine, indicating that GS 386 can enter into the cytoplasm where it may exert secondary actions on internal sites of the muscle, such as sarcoplasmic reticulum. Moreover, GS 386 showed verapamil-resistant component of relaxation and increased cAMP levels in rat trachal smooth muscle. These results suggest that the mechanism of action of GS 386 attributes to not only Ca^{2+} antagonistic action but also weak phosphodiesterase inhibitory action.

Key Words: Calcium channel, Rat trachea, Relaxation, Phosphodiesterase

INTRODUCTION

Calcium antagonists, capable of inhibiting transmembrane influx of extracellular Ca^{2+} through specific Ca^{2+} channels, are useful drugs in the treatment of hypertension, angina pectoris, cardiac arrhythmia, and bronchial asthma (Ahmed *et al.*, 1985; Boner *et al.*, 1987; Conti *et al.*, 1985). Currently, three distinct classes of Ca^{2+} entry blockers of the L-type Ca^{2+} channel are in clinical use, namely, the dihydropyridines, the phenylalkylamines, and the benzothiazepines (Fleckenstein, 1977). In addition, some new structural classes of compounds have recently been reported, some of which are related to the isoquinoline pharmacophore (King *et al.*, 1988; Pierrer *et al.*, 1991, Chang *et al.*, 1992, 1993, 1994). Recently we reported that GS 386 inhibited amplitude of the Ca^{2+} current by reducing the probability of Ca^{2+} channel opening without changing channel kinetics in isolated rabbit atrial myocyte. However effects of GS 386 on bronchial smooth muscle have not been reported. The aim of the present study was to further characterize the mechanism of action of GS 386 using isolated rat trachealis. We compared effects of GS 386 with those of typical calcium channel blocker, verapamil. Because several clinical studies have demonstrated the bronchodilator effects of inhaled verapamil in asthmatic patients after challenge with methacholine, histamine, acetylcholine and antigen

(Ahmed *et al.*, 1985; Boner *et al.*, 1987; Popa *et al.*, 1984). In Ca^{2+}-free media, GS 386 but not verapamil attenuated a phasic contraction not only by carbachol but also by caffeine. Furthermore, GS 386 showed verapamil-resistant component of relaxation, indicating that the mechanism of GS 386 is different from that of typical Ca^{2+} channel blocker, verapamil. We found that GS 386 has weak phosphodiesterase inhibitory action which may be responsible for the mechanism of action of GS 386 along with Ca^{2+} antagonistic action.

METHODS AND MATERIALS

General

The experiments were carried out on thracheas from Sprague-Dawley rats of either sex, weighing 300 to 350 g. Animals were anesthetized with ketamine (75 mg/kg) and xylazine (15 mg/kg) administered intramusculary. The trachea was removed and prepared according to Chang *et al* (1993). The trachea was cut into 4 rings of segment and tissues were set up at 37℃ in a 5 ml muscle chamber, supplied with 95% O_2-5% CO_2 and normal Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25, glucose 11 and EDTA 0.03. The Ca^{2+}-free solution was the same as the normal Krebs-Ringer bicarbonate solution except 1 mM EGTA was used instead of 2.5 mM $CaCl_2$. Isometric tension was recorded on a Grass physiograph (model 7E, Grass Instruments, Quincy, Mass.) via a force transducer (FT-03). The initial tension was adjusted to 1g, followed by equilibration for more than 90 min and washing at 20 min intervals. Cumulative concentration-response curves, with 0.5 log unit concentration intervals, were utilized to quantitate the sensitivity of the tissue to drugs.

Measurement of tracheal relaxation

For measuring tracheal relaxation, contractions were obtained by adding carbachol (0.1~1 μM) in Krebs-Ringer bicarbonate solutions or by changing the bath fluid with 65.4 mM potassium, which was made by substituting equimolar potassium concentrations for sodium from the Krebs-Ringer bicarbonate solutions. After reaching the plateau of contraction, test substances were added. To assess the inhibitory effect of GS 386 or verapamil against carbachol- or KCl-induced contraction, the tissues were exposed to GS 386 or verapamil for 10 min before adding carbachol or KCl, and cumulative concentration-response curves were obtained by a stepwise increase in concentration of carbachol (0.1~100 μM), or KCl (10~180 mM). All experiments were carried out in the presence of indomethacin (1 μM).

Calcium-induced contraction in Ca^{2+}-free media

In Ca^{2+}-induced contraction experiments, the bathing fluid was replaced by a Ca^{2+}-free salt solution for 30 min. After this period, carbachol (1 μM) or high K^+ (65.4 mM) was added. The contractile effects of calcium were studied in tracheal rings by addition of calcium to obtain the desired concentrations, and the cumulative Ca^{2+} concentration-response curves were constructed in the presence or absence of GS 386 or verapamil.

Assessment of inhibitory action of GS 386 on intracellular Ca^{2+} release by carbachol in Ca^{2+}-free media

Effects of GS 386 on intracellular Ca^{2+} release was evaluated as reported previously (Chang *et al.*, 1994, Anireddy, 1991). After recording of the response to the initial carbachol challenge, the tissues were washed until a stable resting tension was reached and the Krebs-Ringer solution was exchanged for a high K^+, Ca^{2+}-free solution (point a). After the tissues were equilibrated for 30 min with high K^+, Ca^{2+}-free solution, a high K^+, Ca^{2+}-containing solution was substituted (point b) in order to replenish the Ca^{2+} in the internal storage sites, in which the contractile effect plateaued, then the high K^+, Ca^{2+}-free solution was returned to the bath (point c) resulting in complete relaxation where carbachol was added in the presence or absence of GS386. GS386 was added simultaneously with the high K^+, Ca^{2+}-free solution at the point c. The results were represented as per centage of the maximum contraction induced by carbachol in the absence of GS 386.

Assessment of inhibitory action of GS 386 on intracellular Ca^{2+} release by caffeine in Ca^{2+}-free media

To know the effect of GS 386 on caffeine-induced Ca^{2+} release in Ca^{2+}-free media, caffeine-induced contractions were recorded, in the presence or absence of GS 386, by replacing the Krebs-Ringer solution with a Ca^{2+}-free solution containing 10 mM caffeine at 25℃. The results were also represented as per centage of the maximum contraction induced by caffeine in the absence of GS 386.

Preparation of phosphodiesterase from rat trachealis

The tracheas were immediately excised and rinsed in ice-cold 50 mM Tris-HCl buffer (pH 7.5) containing 3.75 mM 2-mercaptoethanol (extraction buffer). Then the tissues were homogenized in three to five volume of the same solution, using an Ultra-turrax T-25 (IKA-Labortechnik, Germany). The homogenates were centrifuged at 15,000×g for 60 min, and the supernatants (crude extract) were used as the enzyme source for phosphodiesterase (PDE).

Assay for phosphodiestrase activity

The assay for PDE was described previously by Chang *et al* (1992). In brief, the standard mixture contained, in a final volume of 0.1 ml, Tris-HCl buffer (pH 7.5), 5 mM; EDTA. 0.25 mM; $MgCl_2$, 2 mM; 5'-nucleotidase (*ophiaphagus hannah* snake venom), 10~30 mg; [2,8-^{3}H]-labeled cyclic AMP, 0.1 μM, containing about 1. 10^5 cpm, and the indicated amounts of crude supernatant of tracheal smooth muscle. 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 by using anion exchange (AG 1×2) chromatography.

Measurement of cAMP content

Cyclic AMP contents was measured by radioimmunoassay using a [^{125}I]-labelled cAMP kit (Amersham U.K).

Drugs

Carbachol, verapamil, indomethacin, ophiaphagus hannah snake venom and caffeine were purchased from Sigma Co. Ltd. [2,8-^{3}H]-labeled cyclic AMP and [^{125}I]-labelled cAMP kit were obtained from Amersham (U.K).

RESULTS

Effects of GS 386 on carbachol and KCl-induced contraction

In quiescent tracheal ring preparations GS386 (1~100 μM) evoked no mechanical response (n =4, data not shown). Verapamil and GS 386 inhibited muscle contraction induced by carbachol and high K^+ in a concentration-dependent manner in rat trachea. However, verapamil significantly antagonized the contraction induced by high K^+ more potently than that due to carbachol in terms of IC_{50} values (Table 1). GS 386, concentration-dependently relaxed the tracheal smooth muscle precontracted with carbachol (pD_2 value: 4.69±0.05, Fig. 1). The relaxation to GS 386 was not affected by indomethacin. Carbachol evoked concentration-response curve in rat trachealis caused a significant shift to the right by GS 386 (Fig. 2). Higher concentrations, but not lower concentrations, of GS 386 reduced the maximal response to carbachol (Fig. 2).

Effects of GS 386 on Ca^{2+}-induced contraction in Ca^{2+}-free media

To see more directly the antagonizing action of verapamil and GS 386 on Ca^{2+} influx, we

Table 1. Comparison of pIC_{50} values of GS 386 and verapamil on carbachol and KCl-induced contraction in rat trachealis

Compounds	Carbachol	KCl
	pIC_{50}(M)	
Verapamil	4.76±0.26	6.33±0.12
GS 386	4.69±0.05	4.94±0.15

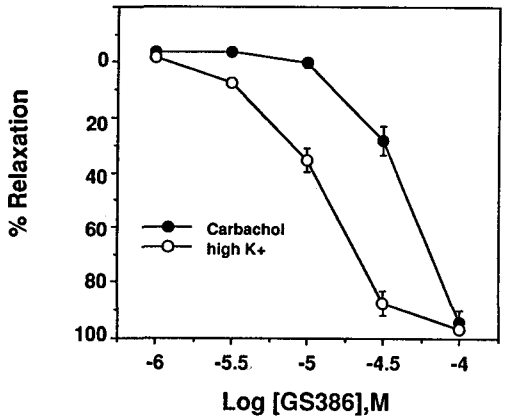

Fig. 1. Concentratin-response curve of GS 386 on carbachol (●) and high K^+(○)-contrated rattracheal smooth muscle.

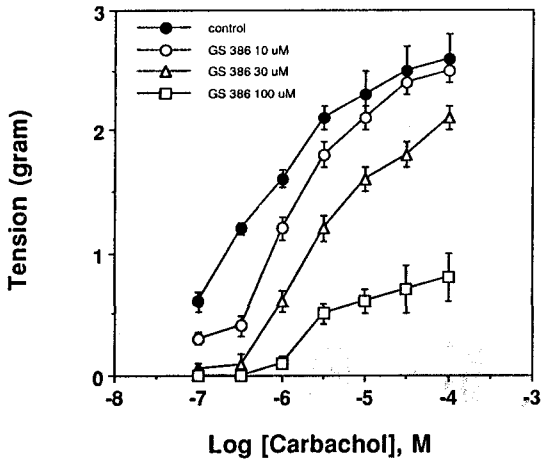

Fig. 2. Inhibitory effect of GS 386 on carbachol-induced contraction in rat tracheal smooth muscle.

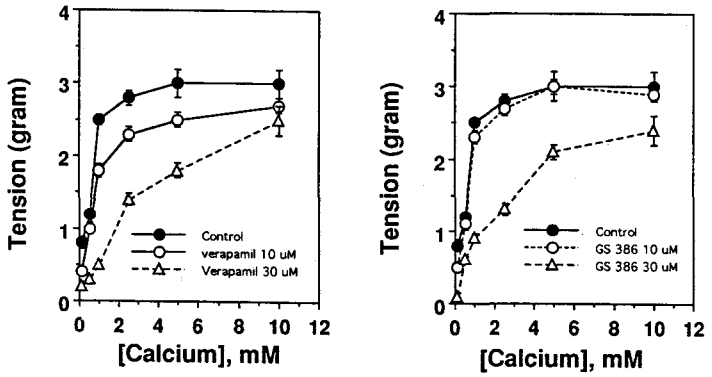

Fig. 3. Effects of verapamil and GS 386 on calcium-induced contraction in calcium-free media.

tested Ca^{2+}-induced contraction in Ca^{2+}-free media. In rat trachea, addition of carbachol caused a transient contraction (0.65±0.04 g) in Ca^{2+}-free media, where cumulative addition of Ca^{2+} (0.1~10 mM) to the external media increased the contraction. Pretreatment of either GS 386 or verapamil inhibited Ca^{2+}-induced contraction (Fig. 3).

Effects of GS 386 on verapamil-resistant component relaxation

Verapamil (Fig. 4), the concentration in which high K^+-induced tone was completely relax, caused a partial inhibition of contraction induced by carbachol. The residual contraction after application of the verapamil (verapamil-insensitive part) was inhibited by cumulative addition of GS 386. The pD_2 value and the maximal response to GS 386 in the condition were not different from those obtained in Fig. 1. (data not shown). Contractions induced by 65.4 mM KCl were totally inhibited by verapamil (Fig. 4). After the tension reached the basal level by verapamil, the trachea was again contracted half-maximally with carbachol and then the relaxation to GS 386 was investigated. In these conditions, GS 386 evoked relaxations

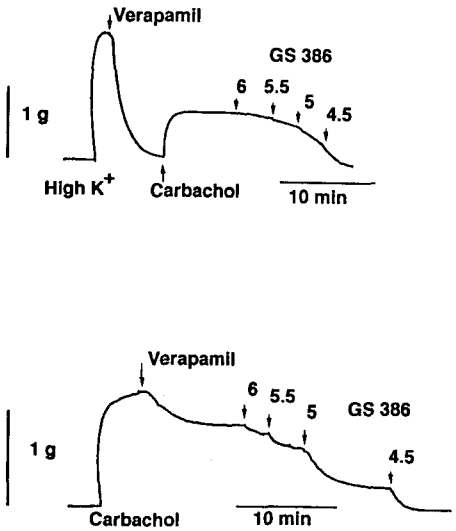

Fig. 4. Effects of GS 386 on verapamil-insensitive component of relaxation in rat tracheal smooth muscle.

with similar pD_2 value to those obtained with carbachol in Krebs solution, while the response to 10 μM GS 386 was different in the two groups. The relaxation was more pronounced in tracheas precontracted with carbachol in the 65.4 mM KCl solution containing verapamil than in those precontracted with 65.4 mM KCl.

Effects of GS 386 on intracellular Ca^{2+} release by carbachol in Ca^{2+}-free media

As shown in Fig. 5, carbachol evoked transient contractions in the Ca^{2+}-free solution. The magnitude of contraction was 58.3±2.3% of the 65.4 mM KCl-induced contraction. GS 386 inhibited carbachol-induced phasic contraction. After washing out the compound, the same protocol was applied without the probe, in which the phasic contraction upon carbachol stimulation was completely restored. However the same concentration of verapamil had no effect on the phasic contraction.

Effects of GS 386 on intracellular Ca^{2+} release by caffeine in Ca^{2+}-free media

Caffeine induced transient contractions in Ca^{2+}-free solution (Fig. 5). The magnitude of the contraction was 42.5±6.3% of the 65.4 mM KCl-induced contraction. GS 386 significantly reduced caffeine-induced initial rapid contraction. However, inhibitory effects of GS 386 on release of intracellulary stored Ca^{2+} by caffeine were less susceptible than those of carbachol.

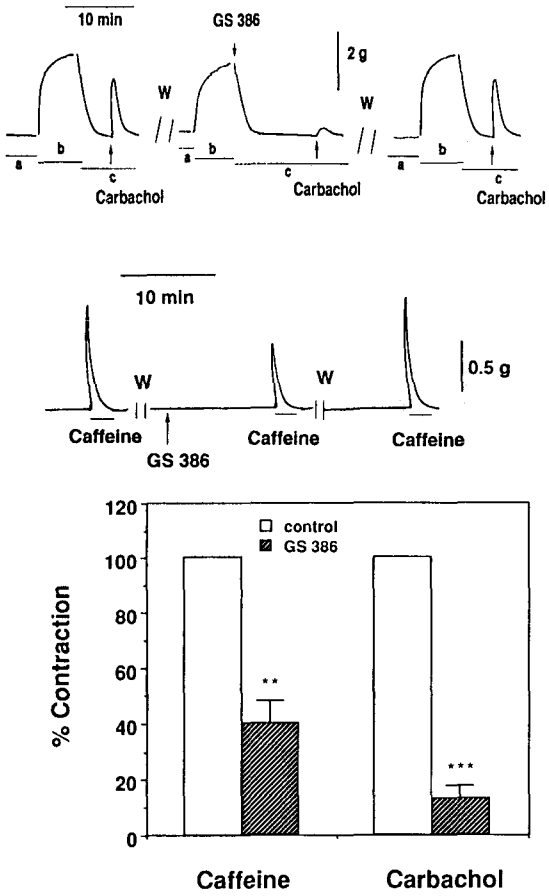

Fig. 5. Effects of GS 386 on intracellular calcium release evoked either by carbachol or caffeine in calcium-free media. ** represent $P<0.05$, ***$P<0.01$.

Effects of GS 386 on cAMP-dependent phosphodiesterase

In rat tracheal homogenates as crude enzyme source, only 100 μM GS 386 increased cAMP levels about 5 fold over the control (Table 2). At lower concentrations it did not affect cAMP levels.

Table 2. Effects of GS 386 on cyclic AMP in isolated tracheal tissues

Treatment	cAMP (pmol/g tissue)	n
Control(noe)	34± 5	3
GS 386(1 μM)	36± 3	4
GS 386(3 μM)	47± 8	3
GS 386(10 μM)	132±11	3
GS 386(30 μM)	197±17	4
GS 386(100 μM)	332±58	3

DISCUSSION

In the present study, we assayed the action of GS 386 on contractions of the rat trachealis which are mediated by: ① Ca^{2+}-entry through potential-operated channels (KCl-induced contraction); ② Ca^{2+}-entry induced by muscarinic-receptor activation (carbachol-induced contraction); ③ release of intracellular Ca^{2+} (carbachol or caffeine in Ca^{2+}-free media). Recently, new structural classes of compounds related to the benzylisoquinoline structure have been reported to be Ca^{2+} channel blockers (King *et al.*, 1988; Triggle *et al.*, 1989; Pierre *et al.*, 1991; Chang *et al.*, 1993, 1994). GS 386 induced concentration-dependent relaxation in rat trachealis precontracted with carbachol and KCl. And GS 386 shifted the concentration-response curves for carbachol to the right. Since the maximal responses to the carbachol were reduced by higher concentrations of GS 386, it seems unlikely that GS 386 acts as a competitive antagonist at muscarinic receptor. Involvement of prostagladins in the relaxation response to GS 386 was excluded since indomethacin, cyclooxygenase inhibitor, did not affect the relaxation. Inhibition of cellular Ca^{2+} uptake induced by depolarization is usually well correlated with the relaxing or antispasmodic effects of many kinds of drugs acting on cardiac or smooth muscle cells (Godfraind, 1981; Hof *et al.*, 1984; Cheng and Townley, 1983). The mechanism of potassium-induced excitation-contraction coupling in smooth muscle involves an increased Ca^{2+} influx through voltage-dependent channels (Bolton, 1979), which is highly sensitive to calcium entry blockers. Verapamil, a typical calcium antagonist, inhibited contractions induced by KCl more strongly ($P<0.001$) than those with carbachol.

Evidence indicates that the release of Ca^{2+} from the sarcoplasmic reticulum and its subsequent refilling are essential links in excitation-contraction coupling (Chen and van Breemen, 1992; Moore *et al.*, 1993). Caffeine induced transient contractions in Ca^{2+}-free solution by releasing Ca^{2+} from intracellular Ca^{2+} store site, sarcoplasmic reticulum (Endo, 1977; Dohi *et al.*, 1990). Recently it has been reported that in tracheal smooth muscle, acetylcholine- and caffeine-releasable Ca^{2+} stores functionally ovelap, however, these stores differ in the mechanisms by which they are refilled (Liu and Farley, 1996). Caffeine-sensitive store is filled through a cyclopiazonic acid -and verapamil- insenstive pathatway. In contrast, acetylcholine-sensitive store is affected not only by cyclopiazonic acid- and verapamil-sensitive mechanism but also cyclopiazonic acid- and verapamil-insensitive pathway (Liu and Farley, 1996). We previously reported that in trachea muscle it is prerequisite that enough Ca^{2+} is being stored before the evoking of contraction by cholinergic stimulation in the Ca^{2+}-free media (Chang *et al.*, 1993). The fact that GS 386 did inhibit the contraction induced not only by KCl but also caffeine and carbachol in Ca^{2+}-free media, indicates that GS 386 may have influence the component of contraction which depends on extracellular as well as intracellular Ca^{2+}. The compound might inhibit Ca^{2+} movement through Ca^{2+} channels in plasmalemma, and sarcoplasmic reticulum. Indeed, GS 386 effectively reduced Ca^{2+} current by reducing the probability of Ca^{2+} channel opening without changing channel knietics in rabbit isolated atrial myocytes (Chang *et al.*, 1994). In the present study, the contraction induced by 65.4 mM K^+ was completely inhibited by verapamil, indicating that the contraction depends on Ca^{2+} influx through verapamil-sensitive (voltage-dependent) Ca^{2+} channels. GS 386 evoked less pronounced relaxations in trachea precontracted with carbachol than those precontracted with KCl. This finding is consistant with the result obtained in rat aorta in

which KCl-induced contractions by GS 386 more strongly inhibited than that induced by phenylephrine (Chang *et al.*, 1994). GS 386 must have a greater inhibitory effect against carbachol-activated, verapamil-insensitive Ca^{2+} channels than against verapamil-sensitive channels since the carbachol-induced contraction in the high KCl-solution containing verapamil depends on Ca^{2+} influx through carbachol-activated, but not verapamil-sensitive Ca^{2+} channels. It seems unlikely that GS 386 affect Ca^{2+} sensitivity, since when tested GS 386 with α-toxin skinned rat mesentery artery, it have no effect on contractile machinery (unpublished observation). Because many benzylisoquinoline derivatives are reported to have cyclic nucleotide-dependent PDE inhibitory action, therefore, we investigated as to whether GS 386 has cyclic nucleotide-dependent phosphodiesterase inhibitory action. In rat trachealis crude homogenates, only at high concentration of GS 386 increased cyclic AMP contents, indicating that it has a weak phosphodiesterase inhibitory action. Furthermore, analysis of IC_{50} (KCl)/IC_{50} (carbachol) ratios provides information on selectivity (Table 1) and indicates that GS 386 exhibits less selectivity in inhibition of the contractile response induced by KCl over that of carbachol. Even though in guinea pig tracheal smooth muscle cells, papaverine effectively inhibited the Ca^{2+} channel current in a way independent from the intracelluar cyclic AMP (Iguchi *et al.*, 1992), however, it remains to be elucidated further as to whether Ca^{2+} channel blocking action of GS 386 is independent of increment of cycic AMP in rat trachea. In conclusion, we investigated the effects of GS 386 by isometric tension recording using isolated rat trachealis. GS 386 concentration-dependently relaxed rat trachealis contracted by carbachol (0.3 μM) and high K^+ (65.4 mM). In Ca^{2+}-free media, not only Ca^{2+}-induced contraction but also caffeine- or carbachol-induced initial phasic contractions were inhibited by GS 386. GS 386 has weak phosphodieasterase inhibitory action in rat trachealis. Therefore, Ca^{2+} antagonistic action along with phosphodiesterase inhibitory action of GS 386 is responsible for the bronchodilating action in rat trachealis.

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=국문초록=

흰쥐 기관평활근에 대한 GS 386의 칼슘억제 및 포스포디에스테라제 억제 작용

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장기철 · 이회영 · 강영진 · 구의본

최근 본 연구실에서는 GS 386인 1-(4'-methoxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline이 적출된 토끼의 심방세포에서 Ca^{++} 채널의 운동성 변화없이 Ca^{++} 채널이 열릴 가능성을 줄임으로써 Ca^{++} 전류의 증폭을 억제한다고 보고하였다. 이번 연구에서는 적출된 쥐의 기관지를 사용하여 GS 386의 작용기전에 대해 연구하였다. GS386은 carbachol (0.3 μM)과 높은 농도의 K^+ (65.4 mM)에 의해 수축된 쥐의 기관지를 용량-의존적으로 이완시켰으며 이때 IC_{50}는 5.24와 5.67 μM이었다. verapamil은 carbachol에 의한 수축시 보다 높은 농도의 K^+에 의해 수축된 조직에 더욱 효과적으로 억제하였다. Ca^{++}이 없는 상태에서 Ca^{++}에 의한 수축은 GS386에 의해 억제되었다. 더욱이 높은 농도의 GS386(100 μM)은 verapamil과는 다르게 carbachol뿐만 아니라 caffeine에 의한 위상성 수축을 억제 시키므로 GS386은 세포질내로 들어가 sarcoplasmic retuculum과 같은 근육 내부에 2차적인 영향을 나타내었다. 더군다나 GS386은 verapamil에 의해 영향을 받지않는 (verapamil-insensitive component)이완을 보였고 쥐 기관지의 평활근에서 cAMP의 양을 증가 시켰다. 이러한 결과는 GS386의 작용기전이 Ca^{++} 길항적인 작용 뿐만 아니라 posphodiesterase억제작용에 기인한다는 사실을 제시한다.