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Effects of the nitric oxide-donor, GEA 3175, on guinea-pig airways

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Abstract

This investigation characterized the smooth muscle relaxing effect of a novel nitric oxide (NO)-releasing substance, GEA 3175 (1,2,3,4-oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulfonyl]amino], hydroxide inner salt) on guinea-pig trachea. GEA 3175 caused a concentration-dependent relaxation of tracheal smooth muscle precontracted with acetylcholine. This effect was reversed by both okadaic acid, an inhibitor of serine/threonine-specific phosphatases, and iberiotoxin, an inhibitor of Ca^{2+}-activated K^+ channels. Furthermore, GEA 3175 had a relaxation potency similar to that of the commonly used NO-donor, *S*-nitroso-*N*-acetyl-penicillamine. On the contractile response provoked by electrical field stimulation, GEA 3175 induced a long-lasting relaxation which persisted even after repeated washing. The relaxing effect of GEA 3175 was associated with rises in guanosine 3′:5′-cyclic monophosphate (cGMP). In time course studies, cGMP continued to increase with incubation time after stimulation with GEA 3175 and there was a significant elevation of cGMP even after washing. In contrast, incubation with *S*-nitroso-*N*-acetyl-penicillamine caused a transient rise in cGMP. The present investigation showed that GEA 3175 evokes long-lasting effects on contractile responses and cGMP levels in guinea-pig trachea. Our results indicate that the relaxing effect of GEA 3175 occurs through a mechanism involving phosphatases and iberiotoxin-sensitive K^+ channels. © 1997 Elsevier Science B.V.

Keywords: K^+ channel, Ca^{2+}-activated; cGMP; Iberiotoxin; Nitric oxide (NO); Okadaic acid; Phosphatase; Smooth muscle

1. Introduction

Nitric oxide (NO) and NO-donors can inhibit contraction of airway smooth muscle both in vitro and in vivo (Jansen et al., 1992; Brown et al., 1994; Gaston et al., 1994). The effect of NO is thought to be mediated primarily through the activation of soluble guanylyl cyclase, leading to increased formation of guanosine 3′:5′-cyclic monophosphate (cGMP) (Ignarro, 1990). However, the exact mechanisms by which NO and cGMP relax smooth muscle are still unclear, but may involve decreased formation of inositol metabolites, reduced concentration of cytosolic Ca^{2+} and inhibition of contractile proteins. It has also been suggested that NO may regulate the activity of K^+ channels (Rapoport, 1986; Felbel et al., 1988; Blatter and Wier, 1994; Ellis and Conanan, 1994). Other smooth muscle relaxants such as β_2-adrenoceptor agonists, commonly used in the treatment of asthma, have been shown to mediate bronchodilation via iberiotoxin-sensitive K^+ channels (Jones et al., 1993). Compounds that liberate NO may in the future represent a new pharmacological tool for the management of asthma.

The novel NO-donor, GEA 3175 (1,2,3,4-Oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulfonyl]amino]-, hydroxide inner salt), is a 4-aryl-substituted oxatriazol derivative. It was recently shown that GEA 3175 and closely related substances are more potent than other, better-known NO-donors with regard to their effects on a variety of cellular functions (Moilanen et al., 1993; Corell et al., 1994).

The aim of the present study was to characterize the effect of GEA 3175 on guinea-pig tracheal preparations contracted with either acetylcholine or electrical field stimulation and to investigate possible mechanisms by which GEA 3175 relaxes airway smooth muscle.

2. Materials and methods

2.1. Tissue preparation

Male guinea pigs weighing 300–600 g were used. The animals were killed by a blow to the neck. The lungs,

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including the trachea and bronchi, were removed and placed in Krebs solution (122 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 15.4 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.5 mM glucose) and equilibrated with 95% O_2 and 5% CO_2 at 37°C, final pH 7.4. Throughout the experiments, the buffer solution contained propranolol (10^{-6} M) to exclude any relaxant effect mediated via β_2-adrenoceptors.

The tracheae were prepared and divided into ring segments, 3 mm in length. The segments were mounted on special holders (Grundström et al., 1981) and immersed in organ baths containing Krebs solution for measurement of isometric tension. The preparations were stretched to an initial tension of 1 g and then equilibrated for 1 h. During the equilibration period and throughout the experiments, the tracheal preparations were subjected to a washing procedure that entailed rinsing the preparations twice and then refilling the organ bath with fresh buffer.

2.2. Experimental protocol

Using tracheal segments precontracted with acetylcholine (5×10^{-7}–10^{-5} M), the relaxing property of the new lipid-soluble NO-donor, GEA 3175 (10^{-9}–10^{-5} M) (Fig. 1), were tested. In some experiments, the effect of GEA 3175 was compared with that of the water-soluble substance, *S*-nitroso-*N*-acetyl-penicillamine (10^{-9}–10^{-4} M). GEA 3175 was dissolved in dimethylsulfoxide (DMSO). All experiments were performed in the dark, because the drugs to be tested are sensitive to light. The protein phosphatase inhibitor, okadaic acid (10^{-8} M), and a selective blocker of the high conductance Ca^{2+}-activated K^+ channels, iberiotoxin (10^{-8}–10^{-7} M), were tested for their influence on the effect of GEA 3175. Okadaic acid was dissolved in DMSO, and iberiotoxin in distilled water. The final concentration of DMSO in the incubation medium did not exceed 0.1%.

To study the effect of GEA 3175 on cholinergic neurotransmission, the tracheal preparations were subjected to electrical field stimulation. Platinum electrodes were placed on each side of the tissue segments and a Grass S88 stimulator was used to produce monophasic square waves of 1 ms duration at 40 V and 5 Hz for 3 s every min. Earlier studies have shown that the contractile responses to this electrical field stimulation are completely abolished by atropine or tetrodotoxin, confirming a cholinergic nerve-mediated response (Grundström et al., 1981).

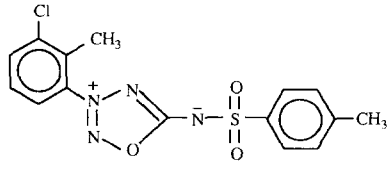

Fig. 1. The chemical structure of GEA 3175.

2.3. CGMP determination

Intracellular cGMP levels were measured in trachea after treatment with GEA 3175 (10^{-5} M) or *S*-nitroso-*N*-acetyl-penicillamine (10^{-5} M). Each trachea was divided into two pieces which were preincubated in oxygenated Krebs buffer for 30 min at 37°C in the dark. After washing, the preparations were incubated with the drugs for different periods of time, i.e., from 1 min up to 90 min, and then immediately frozen in acetone and dry ice. The trachea pieces were homogenized in ice-cold 10% trichloroacetic acid, centrifuged for 15 min at $4000 \times g$, and the supernatant was extracted with (4×3 ml) water-saturated diethylether. The water phase was frozen at −70°C, lyophilized and then dissolved in sodium acetate buffer (50 mM, pH 6.2). The cGMP content was measured with the radioimmunoassay described by Steiner et al. (1972) with antisera prepared in our laboratory (Axelsson et al., 1988).

2.4. Statistical analysis

The results are expressed as mean values ± S.E.M. The number in parentheses (n) in all legends refers to the number of animals. Statistical significance was tested by using Student's unpaired t-test. The pD_2 values were estimated from the concentration–response curves by means of non-linear regression analysis of the experimental data; the values are represented with 95% confidence limits.

2.5. Drugs

The drugs used were obtained from the following sources: GEA 3175, 1,2,3,4-oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[[(4-methylphenyl)sulfonyl]amino], hydroxide inner salt (GEA Pharmaceutical, Copenhagen, Denmark); guanosine 3′:5′-cyclic phosphoric acid, 2′-*O*-succinyl (^{125}I)iodotyrosine methyl ester (Du Pont, Belgium); iberiotoxin (Research Biochemicals International, Natick, MA, USA); okadaic acid (Calbiochem-Novabiochem, La Jolla, CA, USA); propranolol hydrochloride (ICI-Pharma, Macclesfield, UK); *S*-nitroso-*N*-acetyl-penicillamine (GEA Pharmaceutical).

3. Results

3.1. Inhibition of acetylcholine-induced contraction by GEA 3175

The new lipid-soluble NO-donor, GEA 3175, relaxed tracheal ring preparations precontracted with acetylcholine in a dose-dependent (10^{-9}–10^{-5} M) manner. The inhibitory effect of GEA 3175 was compared to that of the more conventional water-soluble NO-donor, *S*-nitroso-*N*-

acetyl-penicillamine (10^{-9}–10^{-4} M). Both substances abolished the contractions, with pD_2 values 6.55 (6.20–6.90) and 5.99 (5.42–6.56), respectively (Fig. 2). During the equilibration period, before addition of acetylcholine, the tracheal preparations reached a low and stable tension with no significant difference between the experimental groups of GEA 3175 (0.37 ± 0.09 g) and *S*-nitroso-*N*-acetyl-penicillamine (0.58 ± 0.19 g). The acetylcholine dose varied between 5×10^{-7} and 10^{-5} M to achieve about the same contractile responses when GEA 3175 and *S*-nitroso-*N*-acetyl-penicillamine were tested, 1.39 ± 0.19 g and 1.23 ± 0.31 g, respectively.

3.2. Influence of okadaic acid and iberiotoxin

To study possible intracellular mechanisms for the action of GEA 3175, we used a protein phosphatase inhibitor, okadaic acid, and a blocker of high conductance Ca^{2+}-activated K^+ channels, iberiotoxin. Okadaic acid is a toxin produced by dinoflagellates and acts as an inhibitor of serine/threonine-specific protein type 1 and 2A phosphatases (Hescheler et al., 1988; Ishihara et al., 1989). Addition of okadaic acid, 20 min prior to acetylcholine-induced contraction, inhibited the effect of GEA 3175 without affecting the basal tension. When the tracheal tissue was exposed to the highest concentration of GEA 3175 in the presence of okadaic acid (10^{-8} M), the reduction of the contractile response was only $55.1 \pm 7.1\%$ (Fig. 3).

Iberiotoxin is present in the venom of the scorpion, *Buthus tamulus*, and is known to inhibit selectively high conductance Ca^{2+}-activated K^+ channels (Galvez et al., 1990). The tracheal preparations were incubated with iberiotoxin (10^{-8} M or 10^{-7} M) 20 min before the addition of acetylcholine. The highest dose of iberiotoxin had a contractile effect on the tracheal preparations while the lower dose only slightly affected the basal tension in some cases. The inhibitory effect of GEA 3175 on acetylcholine-induced contractions was almost abolished by iberiotoxin. The reduction of the contractile response with the highest dose of GEA 3175 was only $41.8 \pm 5.4\%$ when preincubated with 10^{-8} M iberiotoxin and $19.5 \pm 7.2\%$ with 10^{-7} M iberiotoxin (Fig. 4).

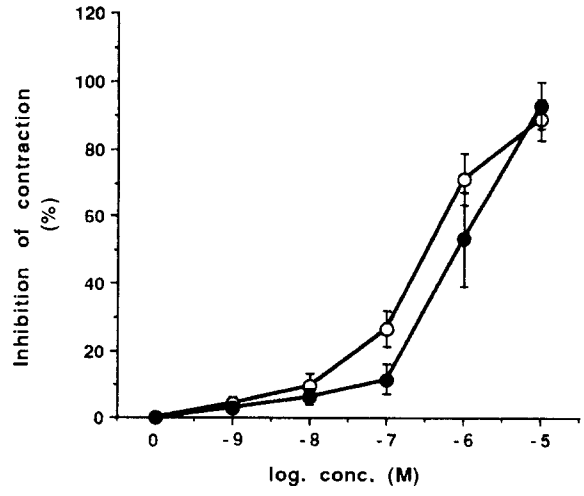

Fig. 2. Relaxation of guinea-pig trachea precontracted with acetylcholine. Symbols: (○) GEA 3175 $n = 11$, (●) S-nitroso-*N*-acetyl-penicillamine $n = 6$. The results are shown as means ± S.E.M.

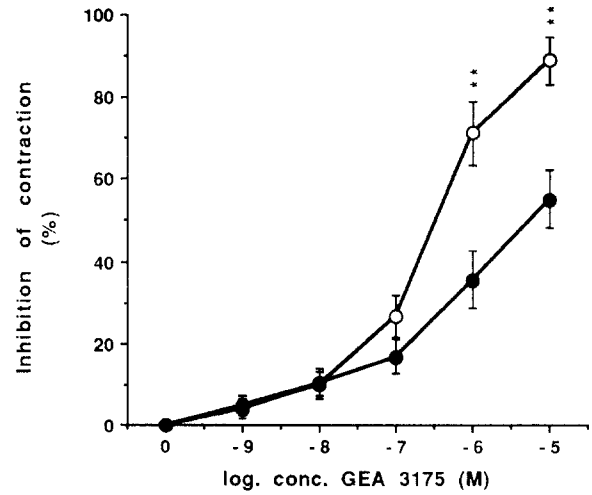

Fig. 3. Influence of okadaic acid on the inhibition by GEA 3175 of acetylcholine-induced contractions of guinea-pig trachea. Symbols: (○) GEA 3175 control $n = 11$; (●) in the presence of 10^{-8} M okadaic acid $n = 8$. The results are shown as means ± S.E.M. Significance is denoted ** $P < 0.01$.

3.3. Long-lasting effect of GEA 3175 on contractile response elicited by electrical field stimulation

Electrical field stimulation of tracheal ring preparations produced stable twitch contractions and the basal tension was 0.42 ± 0.05 g. GEA 3175 dose dependently inhibited

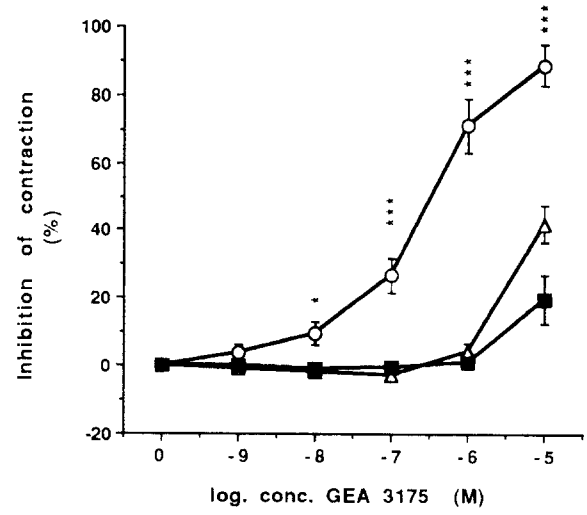

Fig. 4. Influence of iberiotoxin on the inhibitory action of GEA 3175 on acetylcholine-provoked contractions in guinea-pig trachea. Symbols: (○) GEA 3175 control, $n = 11$; (△) in the presence of 10^{-8} M iberiotoxin $n = 7$; (■) in the presence of 10^{-7} M iberiotoxin $n = 5$. Significance is calculated in relation to the iberiotoxin concentration of 10^{-8} M: * $P < 0.05$ and *** $P < 0.001$. The results are shown as means ± S.E.M.

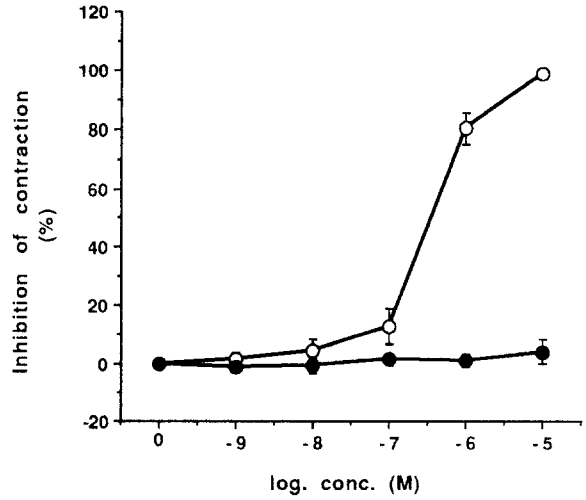

Fig. 5. Inhibition by GEA 3175 (○) of the contractile response elicited by electrical field stimulation in guinea-pig trachea $n = 20$. The solvent, DMSO (●), was tested in comparable concentrations (0.00001–0.1%). The results are shown as means ± S.E.M.

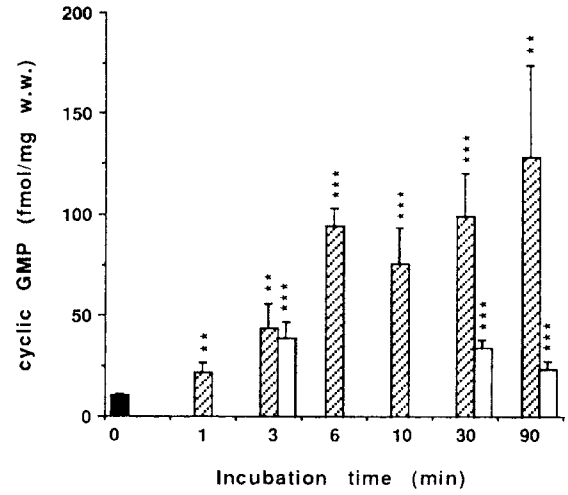

Fig. 7. Intracellular level of cGMP in guinea-pig trachea after incubation with GEA 3175 (hatched column) $n = 5$–6 or S-nitroso-*N*-acetyl-penicillamine (open column) $n = 5$ for different periods of time. The initial control level of cGMP is shown at time 0 (black column) $n = 11$ and significance was calculated versus this value: ** $P < 0.01$ and *** $P < 0.001$. The results are shown as means ± S.E.M.

the contractile response, pD_2 6.45 (6.34–6.57), and the highest concentration (10^{-5} M) completely blocked the contractions (Fig. 5). The medium in which GEA 3175 was dissolved, i.e., DMSO, did not affect the contractions. The block by GEA 3175 was long-lasting and in none of the experiments did the contractile response reappear before washing, although the preparations were subjected to continuous electrical field stimulation. Washing the trachea twice, 30 min after blocking, restored the contractile response in some experiments. The contractions reappeared gradually with some variation in time and sometimes repeated washing was needed. The contractile response could be completely inhibited for up to 2 h (Fig. 6). In an earlier study in our department, *S*-nitroso-*N*-acetyl-penicillamine caused a similar relaxation of the contractile response, but showed no long-lasting inhibitory effect (unpublished results). These experiments were performed under conditions slightly different from those used in the present investigation, i.e., electrical field stimulation was applied for 1 s every min and the incubation medium contained indomethacin.

3.4. Measurement of cGMP

The intracellular level of cGMP was measured by applying a radioimmunoassay technique to tracheal preparations incubated with GEA 3175 for 1, 3, 6, 10, 30, or 90 min. The level of cGMP was significantly elevated after 1 min and became more pronounced with increasing incubation time (Fig. 7). The solvent, DMSO, had no influence on the cGMP level. After incubation with 0.1% DMSO alone for 3 and 90 min, the cGMP levels were 7.0 ± 2.5 and 7.6 ± 3.3 fmol/mg w.w., respectively ($n = 5$), with as control value, 9.8 ± 1.0 fmol/mg w.w. ($n = 11$). Treating the preparations with *S*-nitroso-*N*-acetyl-penicillamine also resulted in an increase of cGMP: as with GEA 3175, a 3-min exposure to *S*-nitroso-*N*-acetyl-penicillamine led to a 4-fold increase in the cGMP level. However, incubation with *S*-nitroso-*N*-acetyl-penicillamine for longer periods, 30 or 90 min, did not further increase the amount of cGMP. After a 90-min exposure to the drugs, *S*-nitroso-*N*-acetyl-penicillamine increased the cGMP content 2-fold, while there was a 12-fold increase on incubation with GEA 3175.

Washing tracheal preparations after 30 min of exposure to GEA 3175 markedly decreased the amount of cGMP, although the level was still significantly high, and remained so even 60 min after washing; 14.6 ± 1.8 fmol/mg

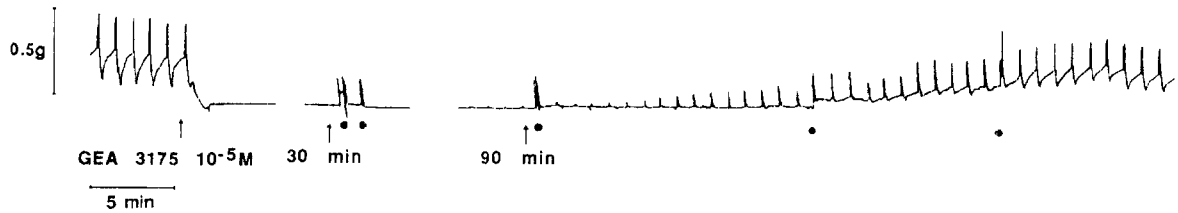

Fig. 6. Recording illustrating the long-lasting inhibitory effect of GEA 3175 on the contractile response provoked by electrical field stimulation in guinea-pig trachea. Dots indicate when washing was performed.

w.w. ($n = 6$; $P < 0.05$) as compared to the control 10.7 ± 0.7 fmol/mg w.w.

4. Discussion

The present investigation showed that GEA 3175 potently relaxes smooth muscle in guinea-pig airways. The compound was equally effective to inhibit acetylcholine- and electrical field stimulation-induced contractile responses in tracheal preparations. This may indicate that GEA 3175 affects mainly the smooth muscle cells and does not inhibit the release of cholinergic neurotransmitters.

GEA 3175 and *S*-nitroso-*N*-acetyl-penicillamine exerted similar relaxing effects on tracheal preparations precontracted with acetylcholine, and GEA 3175 also elicited a sustained inhibitory response during electrical field stimulation. Such a long-lasting inhibition of electrical field stimulation-induced contractile response of the airways, has only been documented for some β_2-adrenoceptor agonists (Ball et al., 1991). Moreover, it is interesting that our results were obtained in the absence of any phosphodiesterase inhibitors. *S*-Nitrosothiols are unstable, especially in the presence of oxygen (Ignarro et al., 1981). These compounds are assumed to release NO spontaneously, but may also be degraded by enzymes on the plasma membrane (Kowaluk and Fung, 1990). In any case, intracellular penetration of intact *S*-nitroso-*N*-acetyl-penicillamine is probably not required for NO release. GEA 3175 is stable and lipid soluble and may accumulate intracellularly or in cellular membranes, possibly in an environment that is better protected from NO-scavengers. This may explain why GEA 3175 exerted a long-lasting inhibitory effect on the contractile response elicited by electrical field stimulation and also caused a sustained increase in cGMP.

Several investigators have shown that commonly used NO-donors, such as *S*-nitrosothiols, relax airway smooth muscle (Jansen et al., 1992; Gaston et al., 1994), although the mechanisms responsible for this relaxation are still not completely understood. The results of the present study indicate that the high conductance Ca^{2+}-activated K^+ channels are one of the major targets of the action of GEA 3175. This hypothesis is based on the essentially complete inhibition of the relaxant response to GEA 3175 in the presence of iberiotoxin. It is possible that GEA 3175 stimulates the activity of these ion channels, which leads to hyperpolarization and relaxation of airway smooth muscle. It has, in fact, been shown that smooth muscle relaxation induced by β-adrenoceptor agonists, sodium nitroprusside or *S*-nitroso-*N*-acetyl-penicillamine is inhibited by iberiotoxin (Jones et al., 1993; Bialecki and Stinson-Fisher, 1995; Kannan and Johnson, 1995). Kannen and Johnson also showed that relaxations induced by electrical field stimulation in pig tracheal smooth muscle can be reduced by iberiotoxin. When electrical field stimulation is applied to tracheal preparations, the response is a combination of excitatory and inhibitory components (Grundström et al., 1981). The inhibitory non-adrenergic non-cholinergic (NANC) response is mediated, at least partly, by NO (Tucker et al., 1990; Kannan and Johnson, 1995).

Consequently, the activity of the high conductance Ca^{2+}-activated K^+ channels may be regulated by both cyclic AMP and cGMP. Archer et al. (1994) suggested that cGMP-dependent protein kinase induces vasorelaxation by activating charybdotoxin-sensitive K^+ channels, which would imply that the effect of GEA 3175 on airway smooth muscle is mediated by an increase in cGMP. However, it has recently been proposed that NO relaxes vascular smooth muscle by stimulating Ca^{2+}-activated K^+ channels in a cGMP-independent way (Bolotina et al., 1994). Nevertheless, our results show that GEA 3175 effectively relaxes smooth muscle and potently activates soluble guanylyl cyclase in guinea-pig trachea.

Okadaic acid, an inhibitor of type 1 and 2A phosphatases, also antagonized the relaxation induced by GEA 3175. The presence of okadaic acid has been shown to induce both contraction and relaxation of smooth muscle preparations (Naline et al., 1994). However, in our study, the low concentration of okadaic acid (10^{-8} M) did not affect smooth muscle tone in guinea-pig trachea and such a low dose of okadaic acid has been reported to be compatible with inhibition of type 2A phosphatase (Hescheler et al., 1988; Ishihara et al., 1989). Accordingly, our findings may indicate that GEA 3175 either stimulates type 2A phosphatases directly or amplifies indirectly the activity of these enzymes. White et al. (1993) studied an atrial natriuretic peptide-stimulated pituitary tumor cell-line and found that cGMP stimulates the activity of K^+ channels, probably by dephosphorylation. This suggests that the effect may have been due to cGMP-dependent activation of type 2A phosphatases. The existence of such a mechanism in airway smooth cells would explain why both iberiotoxin and okadaic acid markedly reduced GEA 3175-induced tracheal relaxation.

In summary and conclusion, the present study showed that GEA 3175 is a potent airway smooth muscle relaxant with remarkably long-lasting effects on both cGMP levels and contractions induced by electrical field stimulation. The relaxation of acetylcholine-contracted tracheal preparations by GEA 3175 was potently reduced by okadaic acid and iberiotoxin, indicating that phosphatases and high conductance Ca^{2+}-activated K^+ channels play important roles in smooth muscle relaxation.

Acknowledgements

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