ELSEVIER

European Journal of Pharmacology 410 (2000) 61–68

ejp

www.elsevier.nl/locate/ejphar

Thapsigargin, a Ca^{2+}-ATPase inhibitor, relaxes guinea pig tracheal smooth muscle by producing epithelium-dependent relaxing factors

Naotsugu Takahashi [a,b], Hisamichi Aizawa [c,*], Satoru Fukuyama [a], Hiromasa Inoue [a], Sankei Nishima [b], Nobuyuki Hara [a]

[a] *Research Institute for Diseases of the Chest, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[b] *National Minami-Fukuoka Hospital, 4-39-1 Yakatabaru, Minami-ku, Fukuoka 815-1139, Japan*
[c] *Clinical Research Institute, National Fukuoka-Higashi Hospital, 1-1-1 Chidori, Koga 811-3195, Japan*

Received 8 September 2000; received in revised form 24 October 2000; accepted 27 October 2000

Abstract

A non-phorbol ester-type tumor promoter, thapsigargin has been reported to deplete Ca^{2+} stores in endothelial cells by inhibiting Ca^{2+}-ATPase, which in turn increases intracellular Ca^{2+} by mobilization of extracellular Ca^{2+}, leading to activation of constitutive nitric oxide synthase (cNOS) and resultant generation of nitric oxide (NO). In the present study, to evaluate the role of Ca^{2+} in the release of epithelium-dependent relaxing factor (EpDRF), we determined the effect of thapsigargin (10^{-6} M) on the contraction evoked by exogenous Ca^{2+} or acetylcholine (10^{-5} M) in epithelium-denuded or epithelium-intact smooth muscle from guinea pig trachea. The following results were obtained: (1) In epithelium-denuded smooth muscle, the contraction evoked by exogenous Ca^{2+} in Ca^{2+}-free solution or by acetylcholine (10^{-5} M) in Ca^{2+}-containing solution did not change within 20 min after thapsigargin application, but the contraction evoked by exogenous Ca^{2+} increased markedly after 120 min, indicating that thapsigargin had no effect on smooth muscle itself within 20 min of application. The following experiments were performed within 20 min of thapsigargin application. (2) In epithelium-intact smooth muscle, thapsigargin significantly suppressed the contraction evoked by acetylcholine, suggesting that thapsigargin stimulate the epithelium to produce EpDRF. N^G-nitro-L-arginine methylester (L-NAME) partly, but significantly, attenuated this inhibitory effect of thapsigargin. (3) In epithelium-denuded smooth muscle, atropine (10^{-6} M) and L-NAME (10^{-5} M) did not change the contraction evoked by exogenous Ca^{2+} after application of thapsigargin, suggesting that thapsigargin did not stimulate acetylcholine and NO release from nerve terminals. These results suggest that thapsigargin (10^{-6} M) may stimulate EpDRF, including NO and other factor(s) by Ca^{2+}-dependent mechanisms.

Keywords: Thapsigargin; Ca^{2+}-ATPase inhibitor; Ca^{2+} store, EpDRF (epithelium-dependent relaxing factor); Airway epithelial cell; (Guinea pig)

1. Introduction

It has been reported that airway hyperresponsiveness can be induced by epithelial damage in animals (Fabbri et al., 1984) and in humans (Seltzer et al., 1986). The removal of airway epithelium increases the responsiveness of smooth muscle, suggesting that the epithelium may be involved in the inhibition of smooth muscle contraction (Flavahan et al., 1985; Aizawa et al., 1988; Nijkamp et al., 1993; Folkerts and Nijkamp, 1998). Airway epithelial cells release various inhibitory factor(s), such as prostaglandin E_2 and epithelium-derived relaxing factor (EpDRF), and these cells also inhibit excitatory vagal neurotransmission, presumably by suppressing the release of acetylcholine (Xie et al., 1992). Airway epithelial cells may therefore play an important role in regulating the responses of airway smooth muscle (Flavahan et al., 1985; Aizawa et al., 1994).

* Corresponding author. Tel.: +81-92-943-2331; fax: +81-92-943-8775.
E-mail address: aizawah@fukuokae.hosp.go.jp (H. Aizawa).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00840-2

In the vascular system, the endothelium-derived relaxing factor produced and released from arteries is reported to be nitric oxide (NO) (Ignarro et al., 1987; Palmer et al., 1987). Endothelial nitric oxide synthase is expressed by cultured human bronchiolar epithelium (Shaul et al., 1994). Recently, it has been reported that bradykinin releases NO from the tracheal epithelium (Figini et al., 1996), and that epithelial depolarization releases a relaxing factor(s) like NO in guinea pig airway (Folkerts and Nijkamp, 1998). It has also been shown that human epithelial cells express nitric oxide synthase (Watkins et al., 1997).

The purpose of the present study was to evaluate the role of Ca^{2+} in the release of EpDRF. We used thapsigargin, which is non-phorbol ester promoter that causes relaxation due to release of NO from endothelial cells in a Ca^{2+}-dependent manner (Moritoki et al., 1994). Thapsigargin directly inhibits endoplasmic reticulum Ca^{2+}-ATPase and thus increases cytosolic Ca^{2+} in sensitive cells by acute blockade of the Ca^{2+} pump in the endoplasmic reticulum, followed by rapid leakage of Ca^{2+} stores (Thastrup et al., 1990). Consequently, the intracellular Ca^{2+} concentration is regulated by the extracellular Ca^{2+} concentration after application of thapsigargin (Amrani et al., 1995; Gonzalez et al., 1995) and can be used as a probe to investigate intracellular Ca^{2+} storage and release processes. The second purpose of this study was to examine the role of NO in the epithelium-dependent relaxation mediated by Ca^{2+}. We obtained evidence that thapsigargin inhibits acetylcholine-induced contraction by promoting the formation of NO and other factor(s) through a process that depends on the intracellular Ca^{2+} concentration of epithelial cells.

2. Material and methods

2.1. General procedure

Male Hartley guinea pigs weighing 500–600 g (Kyudo, Kumamoto, Japan) were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and exsanguinated. The thorax was opened and the heart, lungs, and whole trachea were transferred to a bath containing oxygenated Krebs solution. The trachea was cleaned of fat and connective tissue under a dissecting microscope and separated from the rest of the preparation by cutting the main bronchi, and then was cut into one-cartilage segment. Tracheal ring was opened longitudinally through the anterior aspects, and a dorsal strip was cut transversely at a length of 2–3 mm and a width of 1–1.5 mm.

The preparation was bathed in modified Krebs solution with the following composition (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^-134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. The solution was aerated with 97% O_2 and 3% CO_2 and the pH was 7.3–7.4. Modified Krebs solution contained 2.5 mM Ca^{2+}, while Ca^{2+}-free modified Krebs solution also contained 0.3 mM EGTA.

For measurement of mechanical responses, the tracheal tissue was mounted vertically in a 1-ml organ bath through which the medium (maintained at 35–36°C) flowed continuously at a rate of 2 ml/min. One end of the tracheal ring was tied by fine silk thread to a mechanotransducer (Nihon Kohden, RCA-5734) and the other to a hook at the bottom of the bath. The strips were set up with an optimum initial tension of 0.01–0.02 *g* (Aizawa et al., 1990, 1991; Ito et al., 1989) and mechanical activity was recorded with a pen recorder. The epithelial cells were removed mechanically by rubbing the internal surface of the trachea with fine silver wire (200 μm diameter) or by hypo-osmolarity shock by exposing the inner lumen of the trachea to distilled water for about 30–60 s as has previously been done in vascular tissues for removal of endothelial cells (Bolton et al., 1984; Nagao and Suzuki, 1987).

2.2. Study 1

In order to investigate the time-dependent effect of thapsigargin on smooth muscle and cholinergic nerve fibers, we used epithelium-denuded smooth muscle strips in Ca^{2+}-free modified Krebs solution. After the first contractile responses to Ca^{2+} (2.5 mM)-containing acetylcholine (10^{-5} M) and to Ca^{2+} (2.5 mM) were obtained, the strips were washed out with Ca^{2+}-free Krebs solution, then thapsigargin (10^{-6} M) was added to the organ bath. The contractile responses to Ca^{2+}-containing acetylcholine or Ca^{2+} were measured at 20 min and 120 min after thapsigargin application. We also examined the effect of atropine (10^{-6} M) and N^G-nitro-L-arginine methylester (L-NAME) (10^{-5} M) on the release of acetylcholine and NO at 120 min after thapsigargin application.

2.3. Study 2

In order to investigate the effect of thapsigargin on the airway epithelium, we used epithelium-intact smooth muscle strip. After the first contractile response to Ca^{2+} (2.5 mM)-containing acetylcholine (10^{-5} M) was obtained, thapsigargin (10^{-6} M) was added to the organ bath and the contractile response to Ca^{2+} (2.5 mM)-containing acetylcholine was measured 20 min after thapsigargin application. To examine the contribution of NO to EpDRF during acetylcholine-induced contraction, we investigated the effect of L-NAME. The contractile responses to acetylcholine was measured before and 20 min after application of thapsigargin. The contractile response to acetylcholine

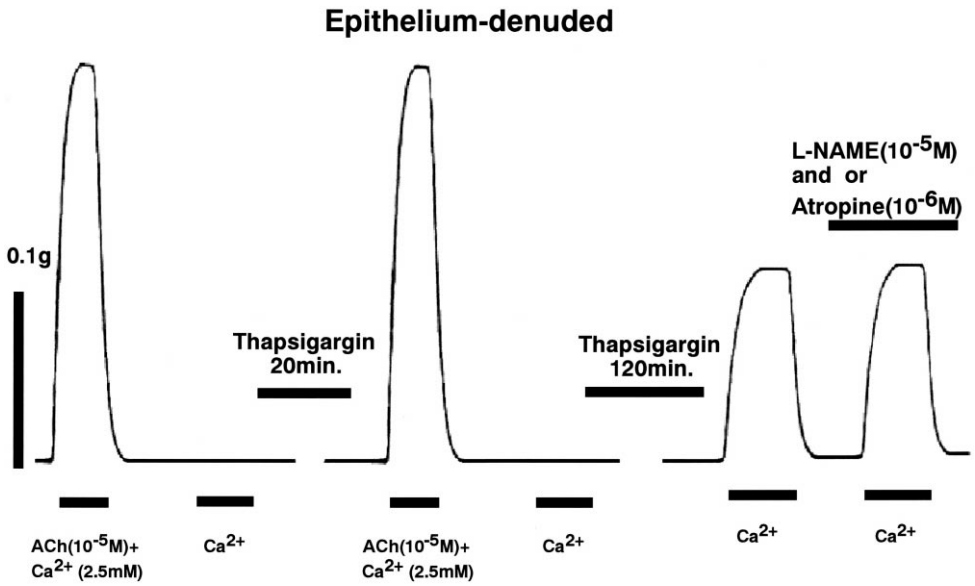

Fig. 1. Effect of thapsigargin (10^{-6} M) on acetylcholine (10^{-5} M) and Ca^{2+} (2.5 mM)-induced contraction of epithelium-denuded smooth muscle from guinea pig trachea in Ca^{2+}-free solution. Ca^{2+} (2.5 mM)-induced contraction was not altered within 20 min after the application of thapsigargin, but it was markedly enhanced after 120 min. This increase in contraction was not affected by atropine and L-NAME.

20 min after application of thapsigargin was measured with and without addition of L-NAME.

2.4. Drugs

Acetylcholine chloride, thapsigargin, N^G-nitro-L-arginine methylester (L-NAME), N^G-nitro-D-arginine methylester (D-NAME), EGTA, dimethyl sulphoxide (DMSO) (all from Sigma, MO, USA), and atropine sulphate (Daiichi Pharmaceutical, Tokyo, Japan) were used in the present study. Thapsigargin was dissolved in DMSO. Each drug was added to the organ bath medium.

2.5. Statistics

Smooth muscle responses were standardized as a percentage of the contraction evoked by Ca^{2+}-containing acetylcholine solution before thapsigargin application, and were expressed as the arithmetic mean and standard error. Differences between mean values were tested for significance using the paired Student's *t*-test. Differences among

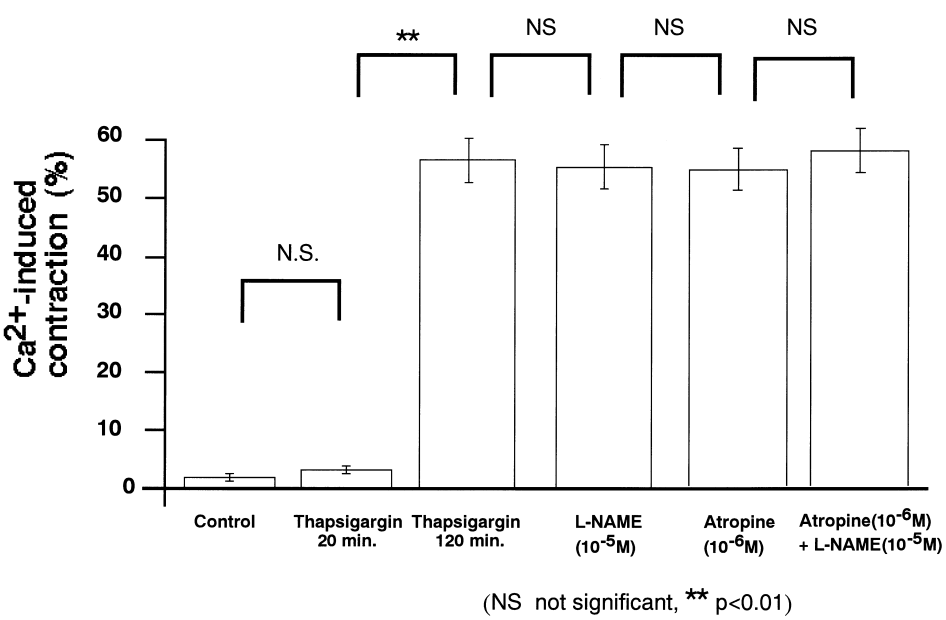

Fig. 2. Effect of thapsigargin at 20 and 120 min after application to epithelium-denuded smooth muscle from guinea pig trachea. The amplitude of the contraction evoked by acetylcholine in the presence of Ca^{2+} before thapsigargin was defined as 100%. Ca^{2+} (2.5 mM)-induced contraction was not altered within 20 min after the application of thapsigargin, but it was significantly enhanced after 120 min. This increase in contraction was not affected by atropine and L-NAME ($n = 8$).

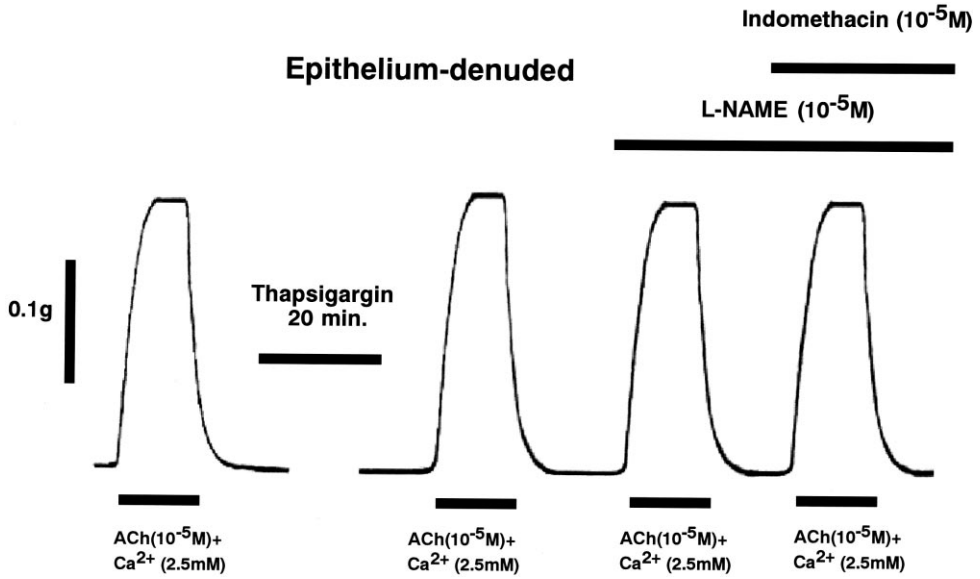

Fig. 3. Effect of thapsigargin (10^{-6} M) on acetylcholine-induced contraction of epithelium-denuded smooth muscle from guinea pig trachea in Ca^{2+}-containing solution. Acetylcholine-induced contraction was not changed within 20 min after application of thapsigargin. This contraction was not affected by L-NAME (10^{-5} M) or indomethacin (10^{-5} M).

more than two groups were compared by one-way analysis of variance followed by Bonferroni's correction. In all analysis, $P < 0.05$ was considered significant.

3. Results

3.1. Effect of thapsigargin on epithelium-denuded tracheal smooth muscle in Ca^{2+}-free solution

Thapsigargin (10^{-6} M) did not influence the Ca^{2+} (2.5 mM)-application in Ca^{2+}-free solution and acetylcholine (10^{-5} M)-application in Ca^{2+}-free solution at 20 min after its application, but markedly contracted the Ca^{2+} (2.5 mM)-application in Ca^{2+}-free solution at 120 min ($n = 6$, $P < 0.01$; Figs. 1 and 2).

To examine the influence of thapsigargin on the release of acetylcholine and NO from peripheral nerve fibers on thapsigargin action, the effect of L-NAME (10^{-5} M) and atropine (10^{-6} M) on this experimental system were investigated. Both agents did not affect the Ca^{2+}-induced contraction at 120 min after thapsigargin application ($n = 6$, $P < 0.01$; Fig. 2).

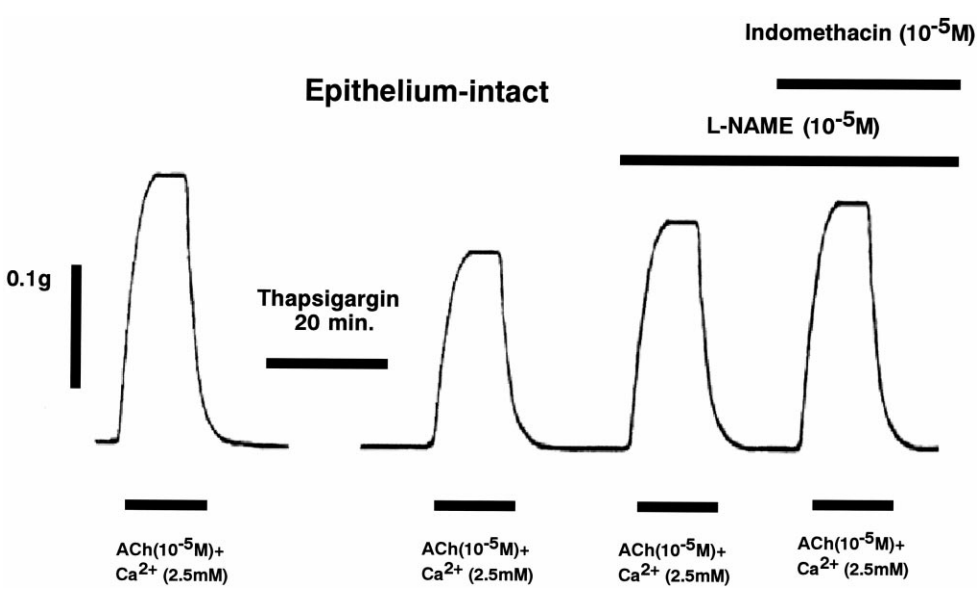

Fig. 4. Effect of thapsigargin (10^{-6} M) on acetylcholine-induced contraction of epithelium-intact smooth muscle from guinea pig trachea in Ca^{2+}-containing solution. Acetylcholine-induced contraction was markedly suppressed 20 min after application of thapsigargin. This inhibition was partially reversed in the presence of L-NAME (10^{-5} M) and indomethacin (10^{-5} M).

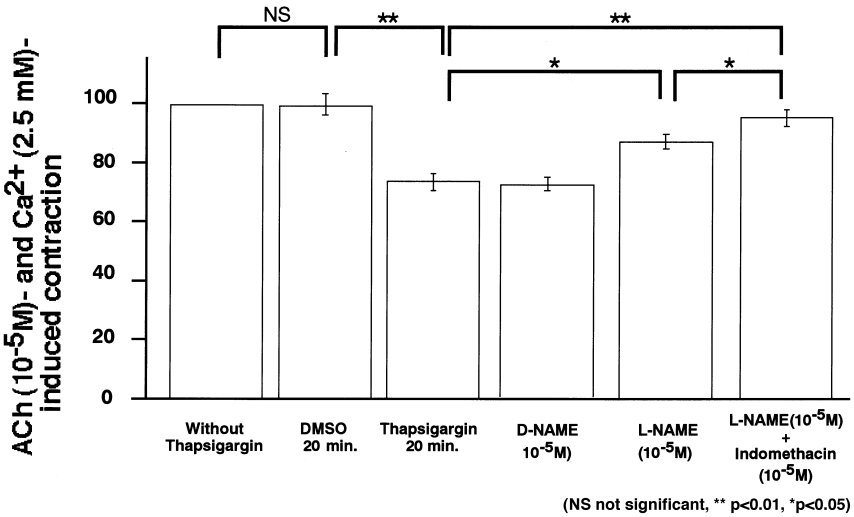

Fig. 5. Effect of thapsigargin on acetylcholine-induced contraction of epithelium-intact smooth muscle from guinea pig trachea at 20 min after application. The amplitude of the contraction evoked by acetylcholine with Ca^{2+} before thapsigargin was defined as 100%. Acetylcholine-induced contraction was significantly suppressed by application of thapsigargin. It was not affected by application of DMSO. This inhibition was partially but significantly reversed by L-NAME (10^{-5} M) and indomethacin (10^{-5} M) ($n = 8$).

These results suggests that release of acetylcholine and NO from peripheral nerve fibers did not play a role in mediating Ca^{2+}-induced contraction in Ca^{2+}-free solution after thapsigargin application.

3.2. Effect of thapsigargin on acetylcholine-induced contraction of epithelium-intact and -denuded tracheal smooth muscle

Thapsigargin did not alter the acetylcholine-induced contraction of epithelium-denuded smooth muscle in Ca^{2+}-containing solution at 20 min after its application (Fig. 3), but significantly suppressed the acetylcholine-induced contraction of epithelium-intact smooth muscle (Figs. 4 and 5). The maximal concentration by DMSO in the bath was $< 0.1\%$ and had no effect on the acetylcholine-induced contraction of epithelium-intact smooth muscle (Fig. 6).

3.3. Effects of thapsigargin on Ca^{2+}-free and Ca^{2+}-containing acetylcholine-induced contraction of epithelium-intact guinea pig trachea

3.3.1. Effect of L-NAME and indomethacin on inhibition by thapsigargin on acetylcholine-induced contraction of epithelium-intact tracheal smooth muscle

To clarify the contribution of NO and prostanoids to epithelium-dependent relaxation, the effect of L-NAME

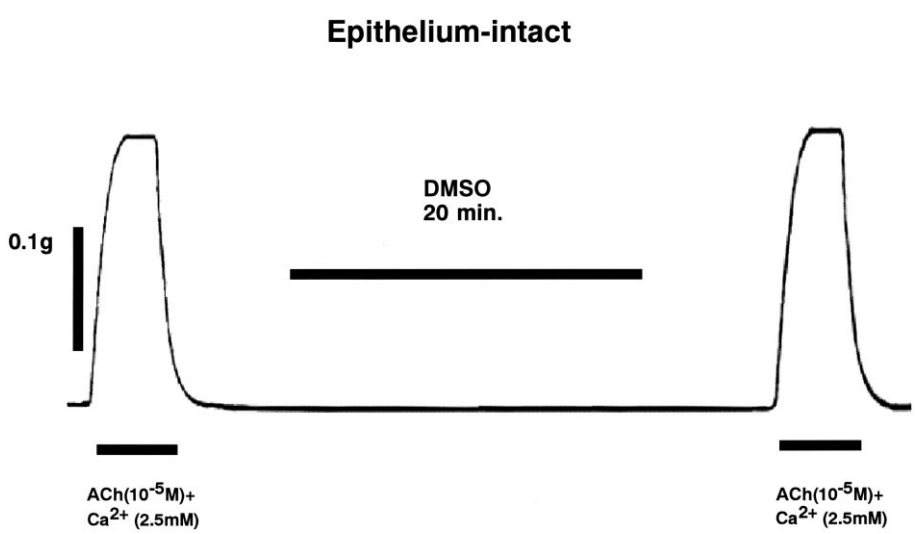

Fig. 6. Effect of DMSO on the Ca^{2+}-containing acetylcholine-induced contraction of epithelium-intact smooth muscle.

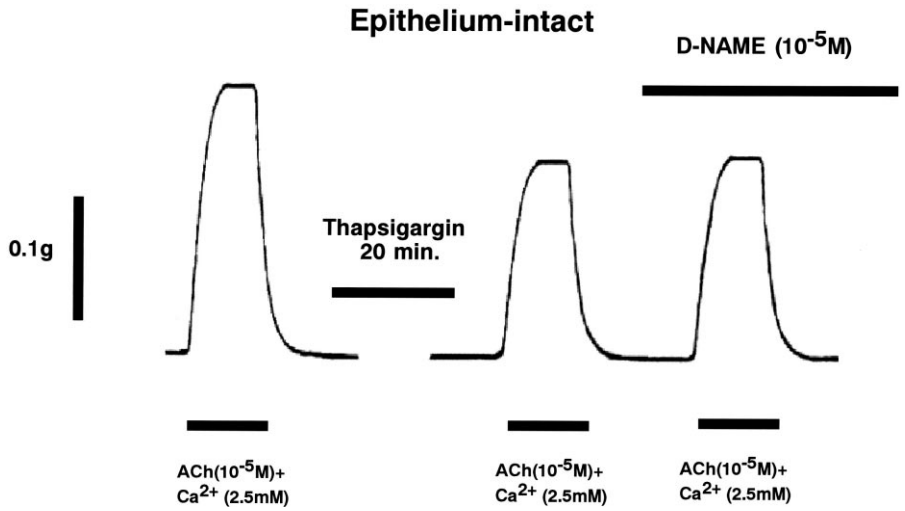

Fig. 7. Effect of thapsigargin (10^{-6} M) on acetylcholine-induced contraction of epithelium-intact smooth muscle from guinea pig trachea in Ca^{2+}-containing solution. Acetylcholine-induced contraction was markedly suppressed 20 min after application of thapsigargin. This inhibition was not reversed in the presence of D-NAME (10^{-5} M).

and indomethacin (10^{-5} M) on the actions of thapsigargin was investigated. The contraction evoked by acetylcholine in the presence of Ca^{2+} before thapsigargin application was defined as 100%. In epithelium-intact smooth muscle, thapsigargin suppressed by acetylcholine induced contraction about 25% at 20 min after application, and L-NAME partly but significantly, reversed the inhibitory effect of thapsigargin compared with D-NAME (Fig. 7). Indomethacin still reversed the inhibitory effect of thapsigargin ($n = 6$, $P < 0.05$; Fig. 5).

4. Discussion

In the present study, we obtained evidence that thapsigargin could suppress the acetylcholine-induced contraction of epithelium-intact smooth muscle in the guinea pig trachea. Inhibition of acetylcholine-induced contraction by thapsigargin was epithelium-dependent, because thapsigargin did not affect the contraction of epithelium-denuded smooth muscle.

Thapsigargin is known to inhibit Ca^{2+}-ATPase and to deplete intracellular Ca^{2+} stores. This results in the subsequent of influx of extracellular Ca^{2+}, so that the intracellular Ca^{2+} concentration becomes regulated by the extracellular Ca^{2+} concentration (Amrani et al., 1995; Gonzalez et al., 1995). In the present study, application of Ca^{2+} to smooth muscle strips in Ca^{2+}-free solution or application of acetylcholine in Ca^{2+}-containing solution evoked contraction after thapsigargin treatment, and this suggested the influx of extracellular Ca^{2+}. The amplitude of acetylcholine-induced contraction was not changed by thapsigargin within 20 min after its application to epithelium-denuded preparations, which indicates that thapsigargin did not have any effect on smooth muscle itself within 20 min. Therefore, we investigated the role of Ca^{2+} in the release of epithelium-dependent relaxing factor from tracheal epithelium using epithelium-intact preparations within 20 min of thapsigargin application. In preparations treated with thapsigargin, intracelluar Ca^{2+} mobilized in the presence of extracellular Ca^{2+} may be available for synthesis of epithelium-dependent relaxing factor, which may then inhibit smooth muscle contraction.

Thapsigargin did not affect the contraction of epithelium-denuded smooth muscle, but significantly suppressed the contraction of epithelium-intact preparations within 20 min, suggesting that thapsigargin caused the release of epithelium-dependent relaxing factor from tracheal epithelial cells. In epithelial cells, thapsigargin increase intracellular Ca^{2+} concentration about 200 s (Kanoh et al., 1999). In smooth muscle cells, thapsigargin increase intracellular Ca^{2+} concentration about 12–15 min (Roux et al., 1997) and about 15–20 min (Kannan et al., 1997). The difference between the affinity of thapsigargin for Ca^{2+}-ATPase in epithelial cells and smooth muscle cells was time-dependent. It was also suggested that inhibition of tracheal smooth muscle contraction by thapsigargin was due to an increase of the intracellular Ca^{2+} concentration in tracheal epithelial cells that was mediated by mobilization of extracellular Ca^{2+}. Another possibility is that the results obtained are due to different release of calcium from intracellular stores. To clarify the role of intracellular Ca^{2+} on EpDRF, the effects of thapsigargin on the action of Ca^{2+}-free and Ca^{2+}-containing acetylcholine-induced contraction of epithelium-intact guinea pig trachea were investigated. Although thapsigargin suppressed the Ca^{2+}-containing acetylcholine-induced contraction, it did not influence Ca^{2+}-free acetylcholine-induced contraction of epithelium-intact guinea pig trachea (data not shown).

The inhibitory effect of thapsigargin was partially but significantly reversed by L-NAME, suggesting that part of the action of thapsigargin was due to blocking of the production of nitric oxide compounds in the epithelial cells. In endothelial cells, NO synthase is Ca^{2+}-calmodulin-dependent, and many studies have shown a crucial role of Ca^{2+} in endothelium-dependent relaxation (Bredt et al., 1991; Bredt and Snyder, 1990; Palmer et al., 1987). It has been concluded that endothelium-dependent relaxing factor is actually nitric oxide (Ignarro et al., 1987). However, few studies have investigated the role of Ca^{2+} in releasing nitric oxide from the tracheal epithelium. Previous studies have shown that airway epithelial cells release at least two factor(s), which may be prostanoid (prostaglandin E_2) and another factor that is distinct from prostanoid (Matsumoto et al., 1996). In the endothelial cells, there is at least one other relaxant factor from, known as hyperpolarizing factor, now known to be K (He et al., 1996; Zygmunt and Hogestatt, 1996). The L-NAME-insensitive component may be attributed to epithelium-dependent relaxing factor(s) other than nitric oxide. Thus, our findings provide evidence that airway epithelial cells may release nitric oxide and other epithelium-dependent relaxing factor(s) by Ca^{2+}-dependent mechanisms.

In conclusion, thapsigargin inhibited tracheal smooth muscle contraction by acting on airway epithelial cells, presumably by promoting the release of epithelium-dependent relaxing factor. Our results also suggested that intracellular Ca^{2+} mobilization in epithelial cells may be crucial for triggering the release of relaxing factor(s). L-NAME and indomethacin partly blocked this inhibitory effect of thapsigargin, suggesting that one relaxing factor is nitric oxide. The L-NAME and indomethacin-insensitive component indicated the presence of other epithelium-dependent relaxing factor. Airway epithelial cells may release nitric oxide and prostanoid(s) and other epithelium-dependent relaxing factor(s) by Ca^{2+}-dependent mechanisms.

References

Aizawa, H., Miyazaki, N., Shigematsu, N., Tomooka, M., 1988. A possible role of airway epithelium in modulating hyperresponsiveness. Br. J. Pharmacol. 93, 139–145.

Aizawa, H., Inoue, H., Miyazaki, N., Ikeda, T., Shigematsu, N., Ito, Y., 1990. Effects of azelastine on vagal neuroeffector transmission in canine and human airway smooth muscle. J. Allergy Clin. Immunol. 86, 171–176.

Aizawa, H., Inoue, H., Ikeda, T., Hirose, T., Ito, Y., 1991. Effects of procaterol, a beta-2-adrenoceptor stimulant, on neuroeffector transmission in human bronchial tissue. Respiration 58, 163–166.

Aizawa, H., Matsumoto, K., Shigyo, M., Inoue, H., Koto, H., Takata, S., Hara, N., 1994. Airway epithelial cells modulate cholinergic neurotransmission in dog trachea. Lung 172, 241–249.

Amrani, Y., Magnier, C., Enouf, J., Wuytack, F., Bronner, C., 1995. Ca^{2+} increase and Ca^{2+}-influx in human tracheal smooth muscle cells: role of Ca^{2+} pools controlled by sarco-endoplasmic reticulum Ca^{2+}-ATPase 2 isoform. Br. J. Pharmacol. 115, 1204–1210.

Bolton, T., Lang, R., Takewaki, T., 1984. Mechanisms of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. J. Physiol. 351, 549–572.

Bredt, D.S., Snyder, S.H., 1990. Isolation of nitric oxide synthetase, a-calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U. S. A. 87, 682–685.

Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R., Snyder, S.H., 1991. Cloned and expressed nitric oxide synthase structurally resembles cytochrome *P*-450 reductase. Nature 351, 714–718.

Fabbri, L.M., Aizawa, H., Alpert, S.E., Walters, H.E., O'Byrne, P.M., Gold, B.D., Holtzman, M.J., Nadel, J.A., 1984. Airway hyperresponsiveness and changes in cell counts in bronchoalveolar lavage after ozone exposure in dogs. Am. Rev. Respir. Dis. 129, 288–291.

Figini, M., Ricciardolo, F.L.M., Javdan, P., Nijkamp, F.P., Emanueli, C., Pradelles, P., Folkerts, G., Geppetti, P., 1996. Evidence that epithelium-derived relaxing factor released by bradykinin in the guinea pig trachea is nitric oxide. Am. J. Respir. Crit. Care Med. 153, 918–923.

Flavahan, N., Aarhus, L., Rimele, T., Vanhoutte, P., 1985. Respiratory epithelium inhibits bronchial smooth muscle tone. J. Appl. Physiol. 58, 834–838.

Folkerts, G., Nijkamp, F.P., 1998. Airway epithelium: more than just a barrier. Trends Pharmacol. Sci. 19, 334–341.

Gonzalez, P.F., Savineau, J.P., Marthan, R., 1995. Control of pulmonary vascular smooth muscle tone by sarcoplasmic reticulum Ca^{2+} pump blockers; thapsigargin and cyclopiazonic acid. Pfluegers Arch.-Eur. J. Physiol. 429, 617–624.

He, G.W., Yang, C.Q., Graier, W.F., Yang, J.A., 1996. Hyperkalemia alters EDHF-mediated hyperpolarization and relaxation in coronary arteries. Am. J. Physiol. 271, H760–767.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G., 1987. endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U. S. A. 84, 9265–9269.

Ito, Y., Suzuki, H., Aizawa, H., Hakoda, H., Hirose, T., 1989. The spontaneous electrical and mechanical activity of human bronchial smooth muscle: its modulation by drugs. Br. J. Pharmacol. 98, 1249–1260.

Kannan, M., Prakash, Y., Johnson, D., Sieck, G., 1997. Nitric oxide inhibits calcium release from sarcoplasmic reticulum of porcine tracheal smooth muscle cells. Am. J. Physiol. 272, L1–L7.

Kanoh, S., Kondo, M., Tamaoki, J., Shirakawa, H., Aoshiba, K., Miyazaki, S., Kobayashi, H., Nagata, N., Nagai, A., 1999. Effect of FK506 on ATP-induced intracellular calcium oscillations in cow tracheal epithelium. Am. J. Physiol. 276, L891–L899.

Matsumoto, K., Aizawa, H., Takata, S., Koto, H., Inoue, H., Hara, N., 1996. Cultured epithelial cells release cyclooxygenase-dependent and cyclooxygenase-independent factors that inhibit cholinergic contraction of canine airway smooth muscle. Respiration 63, 205–212.

Moritoki, H., Hisayama, T., Kondoh, W., Takeuchi, K., 1994. Thapsigargin, a Ca^{2+}-ATPase inhibitor, relaxes rat aorta via nitric oxide formation. Life Sci. 54, 153–158.

Nagao, T., Suzuki, H., 1987. Non-neural electrical responses of smooth muscle cells of the rabbit basilar artery to electrical field stimulation. Jpn. J. Physiol. 37, 497–513.

Nijkamp, F.P., Van der Linde, H.J., Folkerts, G., 1993. Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vivo and in vitro (role of epithelium). Am. Rev. Respir. Dis. 148, 727–734.

Palmer, R.M.J., Ferrige, A.G., Moncada, S., 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524–526.

Roux, E., Guibert, C., Savineau, J., Marthan, R., 1997. [Ca2 +]i oscillations induced by muscarinic stimulation in airway smooth muscle cells: receptor subtypes and correlation with the mechanical activity. Br. J. Pharmacol. 120, 1294–1301.

Seltzer, J., Geffroy, B., Stulbarg, M., Holtzman, M.J., Nadel, J.A., Boushey, H.A., 1986. Ozone induced change in bronchial hyperreac-

tivity to methacholine and airway inflammation in human subjects. J. Appl. Physiol. 60, 1321–1326.

Shaul, P.W., North, A.J., Wu, L.C., Wells, L.B., Brannon, T.S., Lau, K.S., Michel, T., Margraf, L.R., Star, R.A., 1994. Endothelial nitric oxide synthase is expressed in cultured human bronchiolar epithelium. J. Clin. Invest. 94, 2231–2236.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., Dawson, A.P., 1990. Thapsigargin, a tumor promotor, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470.

Watkins, D.N., Peroni, D.J., Basclain, K.A., Garlepp, M.J., Thompson, P.J., 1997. Expression and activity of nitric oxide synthases in human airway epithelium. Am. J. Respir. Cell Mol. Biol. 16, 629–639.

Xie, Z.Q., Hakoda, H., Ito, Y., 1992. Airway epithelial cells regulate membrane potential, neurotransmission and muscle tone of the dog airway smooth muscle. J. Physiol. 449, 619–639.

Zygmunt, P.M., Hogestatt, E.D., 1996. Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. Br. J. Pharmacol. 117, 1006–1600.