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Tumor necrosis factor-α augments contraction and cytosolic Ca^{2+} sensitivity through phospholipase A_2 in bovine tracheal smooth muscle

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Abstract

To elucidate the effects of tumor necrosis factor-α (TNF-α) on tracheal smooth muscle contraction, we simultaneously measured isometric tension and intracellular Ca^{2+} ($[Ca^{2+}]_i$) in fura 2-loaded muscle strips. Smooth muscle force generation was evaluated in a high potassium (K^+; 20.0–80.0 mM) solution and with acetylcholine (3 nM–10 μM). TNF-α (1–100 ng/ml) did not directly contract muscle strips. The contractile response to acetylcholine was enhanced after application of 10 ng/ml of TNF-α for 30 min but not the response of $[Ca^{2+}]_i$. The contractile response and the response of $[Ca^{2+}]_i$ to a high K^+ solution were not altered after application of TNF-α. The $[Ca^{2+}]_i$–tension curve indicated that TNF-α enhanced the responsiveness of tracheal smooth muscle through the acetylcholine-mediated Ca^{2+} sensitivity of intracellular contractile elements. The augmentation of the acetylcholine concentration–response curves for muscle tension in the presence of TNF-α (10 ng/ml) was inhibited in part after application of manoalide, a phospholipase A_2 inhibitor. We conclude that a low concentration of TNF-α enhances smooth muscle responsiveness to acetylcholine by agonist-mediated Ca^{2+} sensitivity facilitated by phospholipase A_2.

Keywords: Cytokine; Bronchial hyperresponsiveness; Bronchial asthma; Arachidonic acid metabolite

1. Introduction

Tumor necrosis factor-α (TNF-α) is a potent cytokine with widespread proinflammatory and antineoplastic activity (Beutler and Cerami, 1989). It is secreted by mast cells and cells of the monocyte/macrophage linkage. TNF-α is stored constitutively within mast cell granules (Gordon and Galli, 1990) and is actively synthesized and secreted de novo by activated monocytes and macrophages (Pennica et al., 1984). In vitro, sensitized rodent mast cells release TNF-α in response to allergen challenge (Gordon and Galli, 1990). TNF-α release from human lung has been demonstrated after sensitization and allergen challenge (Ohkawara et al., 1992). TNF-α may be of importance in asthma because TNF-α is increased in the sputa of patients with bronchial asthma (Taki et al., 1991). TNF-α is also present in the bronchoalveolar lavage fluid of symptomatic asthmatics (Broide et al., 1992). Studies in animals have demonstrated that TNF-α induces airway hyperresponsiveness to aerosolized histamine in vivo (Kips et al., 1992) and inhaled TNF-α increases bronchial responsiveness to methacholine in normal subjects (Thomas et al., 1995). It is not known whether this TNF-α-induced hyperresponsiveness is due to a direct effect of TNF-α on the contractile property of airway smooth muscle. In addition, the mechanism of TNF-α-induced hyperresponsiveness is not yet clear.

An alteration of Ca^{2+} homeostasis in airway smooth muscle may be involved in the airway hyperresponsiveness of bronchial asthma. Only a few reports have shown an abnormality of Ca^{2+} homeostasis associated with hyperresponsiveness in airway smooth muscle (Weiss and Viswanath, 1979; Roder, 1985), because it is difficult to obtain simultaneous measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and muscle tension. A fluorescent indicator, fura 2 (Grynkiewicz et al., 1985), was introduced for simultaneous measurement of $[Ca^{2+}]_i$ and muscle tension in smooth muscle strips (Ozaki et al., 1987, 1990; Himpens and Somlyo, 1988). Simultaneous measurement of $[Ca^{2+}]_i$ and

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muscle tension can be performed in intact smooth muscle. With this method, we previously reported that a low concentration of phospholipase A_2 enhances smooth muscle responsiveness to acetylcholine by agonist-mediated Ca^{2+} mobilization that is facilitated by thromboxane A_2 (Takata et al., 1999). This method is useful for studying agonist-induced changes in the Ca^{2+} sensitivity of the contractile elements (Miwa et al., 1994), because the receptor and the signal transduction system remain intact.

It has been reported that proinflammatory cytokines such as TNF-α and interleukin-1β increase phospholipase A_2 expression and its kinase activity in tracheobronchial smooth muscle cells, and that its expression is downregulated by dexamethasone (Vadas et al., 1996). Phospholipase A_2s catalyze the release of unsaturated fatty acid from the *sn-2* position of phospholipids or phosphatidic acid. While the mechanism of phospholipase A_2 activation through TNF-α stimulation is not completely clear, phospholipase A_2 phosphorylation and its kinase activity may be involved in the signal transduction pathway mediated by TNF-α.

The aims of the present study were to elucidate the effects of TNF-α on smooth muscle contraction and $[Ca^{2+}]_i$ by simultaneous measurement in fura 2-loaded bovine tracheal smooth muscle strips, to clarify how TNF-α influences Ca^{2+} mobilization or Ca^{2+} sensitivity in airway smooth muscle, and to determine whether the effects of TNF-α on smooth muscle involve phospholipase A_2.

2. Methods

2.1. Animals and measurement of $[Ca^{2+}]_i$ and muscle contraction

Segments of bovine tracheae obtained from a local slaughterhouse were dissected, and the epithelium, connective tissues and cartilage were removed. The tissue was cut into small rectangular strips (approximately 1 mm wide and 5 mm long). Each strip was immersed in chilled physiological salt solution (PSS) of the following composition (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.9, glucose 5.5 and EDTA 0.01.

The isometric tension and $[Ca^{2+}]_i$ in the tracheal strips were measured simultaneously as previously reported by Ozaki et al. (1987). Strips were superfused for 3 h under protection from light with PSS (37°C) containing 5 μM of the acetoxymethyl ester of fura 2 (fura 2-AM) gassed with a 95% O_2 and 5% CO_2 mixture at 37°C and pH 7.4. We added a detergent, 0.02% cremophor EL, to solubilize fura 2-AM. This concentration of cremophor EL did not change the contractions. Muscle strips were loaded with fura 2-AM solution for 3 h at room temperature (20–25°C). After loading, the muscle strips were washed with PSS in a tissue bath at 37°C for 20–30 min to remove uncleaved fura 2-AM. The muscle strips were illuminated alternately (48 Hz) at excitation wavelengths 340 and 380 nm and the amount of fluorescence measured at 500 nm induced by excitation at 340 nm (F_{340}) and that induced by excitation at 380 nm (F_{380}) was measured using a fluorimeter (CAF-110, Japan Spectroscopic).

Smooth muscle tissue has pyridine nucleotides that fluoresce in increasing proportion to $[Ca^{2+}]_i$ (Miwa et al., 1994) and interfere with fura 2-Ca^{2+} signals (Ozaki et al., 1990). This fluorescence, at both 340 and 380 nm, increased during muscle contraction by approximately 10% of the total fura 2-AM fluorescence. In contrast to this, the fluorescence due to fura 2-Ca^{2+} changed in the opposite direction as $[Ca^{2+}]_i$ changed. In the present experiments, F_{340} and F_{380} were always monitored to check whether they changed in opposite directions. At the end of the experiments, maximum and minimum fluorescence levels were determined using ionomycin and EGTA, respectively. We used these values for the calculation of $[Ca^{2+}]_i$. However, the absolute Ca^{2+} concentration was not calculated in this experiment because the dissociation constant of fura 2-AM for Ca^{2+} the smooth muscle cytoplasm may be different from that in vitro. Instead, we used the ratio of F_{340}/F_{380} as an indicator of $[Ca^{2+}]_i$, as has been used previously (Vadas et al., 1996; Takata et al., 1999). High-K^+ 72.7 mM-induced sustained changes in the fluorescence ratio and muscle tension were used as references (100%). The threshold concentration, the half maximally effective concentration (EC_{50}) values and maximum responses were determined from the log concentration–response curves for each agent.

2.2. Effects of TNF-α on tracheal smooth muscle contraction

To examine whether TNF-α can contract muscle, the following experiment was performed. The concentration–response relationship of muscle strips for TNF-α (1–100 ng/ml) were determined. We measured the isometric tension and F_{340}/F_{380} after a sustained contraction was obtained with high K^+ solution, and then the strips were washed three times with PSS to return to the resting state. We used two types of contractile agent, high K^+ solution and acetylcholine, to clarify whether TNF-α acts in a voltage-dependent manner or a muscarinic receptor-mediated manner in smooth muscle contraction.

The TNF-α group was defined as follows: first, the tissues were incubated in high K^+ (72.7 mM) solution and then washed. After TNF-α (10 ng/ml) was applied for 30 min, we determined the concentration–response relationship at various concentrations of K^+ solution (20, 40, 60, and 80 mM) and of acetylcholine (3 nM–10 μM) at 5-min intervals. The control group was defined as follows: the same procedure as that used for the TNF-α group was followed, except that TNF-α was not applied before we measured the concentration–response relationship. We measured muscle tension and F_{340}/F_{380} simultaneously

when the muscle strips had reached the sustained phase for each dose of the contractile agent.

2.3. Participation of phospholipase A_2 metabolites

To examine whether phospholipase A_2 participates in the effects of TNF-α on smooth muscle contraction, we performed the isometric tension and $[Ca^{2+}]_i$ experiments after incubation of the strips with a phospholipase A_2 inhibitor (manoalide: 1 μM) (Jacobson et al., 1990). After pretreatment with manoalide for 15 min at room temperature, the tissues were exposed to TNF-α (10 ng/ml) for an additional 30 min, and then isometric muscle tension and F_{340}/F_{380} were measured simultaneously with CAF-110.

2.4. Materials

The following drugs were used: TNF-α acetylcholine chloride (Sigma, St Louis, MO, USA), fura 2-AM (Dojindo Laboratories, Kumamoto, Japan), cremophor EL (Nakarai, Kyoto, Japan); manoalide (Wako, Osaka, Japan)

2.5. Statistical analysis

Data are expressed as means ±S.E.M.. The significance of differences between group means was assessed with analysis of variance and Fisher's protected least-significant differences for unpaired samples using StatView 4.0 (Abacus Concepts, Berkeley, CA) for Macintosh. A value of $P < 0.05$ was taken as significant.

3. Results

We used muscle strips loaded with fura 2 as previously reported (Takata et al., 1999). In fura 2 unloaded tracheal smooth muscle strips, high K^+ or acetylcholine increased both F_{340} and F_{380} with an increase in muscle tension. Because the increments in F_{340} and F_{380} were proportional, F_{340}/F_{380} did not change during the course of the experiment. The changes in the emitted fluorescence at the two excitation wave lengths (340 and 380 nm) were very small, and there was a technical issue in working with a thick strip in that it makes achieving appropriate loading difficult and induces smooth muscle damage. We sometimes observed that the stimulants induced a rapid and transient decrease in fluorescence which then returned to baseline or increased gradually above the resting level. These results seem to have been due to insufficient fura 2 loading. Sufficient fura 2 loading revealed good reproducibility, slow change after stimulation, and stability above the resting level. We observed a preparation successfully loaded with fura 2 and another insufficiently loaded from one animal. Approximately 25% of fura 2-loaded preparations were insufficiently loaded, and thus were unsuitable and discarded. Since the rate of rejection of samples between the TNF-α-treated and control groups was similar, we did not consider the potential for bias to affect data.

3.1. Response of tracheal strips to TNF-α (1–100 ng / ml)

Fig. 1 shows representative tracings of isometric tension and $[Ca^{2+}]_i$ in bovine tracheal strips after the application of TNF-α (1–100 ng/ml) for 10 min each. After the application of TNF-α, muscle tension and $[Ca^{2+}]_i$ did not change significantly.

3.2. Effects of TNF-α (10 ng / ml) on tracheal strip responsiveness to acetylcholine

Fig. 2 shows representative tracings of the change in isometric tension and $[Ca^{2+}]_i$ induced by cumulative in-

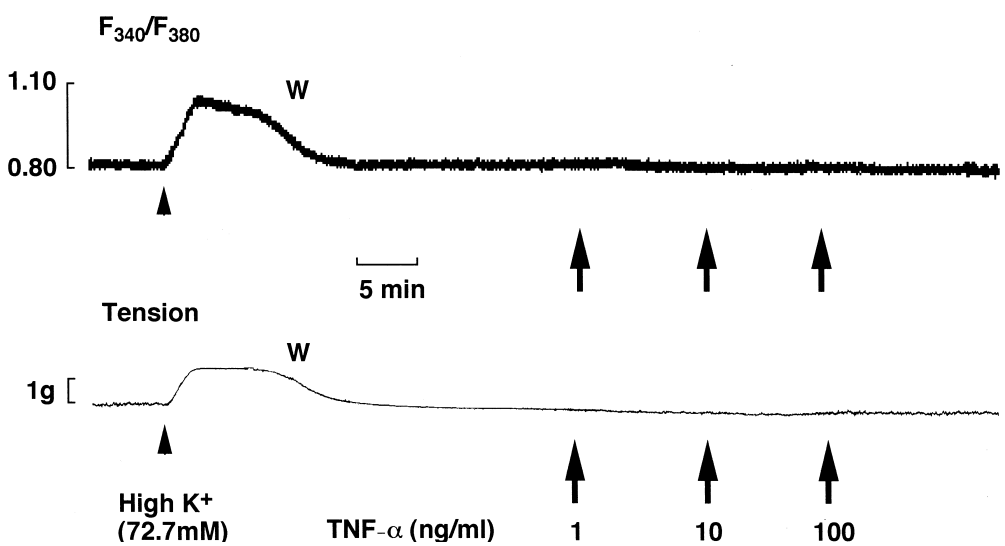

Fig. 1. Representative tracings of changes in $[Ca^{2+}]_i$ (F_{340}/F_{380}) and muscle tension of a fura 2-loaded bovine tracheal strip after the application of TNF-α. w: wash-out PSS.

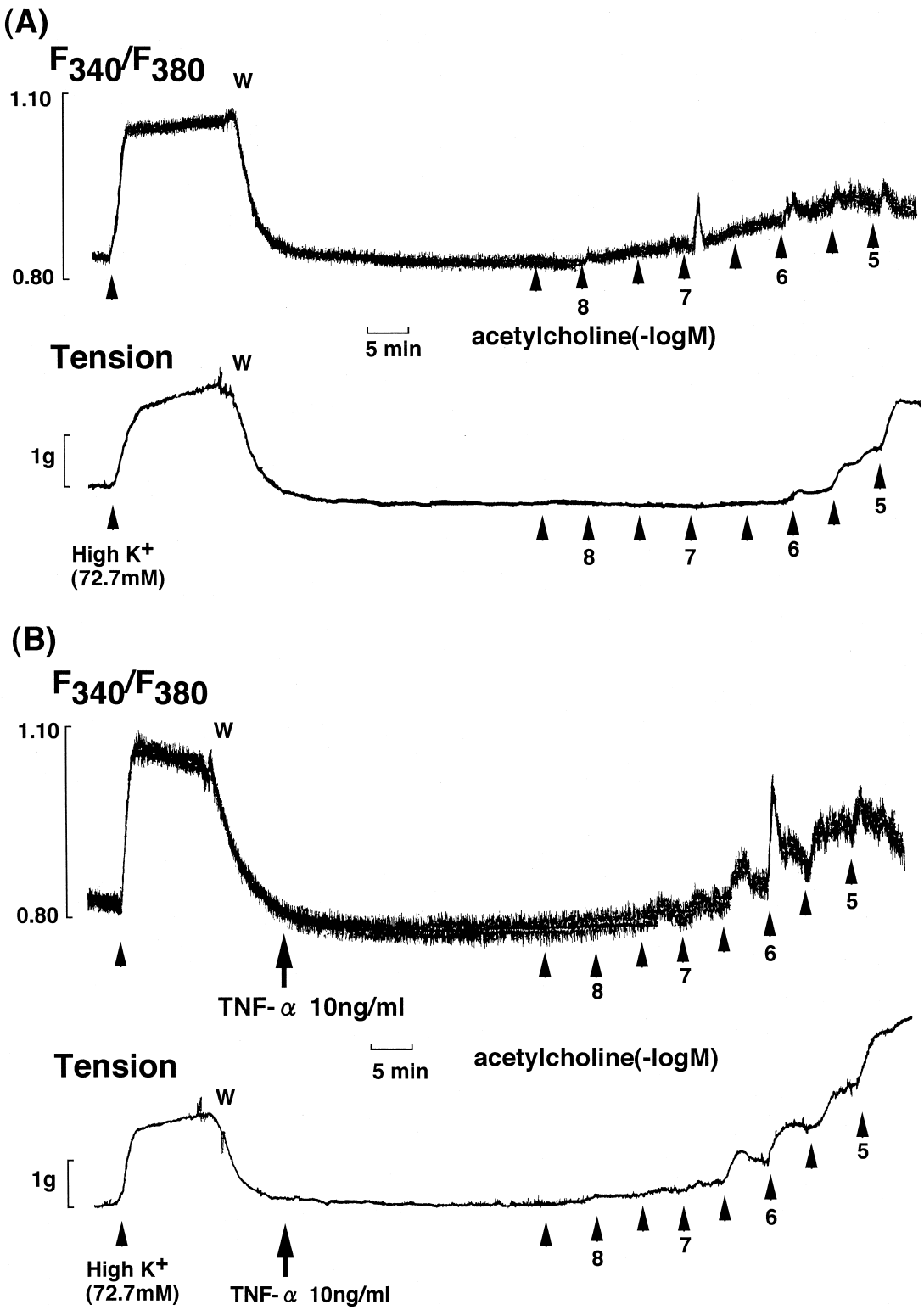

Fig. 2. Representative tracings of changes in $[Ca^{2+}]_i$ (F_{340}/F_{380} and muscle tension induced by cumulative increases in the concentration of acetylcholine in the absence (A) or presence (B) of TNF-α (10 ng/ml) in fura 2-loaded bovine tracheal strips. w: wash-out PSS.

creases in the concentration of acetylcholine without TNF-α (Fig. 2A) and with TNF-α (Fig. 2B).

The concentration–response relationship for isometric tension in response to acetylcholine in the TNF-α group and the control group are shown in Fig. 3a. TNF-α shifted the concentration–response curve for muscle tension induced by acetylcholine to the left. Since a plateau in muscle tension was not obtained in the control group, the tension induced by 10 μM of acetylcholine was used as the maximal response. The threshold concentration, EC_{50},

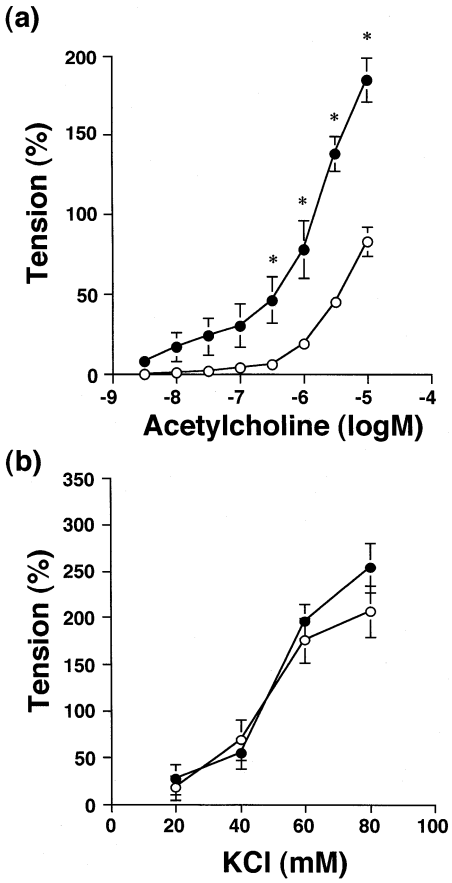

Fig. 3. Effect of 10 ng/ml TNF-α on the log concentration–response relationship for muscle contraction in response to acetylcholine (a) and KCl (b) in bovine tracheal strips. Each point of the control group (○) and the TNF-α-treated group (●) represents the mean of seven preparations, and the S.E.M. is shown by vertical bars. 100% represents the muscle tension induced by 72.7 mM K^+.

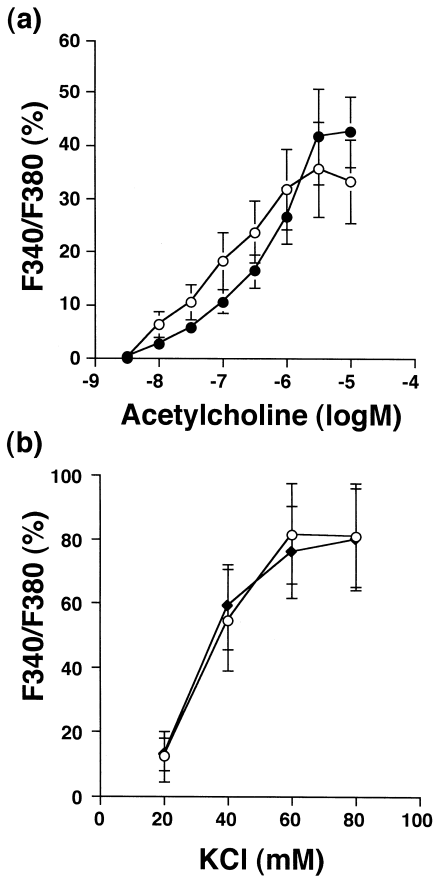

Fig. 4. Effect of 10 ng/ml TNF-α on the log concentration–response relationship for $[Ca^{2+}]_i$ (F_{340}/F_{380}) in response to acetylcholine (a) and KCl (b) in bovine tracheal strips. Each point of the control group (○) and the TNF-α-treated group (●) represents the mean of seven preparations, and the S.E.M. is shown by vertical bars. 100% represents the muscle tension induced by 72.7 mM K^+.

and maximal response in the TNF-α-treated group were significantly lower than those in the control group (Table 1). The concentration–response relationship for isometric tension in response to KCl in the control group and the TNF-α group are shown in Fig. 3b. In contrast to acetylcholine with KCl, the concentration–contraction curves were similar for the two groups. The concentration–response curves for $[Ca^{2+}]_i$ in response to acetylcholine and KCl for both groups are shown in Fig. 4. The concentra-

Table 1
Effect of TNF-α(10 ng/ml) on contractile and $(Ca^{2+})_i$ responses of bovine strips to acetylcholine
TNF-α, tumor necrosis factor-α; Threshold, threshold concentration; EC_{50}, one-half maximally effective concentration; Max, maximum response (percent value of the response induced by 72.7 mM K^+). Values are the mean ± S.E.M. of six preparations.

	Control	TNF-α	
Contraction			
Threshold (−logM)	6.90 ± 0.29	8.00 ± 0.22	$P < 0.05$
EC_{50} (−logM)	5.60 ± 0.03	6.01 ± 0.16	$P < 0.05$
Max (%)	83 ± 9	182 ± 14	$P < 0.01$
$(Ca^{2+})_i$			
Threshold (−logM)	7.90 ± 0.10	7.80 ± 0.20	NS
EC_{50} (−logM)	6.87 ± 0.22	6.46 ± 0.19	NS
Max (%)	38 ± 8	50 ± 5	NS

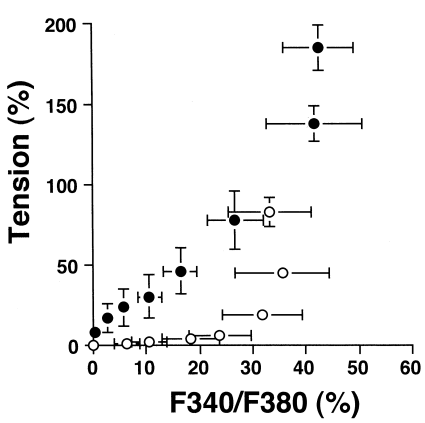

Fig. 5. Effect of TNF-α on the Ca^{2+} sensitivity of contractile elements in bovine tracheal strips, shown by the $[Ca^{2+}]_i$–tension relationship for acetylcholine (1 nM to 10 μM). Each point represents the mean of seven preparations, and the S.E.M. is shown by vertical bars.

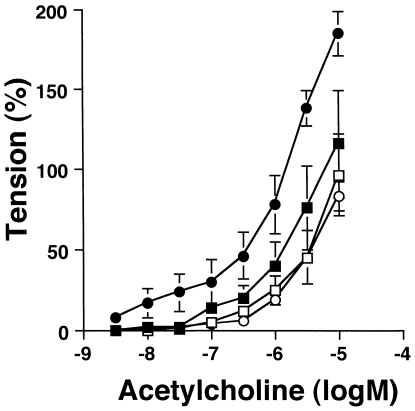

Fig. 6. Effect of manoalide, a PLA_2 inhibitor, on the log concentration–response relationship for contraction of TNF-α-treated bovine tracheal strips in response to acetylcholine. Each point of the control group (○) and the TNF-α-treated group (●), TNF-α-manoalide-treated group (■), manoalide-treated group (□) represents the mean of five preparations, and the S.E.M. is shown by vertical bars. One hundred percent represents the muscle tension induced by 72.7 mM K^+.

tion–response curves for $[Ca^{2+}]_i$ in response to acetylcholine and KCl were similar in the two groups.

Fig. 5 shows the $[Ca^{2+}]_i$–tension relationship for acetylcholine with and without the application of TNF-α. The slope of the $[Ca^{2+}]_i$–tension curve for acetylcholine in the presence of TNF-α was greater than that in the absence of TNF-α. Moreover, the $[Ca^{2+}]_i$–tension relationship after the application of TNF-α shifted to the left compared with the control $[Ca^{2+}]_i$–tension relationship. These findings suggest that the Ca^{2+} sensitivity of the contractile elements in the presence of acetylcholine was significantly different between the TNF-α-treated and control groups.

3.3. Participation of PLA_2

The leftward shift in the concentration–response curve for acetylcholine with TNF-α was inhibited in part by manoalide, a phospholipase A_2 inhibitor (Fig. 6). We preliminarily examined another phospholipase A_2 inhibitor, quinacrine (10 μM). The augmentation of the concentration–response curves for muscle tension in response to acetylcholine in TNF-α (10 ng/ml) was also inhibited in part after application of quinacrine.

4. Discussion

The present study yielded the following results; (1) 1–100 ng/ml of TNF-α did not directly contract bovine smooth muscle; (2) the contractile response to acetylcholine but not to $[Ca^{2+}]_i$ was enhanced after application of 10 ng/ml of TNF-α; (3) the contractile response and the response of $[Ca^{2+}]_i$ to a high K^+ solution were not altered after application of TNF-α; and (4) the augmentation of the contractile response to acetylcholine after application of TNF-α was inhibited by pretreatment with manoalide, a phospholipase A_2 inhibitor.

In our study, TNF-α increased the sensitivity of tracheal smooth muscle more than 10-fold. This concentration of TNF-α, however, did not cause the contraction of tracheal smooth muscle. This hypersensitivity might result from increased sensitivity of contractile elements to Ca^{2+}. We suspect that the muscarinic receptor-coupled pathway may be selectively augmented by TNF-α, because contraction and increases in intracellular Ca^{2+} evoked by a high K^+ solution were not altered after treatment with TNF-α. While we did not show the $[Ca^{2+}]_i$–tension curves for high K^+, the sensitivity of intracellular contractile elements to Ca^{2+} was greater in the presence of acetylcholine than in the presence of a high K^+ medium. This suggests that hypersensitivity to acetylcholine in bovine tracheal smooth muscle after the application of TNF-α may be evoked by an alteration of the function of the muscarinic receptor or of the muscarinic receptor-coupled signal transduction system. To our knowledge, this is the first report that TNF-α increases airway hyperresponsiveness to acetylcholine by changing Ca^{2+} sensitivity.

The mechanism whereby 10 ng/ml of TNF-α augmented airway sensitivity to acetylcholine should be discussed. Proinflammatory cytokines such as TNF-α and interleukin-1β increase phospholipase A_2 expression and its kinase activity in tracheal smooth muscle cells (Vadas et al., 1996). Previous studies also demonstrated that the cytotoxicity and mitogenicity of TNF-α is reduced or blocked by inhibitors of phospholipase A_2 (Palombella and Vilcek, 1989). Hayakawa et al. (1993) used TNF-resistant cell lines and showed the crucial role of high-molecular weight cytosolic phospholipase A_2 in TNF-induced cytolysis. In addition, recent data showed that phospholipase A_2 inhibitors blocked arachidonic acid release, nuclear factor-κB activation and expression of genes involved in inflammation (Thommesen et al., 1998). In this study, the augmentation of the concentration–response curves for muscle tension in response to acetylcholine in the presence of TNF-α was inhibited in part after application of manoalide, a phospholipase A_2 inhibitor. We suggest that TNF-α-induced airway hypersensitivity is mediated through phospholipase A_2 activity.

The $[Ca^{2+}]_i$–tension relationship after the application of TNF-α shifted to the left of the control $[Ca^{2+}]_i$–tension relationship. Thus, the sensitivity of the contractile elements to Ca^{2+} was increased. Myofilament Ca^{2+} hypersensitivity has been reported in canine and swine tracheal smooth muscle (Gerthoffer et al., 1989; Shieh et al., 1992). Kitagawa et al. (1991) suggested that agonist-induced increases in Ca^{2+} sensitization of force in smooth muscle might be due to modulation of the myosin light chain kinase/phosphatase activity ratio mediated through G protein. Furthermore, Nishimura et al. (1990) showed that increased myofilament Ca^{2+} sensitivity in norepineph-

rine-activated vascular smooth muscle is responsible for activation of protein kinase C.

The effects of TNF-α on tracheal smooth muscle are controversial. Penning et al. (1993) reported that pretreatment of guinea-pig trachea for 30 min with TNF-α (1 pM, 30 pM and 100 pM) increases the maximum contractile response to methacholine without changing the *p*D2 values. This result is different from ours, concerning the *p*D2 value. We used bovine tracheal smooth muscle from which the epithelium, connective tissues and cartilage were removed, while they used guinea-pig tracheal strips with epithelium and cartilage. Munakata et al. (1994) reported that TNF-α is involved in smooth muscle relaxation induced by nitric oxide which is released by epithelium, indicating that TNF-α affects smooth muscle contraction directly and epithelium-associated muscle relaxation. Amrani et al. (1995, 1997) reported that pretreatment of human tracheal smooth cells with TNF-α (10 ng/ml) for 24 h increases $[Ca^{2+}]_i$ induced by bradykinin and carbachol without changing the *p*D2 values. These results showed that TNF-α potently regulates G-protein-mediated signal transduction in airway smooth muscle cells by activating pathways dependent on protein synthesis. These results are different from ours concerning *p*D2 values. In our study, the pretreatment time was 30 min, while that in their study it was 24 h. This suggests that long-term pretreatment with TNF-α induces protein synthesis, whereas short-term pretreatment with TNF-α directly stimulates airway smooth muscle cells through the activation of phospholipase A_2.

Phospholipase A_2 plays an essential role in the synthesis of eicosanoids by releasing arachidonic acid metabolites from the membrane phospholipids of various inflammatory cells. Two principal phospholipase A_2 enzymes, cytosolic phospholipase A_2 and secretory phospholipase A_2, have been implicated in the inflammatory process. Secretory phospholipase A_2 is further classified as phospholipase A_2-I when found in the pancreas, lung and spleen (Sakata et al., 1989) and as phospholipase A_2-II when found in inflammatory sites and platelet granules (Vadas et al., 1988). We previously reported that a low concentration of phospholipase A_2-II enhances smooth muscle responsiveness to acetylcholine by agonist-mediated Ca^{2+} mobilization that is facilitated by thromboxane A_2 (Takata et al., 1999). In our present study, we demonstrated that TNF-α plays a role in airway hypersensitivity through Ca^{2+} sensitivity involving phospholipase A_2 activity but not through Ca^{2+} mobilization. We propose two reasons why our two studies produced different results. First, there is a difference in phospholipase A_2 type. In our previous study, we used bee venom phospholipase A_2 (phospholipase A_2-II), while we used endogenous cytosolic phospholipase A_2 in the present study. Second, manoalide, a phospholipase A_2 inhibitor, only partially inhibited the leftward shift in the concentration–response curve for acetylcholine. At least one more mechanism may be involved in the hypersensitivity induced by the application of TNF-α.

The simultaneous measurement of $[Ca^{2+}]_i$ and muscle tension with a CAF-110 fluorimeter in the present study required large amounts of smooth muscle. It is difficult to use this method to study samples of airway smooth muscle obtained from guinea-pigs, which are frequently used in various airway allergic models. We expect further studies using the present method with experimental asthma models or asthmatic patients to clarify whether an abnormality of Ca^{2+} homeostasis in airway smooth muscle cells participates in the pathogenesis of asthma. This clarification could be helpful in the development of novel therapies for bronchial asthma

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