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Phospholipase A_2 augments contraction and intracellular calcium mobilization through thromboxane A_2 in bovine tracheal smooth muscle

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Phospholipase A_2 augments contraction and intracellular calcium mobilization through thromboxane A_2 in bovine tracheal smooth muscle. Y. Takata, Y. Nishimura, H. Maeda, M. Yokoyama. ©ERS Journals Ltd 1999.

ABSTRACT: Phospholipase A_2 (PLA_2) induces hyper-sensitivity to muscarinic agonists in airway smooth muscle *in vitro*. The precise mechanism of this is unknown, but might involve altered calcium homeostasis. In order to elucidate the effects of PLA_2, on bovine tracheal smooth muscle contraction, isometric tension and intracellular calcium concentration ($[Ca^{2+}]i$) were simultaneously measured in fura 2-loaded muscle strips.

A high concentration of PLA_2 (0.5 $\mu g \cdot mL^{-1}$) caused the muscle strips to contract, and this contractile response was significantly attenuated by pretreatment with indomethacin (IND; 10 μM), but not by nordihydroguaiaretic acid (NDGA; 10 μM). A low concentration of PLA_2 (0.02 $\mu g \cdot mL^{-1}$) did not directly contract muscle strips. However a low concentration PLA_2 significantly enhanced the threshold of the contractile response and that of the $[Ca^{2+}]i$ response to acetylcholine (ACh), but not that of the response to a high K^+ concentration. These augmented responses to ACh returned to control levels after pretreatment with IND, a thromboxane (TX) synthetase inhibitor (OKY-046; 10 μM) or a TXA_2 receptor antagonist (ONO-3708; 10 μM), but not after NDGA pretreatment.

These results suggest that a low concentration of phospholipase A_2 enhances smooth muscle responsiveness to acetylcholine by agonist-mediated Ca^{2+} mobilization facilitated by thromboxane A_2. It is concluded that phospholipase A_2 plays an important role in bronchial hypersensitivity involving thromboxane A_2. It remains to be examined whether similar abnormalities in calcium homeostasis and muscarinic receptor function or coupling are involved in the pathogenesis of asthma.

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Phospholipase A_2 (PLA_2) plays an essential role in the synthesis of eicosanoids by releasing arachidonic acid (AA) metabolites from the membrane phospholipids of various inflammatory cells [1, 2]. Two principal PLA_2 cytosolic PLA_2 and secretory PLA_2 ($sPLA_2$), have been implicated in the inflammatory process. $sPLA_2$ is further classified as PLA_2-I when found in the pancreas, lung and spleen [3] and PLA_2-II when found at inflammatory sites and in platelet granules [4]. The involvement of PLA_2-II has been demonstrated in various human inflammatory disease conditions such as endotoxaemia [5], sepsis [6], rheumatoid arthritis [7] and uraemia [8]. Activated inflammatory cells, such as neutrophils and alveolar macrophages, and tracheal epithelial cells release lysosomal and granular PLA_2 into interstitial or intravascular compartments [1].

High concentrations of PLA_2-II have been detected in the exudative fluid of inflammatory sites [9], and PLA_2 has been shown to be released from activated mast cells, which are mainly involved in the allergic inflammation of bronchial asthma [10]. Increased PLA_2 activity has been demonstrated in bronchoalveolar lavage fluid from human asthmatics [1]. The concentration of serum PLA_2 is higher not only in asthmatic patients during asthmatic attacks but also in asthmatic subjects not having an attack compared to nonasthmatics [11]. In *in vitro* experimental studies, PLA_2 induced hyperreactivity of airway smooth muscle to muscarinic agonists [12], and intratracheally-administered PLA_2 increased intratracheal pressure in perfused guinea-pig lungs [13]. Although the PLA_2 - catalysed production of newly synthesized AA metabolites has been observed to induce bronchoconstriction and increased activity of PLA_2 has been closely associated with bronchial tone, [14, 15] the precise mechanism of airway hyperresponsiveness induced by PLA_2 is still undetermined.

An alteration in Ca^{2+} homeostasis in airway smooth muscle may be involved in the airway hyperresponsiveness in bronchial asthma. Few reports have shown an abnormality of Ca^{2+} homeostasis to be associated with hyperresponsiveness in airway smooth muscle [16, 17], probably because it is difficult to obtain simultaneous measurements of intracellular calcium ($[Ca^{2+}]i$) and muscle tension. The simultaneous measurement of $[Ca^{2+}]i$ and muscle tension was recently performed in intact smooth muscle, when a new fluorescent indicator, fura 2 [18], was introduced

[19–21]. This method is very useful for studying agonist-induced changes in the Ca^{2+}-sensitivity of contractile elements, and has been applied in the authors′ laboratory in the evaluation of supersensitivity to serotonin in atherosclerotic arteries [22].

The purposes of the present study were to: 1) elucidate the effects of bee venom PLA_2 (PLA_2-II) on tracheal smooth muscle $[Ca^{2+}]_i$ with the simultaneous measurement of isometric muscle tension, 2) determine whether the effects of PLA_2 on smooth muscle involve AA metabolites, and 3) clarify how PLA_2-II influences calcium mobilization or Ca^{2+}-sensitivity in airway smooth muscle.

Methods

Tissue preparation

Segments of bovine trachea obtained from a local slaughterhouse were dissected, and the epithelium, connective tissues and cartilage removed. The tissue was cut into small rectangular strips (approximately 1 mm wide and 5 mm long). Each strip was immersed in chilled physiological saline 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

Fura 2 loading and measurements of isometric tension and intracellular calcium concentration

Isometric tension and $[Ca^{2+}]_i$ were measured simultaneously in the tracheal strips, as previously reported by OZAKI *et al.* [21]. Strips were superfused with PSS containing 5 μM acetoxymethyl ester of fura 2 (fura 2-AM) gassed with a 95% O_2 5% CO_2 mixture at 37°C and pH 7.4 for 3 h under protection from light. The fura 2-AM was dissolved in dimethyl sulphoxide (DMSO) and 0.02% Cremophor EL, a noncytotoxic detergent. This concentration of Cremophor EL did not change the characteristics of smooth muscle responsiveness. After the fura 2 loading, the muscle strips were washed with PSS (37°C) for 20–30 min to remove all extracellular fura 2-AM and DMSO and to allow the de-esterification of any remaining fura 2-AM in the cytosol to occur. Each tracheal strip was mounted horizontally in the bottom of a 5-mL organ bath made of a quartz glass (37°C) and attached to a fluorimeter (CAF-110; Japan Spectroscopic, Tokyo, Japan). One end of the strip was connected to a strain gauge transducer (Orientec, Tokyo, Japan) monitoring isometric tension. Through the bottom of the bath, an excitation light (a spot of 2–3 mm in diameter) from a xenon high-pressure lamp (75 W) equipped with a rotating wheel holding 340-nm and 380-nm interference filters was focused on part of the strip. The muscle strip was illuminated alternately, at a cycle of 48 Hz, with the two excitation wavelengths (340 nm and 380 nm), and fluorescence emitted from the strip was collected using a photomultiplier, through a 500-nm filter. The intensity of fluorescence at 340 nm (F340) and 380 nm (F380) was measured, and the ratio F340/F380) calculated automatically as an index of $[Ca^{2+}]_i$ [21]. In the muscle strips successfully loaded with fura 2, an increase in F340 and a decrease in F380 represented an increase in $[Ca^{2+}]_i$. To distinguish the fura 2 Ca^{2+} signal from autofluorescence or movement artefact, F340 and F380 were continuously monitored. Only the preparations in which F340 and F380 changed in a mirror image manner were used. Absolute values of $[Ca^{2+}]_i$ were not calculated, since the dissociation constant of fura 2 for Ca^{2+} in the smooth muscle cytosol may be different from that *in vitro* [23]. Instead, the ratio F340/F380) was used as an index of $[Ca^{2+}]_i$ as previously reported [19–21]. After equilibration to stabilize muscle tension and fluorescence (F340, F380 and F340/F380 ratio) under a resting tension of 1 g for 60–90 min, the following experimental protocol was performed.

Experimental protocol

Muscle tension and $[Ca^{2+}]_i$ (F340/F380) were measured before and after tissue exposure to a solution containing a

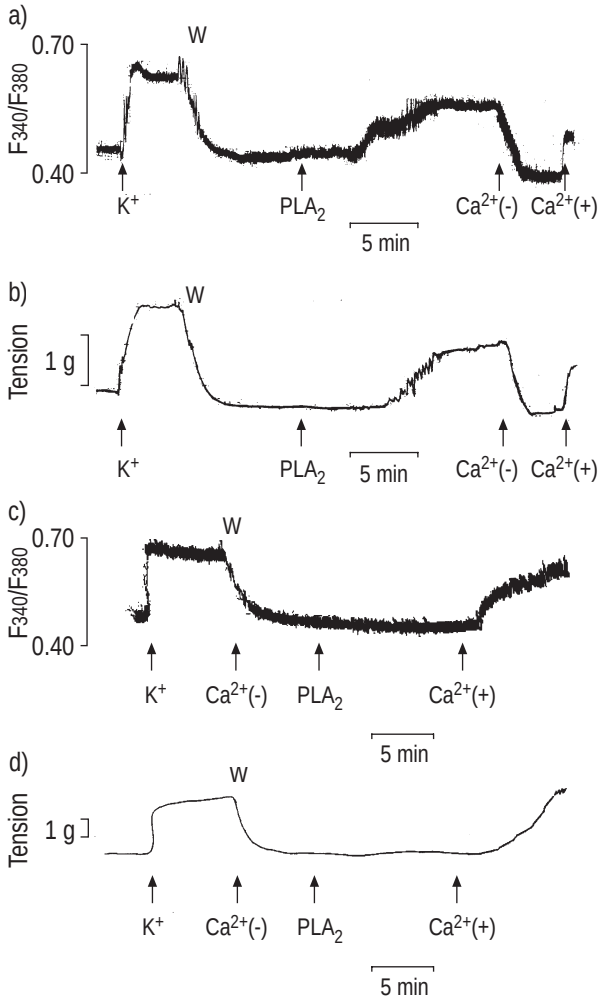

Fig. 1 – Representative trace of changes in: a, c) intracellular calcium concentration (fluorescence at 340 nm (F_{340})/fluorescence at 380 nm (F_{380})); and b, d) muscle tension of a fura 2-loaded bovine tracheal strip. Strips were treated with a high concentration of K^+ (72.7 mM) followed by the application of a high concentration (0.5 $\mu g \cdot mL^{-1}$) of phospholipous A_2 (PLA_2) in the presence or absence of extracellular Ca^{2+}. a, b) Response to 0.5 $\mu g \cdot mL^{-1}$ PLA_2 followed by Ca^{2+}-free physiological saline solution (PSS) containing 0.1 μM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid. c, d) Response to 0.5 $\mu g \cdot mL^{-1}$ PLA_2 following tissue exposure to Ca^{2+}-free PSS containing 0.1 μM EGTA. W: wash-out with PSS; Ca^{2+}: (-): the exchange of Ca^{2+}-containing PSS for Ca^{2+} - free PSS containing 0.1 μM EGTA; Ca^{2+} (+): the replacement of Ca^{2+} -free PSS with Ca^{2+} containing PSS.

high K^+ concentration (72.7 mM) (made by substituting equimolar KCl for the NaCl of the PSS). Measurements at the high K^+ concentration were made when muscle contraction had attained a steady state. Muscle tension and $[Ca^{2+}]_i$ in the resting state were taken as 0%, and those in the high K^+ concentration (72.7 mM)-stimulated states were taken as 100%. The muscle strip was next washed three times with PSS to return it to the resting state.

The concentration/response relations of muscle strips to PLA_2 were determined, and the threshold concentration for PLA_2 found to be 0.2 $\mu g \cdot mL^{-1}$. Two types of contractile agent were used, a high concentration of K^+ and acetylcholine (ACh), to clarify whether PLA_2 interacts with voltage-dependent agonists or muscarinic agonists as regards the contraction of tracheal smooth muscle. The threshold concentration, the half maximally effective concentration (EC_{50}) and the maximum response were determined from the log concentration/response curves for each agent. The PLA_2-treated group was defined as follows. First, the tissues were incubated at high K^+ concentration (72.7 mM) and then washed. Then PLA_2 (0.02 $\mu g \cdot mL^{-1}$, a concentration that did not itself cause contraction) was applied, and, in its presence, the concentration/response relations for a high K^+ concentration (5.4, 18.2, 36.4 and 72.7 mM) and for ACh (1 nM–10 μM) were determined. Each concentration of spasmogen was allowed to equilibrate for 5–10 min. The control group was treated as follows. The same procedure as that used for the PLA_2- treated group was followed, except that no PLA_2 was applied before obtain-ing the concentration/response relations for K^+ or ACh. Muscle tension and F340/F380 were measured simultaneously when the muscle strips had reached the steady state for each concentration of contractile agent.

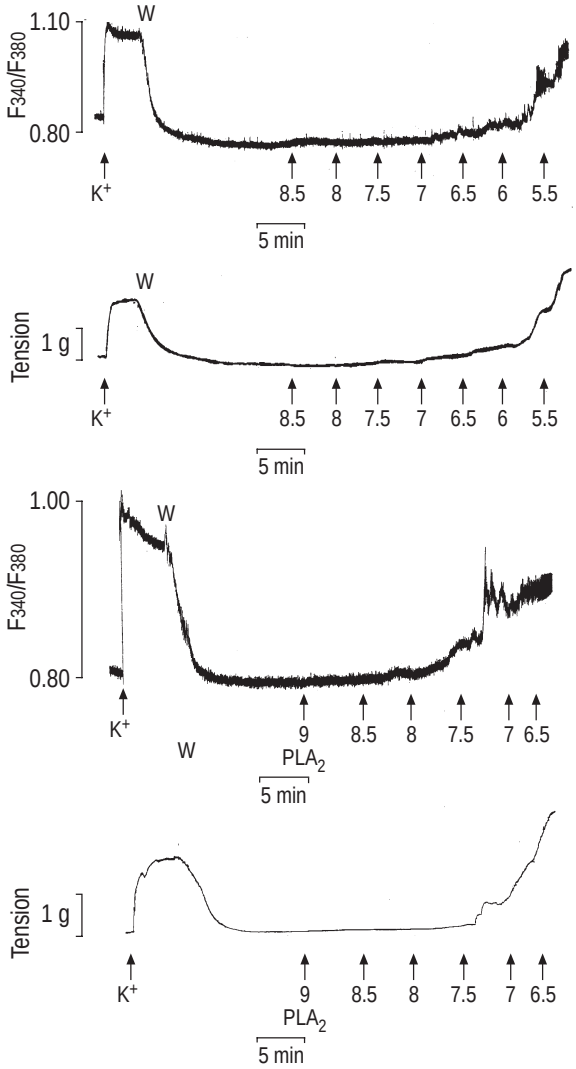

Fig. 2 – Representative trace of changes in: a, c) intracellular calcium concentration (fluorescence at 340 nm (F_{340})/fluorescence at 380 nm (F_{380})); and b, d) muscle tension induced by cumulative increases in the concentration of acetylcholine (ACh) in the absence (a, b) or presence (c, d) of a low concentration of phospholipase A_2 (0.02 $\mu g \cdot mL^{-1}$) in fura 2-loaded bovine tracheal strips. The numbers by the arrows represent the logM concentration of ACh. Before wash-out the strips were treated with a high concentration of K^+ (72.7 mM).

Participation of arachidonic acid metabolites

In order to determine whether AA metabolites participate in the effects of PLA_2 on muscle contraction, isometric tension and $[Ca^{2+}]_i$ experiments were performed following tissue incubation with the cyclo-oxygenase inhibitor indomethacin (IND; 10 μM), the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; 10 μM), a thromboxane (TX) synthetase inhibitor (OKY-046; 10 μM) and a TXA_2 receptor antagonist (ONO-3708; 10 μM). After pretreatment with IND, NDGA, OKY-046 or ONO-3708 for 15 min at room temperature, the muscle strips were challenged with PLA_2 (0.02 or 0.5 $\mu g \cdot mL^{-1}$) for an additional 5 min, and then isometric muscle tension and $[Ca^{2+}]_i$ measured simultaneously.

Data analysis and statistics

Data are expressed as mean±SEM. The significance of differences between group means was assessed using analysis of variance and Fisher's protected least significant differences for unpaired samples. A p-value <0.05 was taken as significant.

Table 1. – Contractile and intracellular calcium concentration ($[Ca^{2+}]_i$) responses of bovine tracheal strips to a high concentration of phospholipase A_2 (0.5 $\mu g \cdot mL^{-1}$) after pretreatment with indomethacin (IND), nordihydroguaiaretic acid (NDGA), OKY-046 or ONO-3708

	No pretreatment	IND^+	$NDGA^+$	$OKY\text{-}046^+$	$ONO\text{-}3708^+$
Contraction %	64.5±12.1	21.5±9.5*	57.2±26.1	19.8±9.8*	22.3±9.0*
$[Ca^{2+}]_i$ %	57.3±10.1	29.2±6.6*	37.5±10.1	23.5±7.3*	29.8±6.4*

+: 10 m. Values are presented as mean±SEM (n=6) and are a percentage of the response induced by 72.7 mM K^+. *: p<0.05 *versus* phospholipase A_2.

Drugs

The following drugs were used: PLA_2 (bee venom), IND, NDGA, ACh chloride (Sigma Chemical Co., St Louis, MO, USA); fura 2-AM (Dojindo Laboratories, Kumamoto, Japan); Cremophor EL (Nakarai Chemicals, Kyoto, Japan); OKY-046 ((*E*)-3 [P-(1H-imidazole-1-methyl)phenyl]-2-propanoic acid hydrochloride monohydrate) (Kissei Pharmaceutical Co., Matsumoto, Japan, and Ono Pharmaceutical Co., Osaka, Japan); and ONO-3708 (7[2α4α-(dimethylmethano)-6β-(2-cyclopentyl-2β-hydroxyacetamido)1-α-cyclohexyll-5(Z)-heptenoic acid) (Ono Pharmaceutical Co.).

Results

In fura 2-unloaded tracheal muscle strips, high K^+ concentration or ACh increased both F340 and F380 with an increase in muscle tension. Because the increments in F340 and F380 were proportional, F340/F380 did not change during the course of the experiment. It was sometimes observed that the stimulants induced a rapid and transient decrease, which returned to baseline or gradually increased above the resting level. These results seemed to be due to insufficient fura 2-loading. Sufficient fura-2 loading revealed good reproducibility and slow change. It was observed that - one preparation was successfully loaded with fura 2 and another from the same animal insufficiently loaded. Approximately 25% of fura 2-loaded preparations were insufficiently loaded, and thus were unsuitable and discarded.

Response of tracheal strips to a high concentration of phospholipase A_2

Figure 1 shows representative traces of isometric tension and $[Ca^{2+}]_i$ in bovine tracheal strips after the application of a high concentration of PLA_2 (0.5 $\mu g \cdot mL^{-1}$). A few minutes after the application of PLA_2, the $[Ca^{2+}]_i$ gradually increased, and then the tracheal strips started to contract. The contractile response disappeared when the PSS in the organ bath was replaced with Ca^{2+}-free PSS containing 0.1 μM EGTA for 5–10 min. After the Ca^{2+}-free PSS containing EGTA was replaced with PSS, the muscle strips again contracted (fig. 1a and b). Figure 1c and d show the effect of withdrawing the extracellular Ca^{2+} before the addition of a high concentration of PLA_2 (0.5 $\mu g \cdot mL^{-1}$). Figure 1 illustrates that the contractile response induced by PLA_2 (0.5 $\mu g \cdot mL^{-1}$) completely depends on the extracellular Ca^{2+} concentration. After the application of 0.5 $\mu g \cdot mL^{-1}$ PLA_2 in the presence of extracellular Ca^{2+}, the muscle tension was 64.5±12.1%, and the $[Ca^{2+}]_i$ was 57.3±10.1%, compared with the values obtained at high K^+ concentration (taken as 100%) (table 1).

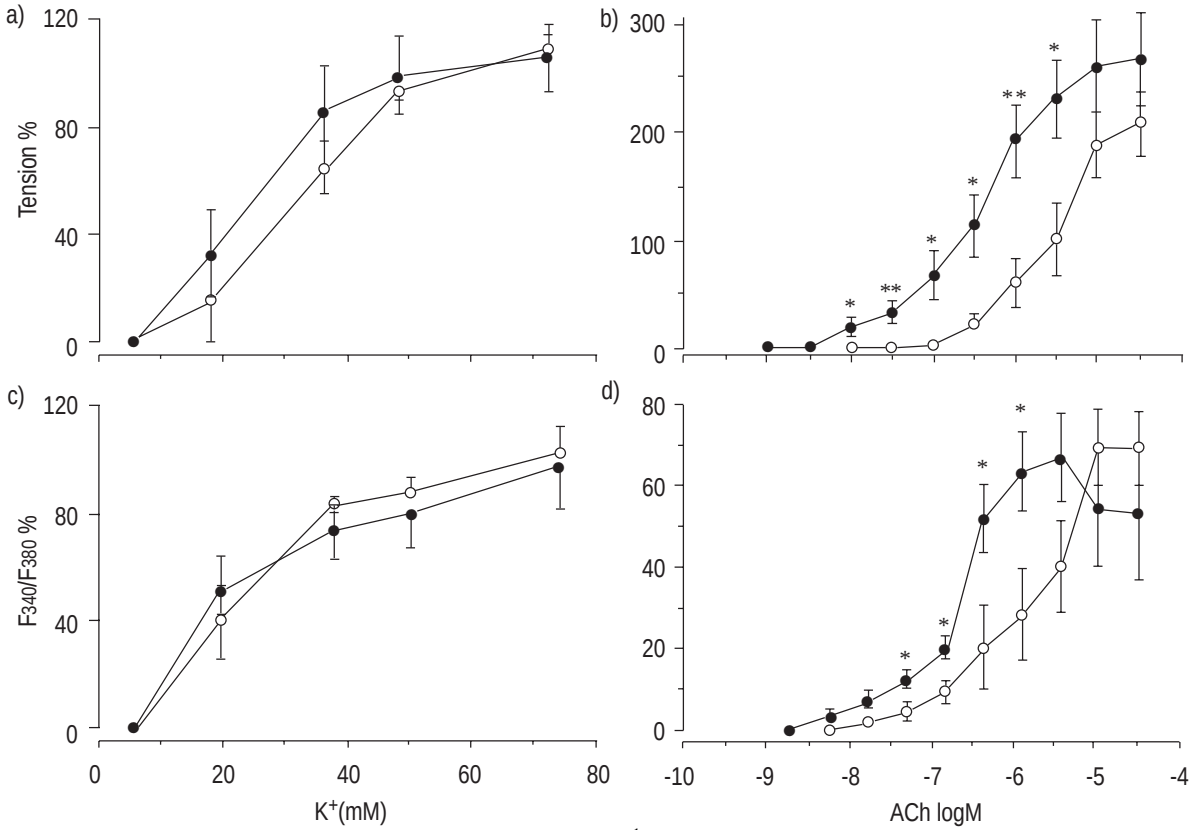

Fig. 3 – Effect of a low concentration of phospholipase A_2 (PLA_2; 0.02 $\mu g \cdot mL^{-1}$) on the concentration/response relations of: a) muscle contraction; and c) intracellular calcium concentration $[Ca^{2+}]$ (fluorescence at 340 nm (F_{340})/fluorescence at 380 nm (F_{380})) responses to a high concentration of K^+, and on the log concentration/response relations of: b) muscle contraction; and d) $[Ca^{2+}]_i$ responses to acetylcholine (ACh) in bovine tracheal strips. Each data point of the control group (○) and PLA_2-treated group (●) represents the mean of six preparations, and SEM is shown by vertical bars. The muscle tension and $[Ca^{2+}]_i$ induced by 72.7 mM K^+ were taken as 100%. *: $p<0.05$. **: $p<0.01$ *versus* control.

Response of tracheal strips to a low concentration of phospholipase A_2

Figure 2 shows representative traces of the changes in isometric tension and $[Ca^{2+}]_i$ induced by cumulative increases in the concentration of (ACh) in the absence (control group) (fig. 2a and b) and presence of PLA_2 (PLA_2-treated group) (fig. 2c and d). In the PLA_2-treated group, the threshold concentrations for contractile responses and the elevation of $[Ca^{2+}]_i$ were lower than those in the control group.

The effects of PLA_2 on the concentration/response relations of isometric tension and $[Ca^{2+}]_i$ to the high K^+ solution are shown in figure 3a and b. The concentration/response relations of both parameters did not differ significantly between the PLA_2-treated and control groups. In contrast, as shown in figure 3c and d, a low concentration (0.02 $\mu g \cdot mL^{-1}$) of PLA_2 shifted the concentration/response curves of both muscle tension and $[Ca^{2+}]_i$ to ACh to the left. The threshold concentration and EC50 of the PLA_2-treated group were significantly lower than those of the control group. There were no significant differences in the maximum responses of both muscle tension and $[Ca^{2+}]_i$ induced by ACh between the two groups (table 2).

As shown in figure 4, the Ca^{2+} sensitivity of intracellular contractile elements was evaluated using the $[Ca^{2+}]_i$/tension relationship. The slope of the $[Ca^{2+}]_i$/tension relation curve in the presence of ACh was greater than that in the presence of a high concentration of K^+, *i.e.* ACh induced stronger contractions at the same level of $[Ca^{2+}]_i$ than did a high concentration of K^+ in both the control and PLA_2-treated groups. These results suggest that agonists such as ACh sensitized the contractile elements to Ca^{2+}. However, the slopes of the curves for both the high concentration of K^+ and ACh for the PLA_2-treated group were not significantly increased compared with those for the control group (analysis of covariance). This suggests that the Ca^{2+} sensitivity of the contractile elements to ACh was not significantly different between the PLA_2-treated and control groups.

Participation of arachidonic acid metabolites

With the high concentration of PLA_2 (0.5 $\mu g \cdot mL^{-1}$), IND pretreatment significantly inhibited both muscle contraction and increase in $[Ca^{2+}]_i$, but NDGA pretreatment did not (table 1). The application of OKY-046 and of ONO-3708 significantly inhibited the responses of smooth muscle contraction and Ca^{2+} mobilization to PLA_2.

The leftward shift of the concentration/response curves of both muscle tension and $[Ca^{2+}]_i$ for ACh in the presence of the low concentration of PLA_2 (0.02 $\mu g \cdot mL^{-1}$) almost disappeared after pretreatment with IND (fig. 5a and b), but persisted after pretreatment with NDGA (fig. 5c and d). The IND pretreatment, however, did not influence the concentration/response curve of muscle tension or $[Ca^{2+}]_i$ for ACh in the control group. The leftward shift in the concentration/response curves for ACh in the presence of the low concentration of PLA_2 was inhibited by OKY-046 (fig. 6a and b) and by ONO-3708 (fig. 6c and d), and the log concentration/response curves of tension and $[Ca^{2+}]_i$ in the presence and absence of PLA_2 were not significantly different.

Table 2. – Effect of a low concentration of phospholipase A_2 (PLA_2); 0.02 $\mu g \cdot mL^{-1}$) on contractile and intracellular calcium concentration ($[Ca^{2+}]_i$) responses of bovine tracheal strips to acetylcholine

	Control	PLA_2	p-value
Contraction			
Threshold -logM	7.17±0.41	8.55±0.28	<0.01
EC50 -logM	5.71±0.36	6.44±0.19	<0.01
Max %	218±29	259±43	NS
$[Ca^{2+}]_i$			
Threshold -logM	8.28±0.42	8.87±0.32	<0.05
EC50 -logM	5.72±0.66	6.84±0.21	<0.05
Max %	69±9	66±11	NS

Data are presented as mean±SEM (n=6). Threshold: threshold concentration; EC50: half maximally effective concentration; Max: maximum response (percentage of response induced by 72.7 mM K^+.)

Discussion

The present study obtained the following findings. 1) A high concentration (0.5 $\mu g \cdot mL^{-1}$) of bee venom PLA_2 caused bovine smooth muscle to contract by promoting the

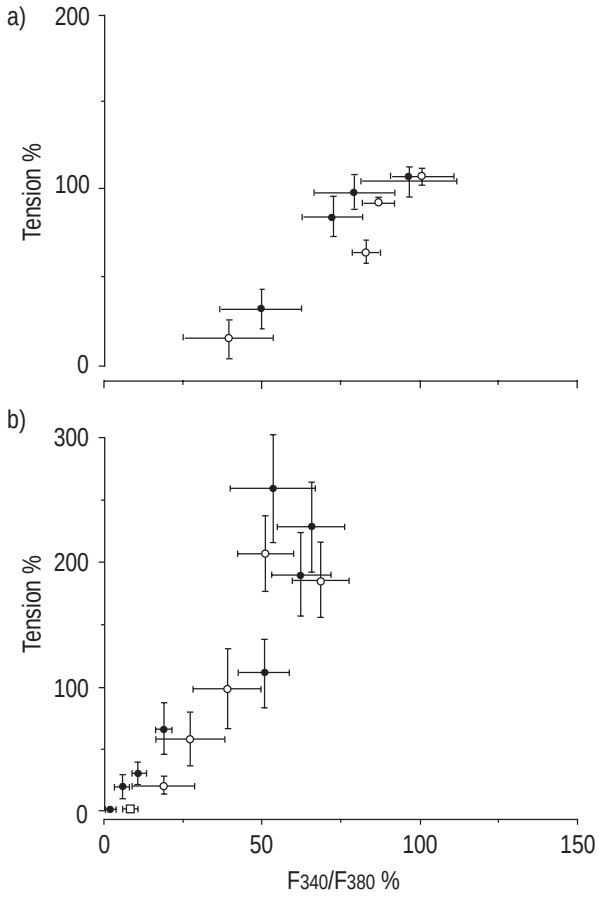

Fig. 4 – Effect of phospholipase A_2 (PLA_2) on the Ca^{2+} sensitivity of the contractile elements in bovine tracheal strips, shown by the intracellular calcium concentration $[Ca^{2+}]_i$/tension relation induced by: a) a high concentration of K^+ (5.4–72.7 mM); and b) ACh (1 nM–10 μM) in the control (○) and PLA_2-treated groups (●). The muscle tension and $[Ca^{2+}]_i$ (fluorescence at 340 nm (F_{340})/fluorescence at 380 nm (F_{380})) induced by 72.7 mM K^+ were taken as 100%. Vertical and horizontal bars represent mean±SEM.

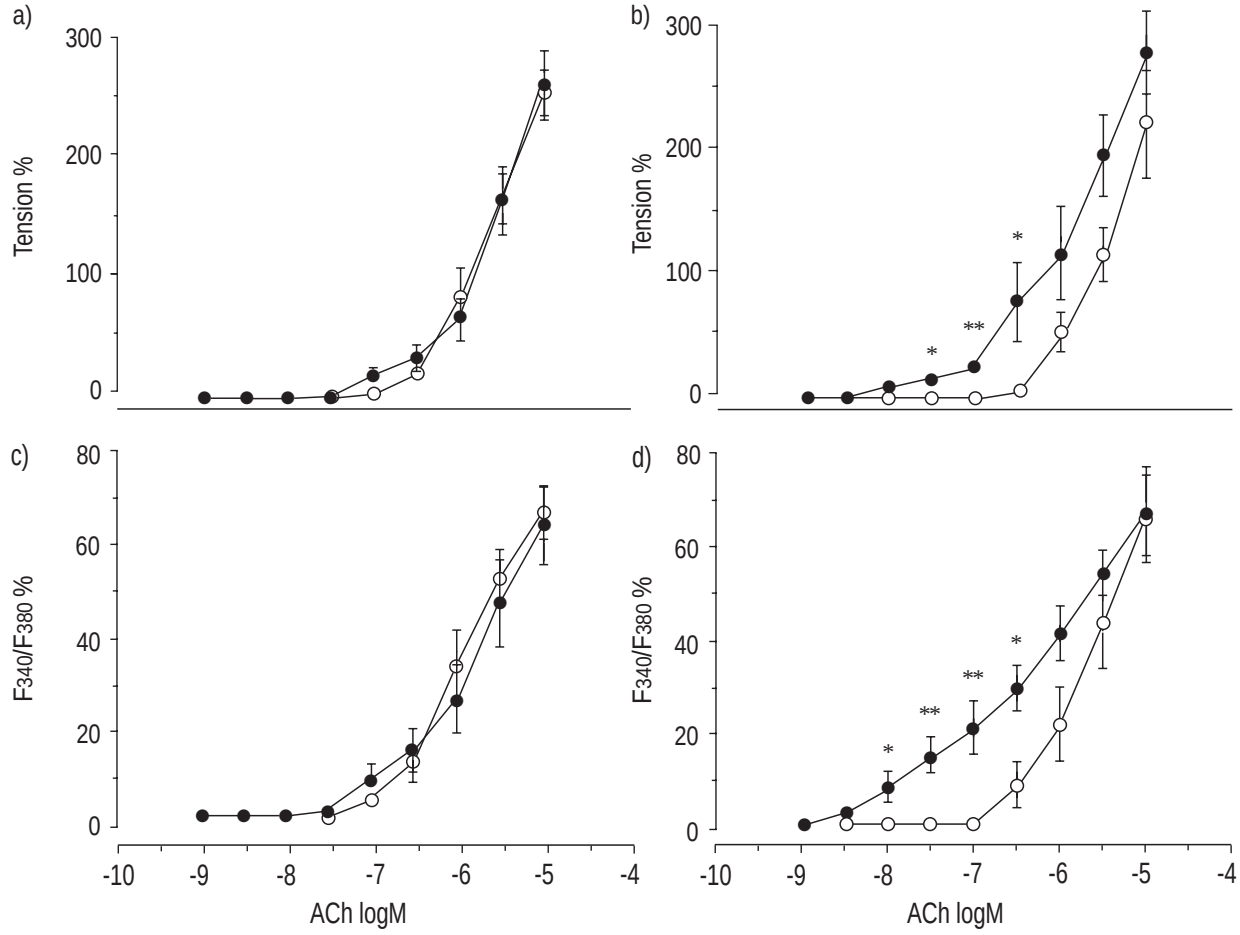

Fig. 5 – Effect of pretreatment with: a, b) indomethacin (10 μM); and c, d) nordihydroguaiaretic acid (10 μm) on concentration/response relations in: a, c) muscle contraction; b, d) intracellular calcium concentration (fluorescence at 340 nm (F340)/fluorescence at 380 nm (F380)) responses to acetylcholine (ACh) in bovine tracheal strips incubated with a low concentration (0.02 $\mu g \cdot mL^{-1}$) of phospholipase A_2. The muscle tension and $(Ca^{2+})i$ induced by 72.7 mM K^+ were taken as 100%. *: $p<0.05$, **: $p<0.01$ *versus* control.

production of AA metabolites. This action was inhibited by IND, a cyclooxygenase inhibitor, but not by NDGA, a lipoxygenase inhibitor. 2) The contractile response and increase in ($[Ca^{2+}]i$) induced by ACh were enhanced after the application of a low concentration of PLA_2 (0.02 $\mu g \cdot mL^{-1}$). PLA_2 failed to modify the equivalent responses to a high concentration of K^+. 3) The altered $[Ca^{2+}]i$/tension relationship for ACh in the presence of PLA_2 showed that the augmented muscle contraction was the result not of enhanced Ca^{2+} sensitivity but rather of increased Ca^{2+} mobilization. The augmentation of both the contractile response and the $[Ca^{2+}]i$ response to ACh after the application of PLA_2 was inhibited by IND, OKY-046 (a TX synthase inhibitor) and ONO-3708 (a TX receptor antagonist), but not by NDGA.

The mechanism by which PLA_2 augments airway sensitivity to ACh is not known. In the present study, the low concentration of PLA_2 may have increased the sensitivity of tracheal smooth muscle as much as 100-2 fold. This PLA_2 concentration, however, did not cause the tracheal smooth muscle to contract. This hypersensitivity might result from augmented intracellular Ca^{2+} mobilization, but not from increased sensitivity of the contractile elements to Ca^{2+}. The mechanism by which Ca^{2+} mobilization was augmented in bovine tracheal smooth muscle in the presence of PLA_2-II was not determined in the present study. It is suspected that the muscarinic receptor-coupled pathway may be selectively augmented by PLA_2, because contraction and increases in $[Ca^{2+}]i$ evoked by a high K^+ concentration were not altered after treatment with PLA_2-II. According to the $[Ca^{2+}]i$/tension curves, the sensitivity of intracellular contractile elements to Ca^{2+} was greater in the presence of ACh than in the presence of a K^+-rich medium. Furthermore PLA_2-II did not seem to influence the Ca^{2+} sensitivity of the intracellular contractile elements. This suggests that hypersensitivity to ACh in bovine tracheal smooth muscle after application of PLA_2 may be evoked by an alteration of the function of the muscarinic receptor or of the muscarinic receptor-coupled signal transduction system.

PLA_2-II induced augmentation of contraction and Ca^{2+} mobilization by ACh in tracheal smooth muscle might be mediated by TXA_2, although the source of the TXA_2 was not determined in this study. STREK *et al.* [24] reported that epithelial metabolism is very important for producing the TXA_2 necessary to elicit airway smooth muscle contraction and to augment its sensitivity to muscarinic agonists. In the present study, the muscle strips were devoid of epithelium but PLA_2-II nevertheless induced hypersensitivity to ACh. There are many kinds of lung cell

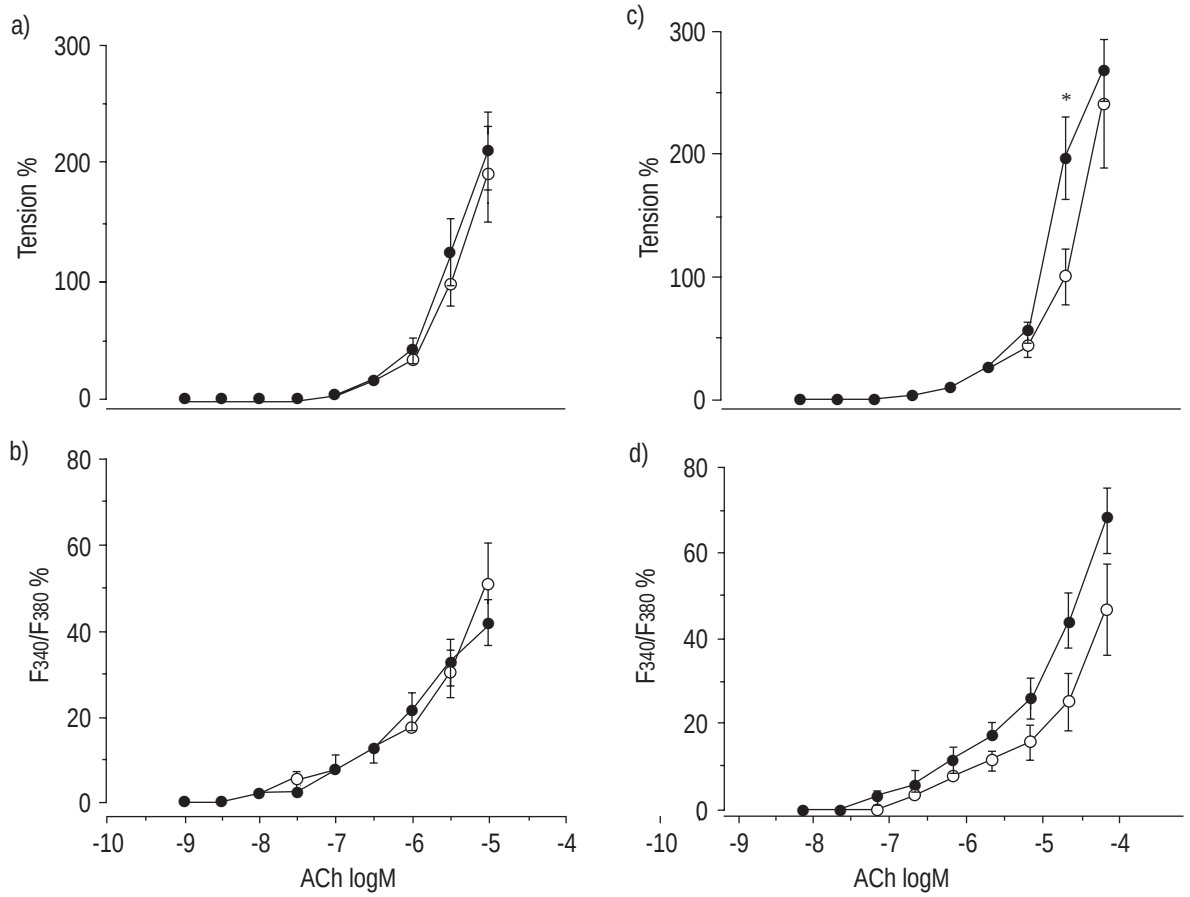

Fig. 6 – Effect of pretreatment with: a, b) OKY-046 (10 μM); and c, d) ONO-3708 (10 μM) on concentration/response relations in: a, c) muscle contraction; b, d) intracellular calcium concentration (fluorescence at 340 nm (F_{340})/fluorescence at 380 nm (F_{380})) responses to acetylcholine (ACh) in bovine tracheal strips incubated with a low concentration (0.02 $\mu g \cdot mL^{-1}$) of phospholipase A_2. The muscle tension and $[Ca^{2+}]_i$ induced by 72.7 mM K^+ were taken as 100%. *: $p<0.05$ *versus* control.

phospholipids. Snyder *et al*. [25] showed that PLA_2-II had contractile effects on guinea-pig lung pleural strips which had no epithelial component. Kanemasa *et al*. [26] reported the augmented contraction of tracheal smooth muscle without epithelium in response to PLA_2-I. Therefore, cells composing the subepithelium and submucosa or a smooth muscle layer in the prepared tracheal muscle might be important for the release of AA metabolites after the application of PLA_2.

In the present study, a high concentration of bee venom PLA_2 directly caused bovine tracheal smooth muscle to contract following an increase in $[Ca^{2+}]_i$. This contractile response depended completely on the extracellular Ca^{2+} concentration. This result is compatible with that found in a study of guinea-pig lung tissue [26]. The level of serum PLA_2 in asthmatic patients was found to be high compared to that found in healthy nonasthmatic volunteers [11]. PLA_2 catalyses the first step in the synthesis of inflammatory mediators that have been associated with bronchoconstriction [14, 15]. Bronchial asthma was recently defined as eosinophilic bronchial inflammatory changes [27], and PLA_2 might therefore play an important role in the pathogenesis of bronchial asthma.

It was found that the direct contractile responses of smooth muscle to bee venom PLA_2-II were inhibited after pretreatment with OKY-046 and ONO-3708 as well as IND, suggesting that newly synthesized TXA_2 might play a role mediated by PLA_2-II in smooth muscle contraction and Ca^{2+} mobilization in bovine tracheal smooth muscle. Kanemasa *et al*. [26] have shown that the smooth muscle contraction and calcium mobilization induced by a high concentration of PLA_2-I were inhibited after treatment with IND, but not with NDGA, which implicated the contribution of some kind of prostaglandin in smooth muscle contraction and Ca^{2+} mobilization. The present data are compatible with those of Kanemasa *et al*. [26] despite the use of a different type of PLA_2. AA metabolites, especially TXA_2, might play an important role in muscle contraction and Ca^{2+} mobilization after application of bee venom PLA_2. In fact, subthreshold concentrations of TXA_2 mimetic agents (STA2 or U-46619) have been shown to augment bronchial responsiveness to ACh or histamine [28, 29]. TXA_2-releasing cells induced by PLA_2-II have not yet been detected in the lung, although PLA_2-I was shown initially to be bound to specific binding sites of epithelial cells, macrophages and polymorphonuclear leukocytes and then to release TXA_2. The contractile response may thus be evoked as a consequence of TXA_2 production in guinea-pig lung parenchyma [26].

Permeabilization with saponin has been the most commonly used method for the quantitative evaluation

of the $[Ca^{2+}]i$/tension relation in smooth muscle [22]. However, saponin-permeabilized preparations do not respond to agonists because of the disruption of receptor function. Moreover, the standard method of measuring Ca^{2+} sensitivity is to fix the intracellular calcium at various levels, add a drug and observe, when the Ca^{2+} level is clamped, whether the tension generated by the smooth muscle changes. The simultaneous measurement of $[Ca^{2+}]i$ and muscle tension was recently performed in intact smooth muscle. This method, used in the present study, is very useful for studying agonist-induced changes in the Ca^{2+} sensitivity of contractile elements, because receptor and signal transduction systems remain intact.

In this study, the effects of K^+-induced depolarization were not studied. Certainly, it seems that depolarization induced by a high concentration of K^+ releases endogenous ACh by affecting presynaptic nerve axons and nerve terminals as well as postsynaptic smooth muscle cells. The effects of K^+-induced depolarization on cholinergic nerve/airway smooth muscle preparations have not been studied in detail and are complex [21, 30]. Small elevations in extracellular K^+ concentration augment the responsiveness of the airways, by increasing the release of ACh from intramural cholinergic nerve terminals [31]. Larger increases in K^+ appear to be inhibitory, possibly due to voltage-dependent effects that occur both pre- and postsynaptically. However, their report [31] indicated that K^+-induced depolarization did not increase the contractile responses of airway smooth muscle to exogenous ACh. Moreover, levels of endogenous ACh in both normal and denervated nerve termini were pM [32]. As compared with exogenous ACh in this study, the amount of endogenous ACh induced by a high concentration of K^+ was much smaller. In addition, there is a possibility that mast cells activated by a high K^+ concentration may release chemical mediators. However, some papers showed that elevated K^+ levels in the external environment do not activate mast cell secretion [33, 34].

The pathogenesis of altered Ca^+ handling of airway smooth muscle in bronchial asthma has not been determined [16, 17]. It is anticipated that further studies using the present method with experimental asthma models or asthmatic patients would clarify whether an abnormality of Ca^+ homeostasis in airway smooth muscle cells is involved in the pathogenesis of asthma. If so, this might provide a new approach to the therapy of bronchial asthma.

In summary, a low concentration of phospholipase A_2 enhanced smooth muscle responsiveness to acetylcholine by agonist-mediated calcium mobilization *via* a thromboxane A_2-mediated pathway. The alteration of the responses of the muscarinic receptor and signal transduction systems to PLA_2-II in tracheal smooth muscle might play an important role in the pathogenesis of bronchial hyperresponsiveness.

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