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# Theophylline attenuates $Ca^{2+}$ sensitivity and modulates BK channels in porcine tracheal smooth muscle

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**1** Theophylline, a nonselective phosphodiesterase inhibitor, has long been regarded as a major bronchodilator in the treatment of human asthma. Using front-surface fluorometry with fura-2 and α-toxin permeabilization, the effects of theophylline on intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), tension development and $Ca^{2+}$ sensitivity of the contractile apparatus were investigated in porcine tracheal smooth muscle strips.

**2** Application of theophylline induced a relaxation without a significant decrease in $[Ca^{2+}]_i$ when strips were precontracted by 40 mM $K^+$ depolarization, while theophylline significantly decreased both $[Ca^{2+}]_i$ and tension induced by carbachol.

**3** The effects of theophylline on the increases in $[Ca^{2+}]_i$ and tension induced by carbachol were significantly inhibited by iberiotoxin, an inhibitor of large-conductance $Ca^{2+}$-activated $K^+$ channels.

**4** In the absence of extracellular $Ca^{2+}$, theophylline significantly attenuated carbachol-induced transient increases in tension development, while it did not affect carbachol-induced transient increase in $[Ca^{2+}]_i$.

**5** The $[Ca^{2+}]_i$–force relationship, which was determined by cumulative applications of extracellular $Ca^{2+}$ (0–5 mM) during 40 mM $K^+$ depolarization, was significantly shifted to the right by theophylline.

**6** In α-toxin permeabilized strips, theophylline significantly increased the $EC_{50}$ value of $[Ca^{2+}]_i$ for contraction and enhanced the effect of cAMP, but not of cGMP.

**7** These results indicate that theophylline induces relaxation of the porcine tracheal smooth muscle through an activation of BK channels, and a resultant decrease in $[Ca^{2+}]_i$ and an attenuation of $Ca^{2+}$ sensitivity, presumably through the action of cAMP.
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**Keywords:** Theophylline; porcine tracheal smooth muscle; intracellular $Ca^{2+}$ concentration; $Ca^{2+}$ sensitivity; large-conductance $Ca^{2+}$-activated $K^+$ channel; iberiotoxin; cAMP; cGMP

**Abbreviations:** BK channel, large-conductance $Ca^{2+}$-activated $K^+$ channel; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; CCh, carbachol; CSS, cytosolic substitution solution; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-bis(beta-aminoethyl ether) *N*,*N*,*N*′,*N*-tetraacetic acid; fura-2/AM, fura-2 acetoxymethyl ester; IbTX, iberiotoxin; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PSS, physiological salt solution

## Introduction

Theophylline has been used worldwide for the treatment of asthma and chronic obstructive pulmonary diseases for several decades, due in part to its low cost and its ease of administration. Theophylline is a bronchodilator that may also be used to enhance respiratory muscle function and mucociliary clearance; it also acts in the central nervous system to enhance ventilation. Theophylline has been shown to relax an isolated airway smooth muscle preparation. The mechanisms whereby theophylline causes tracheal smooth muscle relaxation are not well understood.

Theophylline-induced airway smooth muscle relaxation has been attributed to the increased cellular cAMP and cGMP levels, because theophylline is known to be a nonselective phosphodiesterase (PDE) inhibitor. It is thought that cyclic nucleotides such as cAMP and cGMP cause smooth muscle relaxation mainly by decreasing intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$), as well by attenuating $Ca^{2+}$ sensitivity of the contractile apparatus (Nishimura & van Breemen, 1989; Ushio-Fukai *et al*., 1993). To explain the cAMP- and/or cGMP-induced decrease in $[Ca^{2+}]_i$, several mechanisms have been proposed, including inhibition of $Ca^{2+}$ influx due to hyperpolarization *via* stimulation of $Ca^{2+}$-activated $K^+$ channels (Sadoshima *et al*., 1988), a stimulation of $Ca^{2+}$ uptake into the intracellular stores (Mueller & van Breemen, 1979) and an increase in $Ca^{2+}$ extrusion from cells through the salcolemmal $Ca^{2+}$ pump (Bülbring & Tomita, 1987). However, the relative importance of these various mechanisms in theophylline-induced relaxation of the airway smooth muscle

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remains unclear. Moreover, it is not known whether theophylline decreases the $Ca^{2+}$ sensitivity of the contractile apparatus in airway smooth muscle.

In this study using simultaneous measurements of $[Ca^{2+}]_i$ and tension in fura-2-loaded intact muscle strips, as well as the receptor-coupled permeabilization by the α-toxin technique, we examined the mechanisms underlying theophylline-induced relaxation of the porcine tracheal smooth muscle. We determined that theophylline decreases the $Ca^{2+}$ sensitivity of the contractile apparatus and also reduces $[Ca^{2+}]_i$ through opening of iberiotoxin (IbTX)-sensitive channels, presumably large-conductance $Ca^{2+}$-activated $K^+$ channels (BK channels). The combined effects of reduction in $Ca^{2+}$ sensitivity and inhibition of $Ca^{2+}$ entry would promote smooth muscle relaxation.

## Methods

### *Tissue preparation*

The tracheas were dissected from adult pigs at a local slaughterhouse, using a protocol approved by the Animal Research Committee of the Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University. The tracheas were placed in ice-cold physiological salt solution (PSS). The lower end of the trachea (just above the first branching of the bronchus branching), comprising three tracheal rings in length, was used for the experiments. The posterior portion of the trachea was excised longitudinally, and all cartilage was detached. Both the mucosal and adventitial tissues were carefully removed under microscopic observation. The muscle sheets were transversely cut into rectangular strips measuring approximately 3 mm in length and 1 mm in width (Kai *et al*., 1993).

### *Fura-2 loading*

Tracheal strips were loaded with the $Ca^{2+}$ indicator dye fura-2 in the form of acetoxymethyl ester (fura-2/AM), as previously described (Kai *et al*., 1993). The strips were incubated in 1 ml aerated (95% $O_2$ : 5% $CO_2$) Dulbecco's modified Eagle's medium (DMEM) containing 50 μM fura-2/AM and 5% fetal bovine serum for 3 h at 37°C. After loading with fura-2, the strips were washed with normal PSS to remove excess dye from the extracellular space, and were then equilibrated in normal PSS for at least 1 h.

### *Tension recordings*

Each strip was mounted vertically in a 6 ml quartz organ bath containing PSS maintained at 37°C and bubbled with 95% $O_2$ and 5% $CO_2$. The lower end of the strip was fixed, and the upper end of the strip was connected to a force transducer (TB-612T, Nihon Koden, Japan) to record isometric tension. During the 1 h post-fura-2 loading equilibration period, strips were stimulated with 40 mM $K^+$ PSS at 5–10 min intervals, and the muscle length increased in a stepwise manner after each stimulation until the developed tension reached a maximum. When exposed to 40 mM $K^+$ PSS, most strips produced a stable tension within 10 min, with or without an initial transient force response (Kai *et al*., 1993; 1996; Yoshimura *et al*., 1995; Setoguchi *et al*., 2001). Any strips showing instability in tension, as induced by 40 mM $K^+$ PSS, were excluded from the study. The responsiveness of each strip to 40 mM $K^+$ PSS was then recorded before starting the experimental protocol, since maximal contractile responses to high $K^+$ depolarization were obtained at this concentration of $K^+$. In experiments to determine the effects of IbTX on the theophylline-induced decreases in tension, the developed tension was expressed as a percentage, assigning the values in normal (5.9 mM $K^+$) PSS and the steady state of the contraction induced by 100 nM carbachol (CCh) as 0 and 100%, respectively. For the rest of the experiments, the developed tension was expressed as a percentage, assigning the values in normal PSS and the steady-state contraction induced by 40 mM $K^+$ PSS to be 0 and 100%, respectively.

### *Measurements of fura-2 fluorescence*

Changes in the fluorescence intensity of the fura-2–$Ca^{2+}$ complex were monitored using a front-surface fura-2 fluorometer (model CAM-OF). The details of our front-surface fluorometry system have been described elsewhere (Hirano *et al*., 1990; Kai *et al*., 1993; Kanaide, 1999). In brief, two wavelengths of excitation light (340 and 380 nm) were obtained spectroscopically from a Xenon light source. The strips were illuminated by guiding the two alternating (400 Hz) wavelengths of excitation light through quartz optic fibers. The surface fluorescence of the strip was collected by glass optic fibers and introduced through a 500 nm band pass filter into a photomultiplier. We thus measured the fura-2 fluorescence intensity emission, which was induced by alternating two wavelengths of excitation light (340 and 380 nm), at 500 nm emitting light.

The ratio of the fluorescence intensities (fluorescence ratio) at 340 nm excitation to that at 380 nm excitation was monitored to estimate the changes in $[Ca^{2+}]_i$. In experiments to determine the effects of IbTX on theophylline-induced decreases in $[Ca^{2+}]_i$, the ratio was expressed as a percentage, assigning the values in normal PSS and a steady state of the $[Ca^{2+}]_i$ induced by 100 nM CCh to be 0 and 100%, respectively. For the rest of experiments, the ratio was expressed as a percentage, assigning the values in normal PSS and a steady state of the $[Ca^{2+}]_i$ induced by 40 mM $K^+$ PSS to be 0 and 100%, respectively.

### *Tension measurement in α-toxin permeabilized tracheal strips*

Permeabilization of tracheal strips by α-toxin was performed according to previously described methods (Nishimura *et al*., 1988), with minor modifications. In brief, thin strips (about 0.5 mm in width and 2 mm in length) of the porcine tracheal smooth muscle were mounted between two tungsten wires, one of which was fixed and the other one was attached to a force transducer (UL2; Minebea Co., Japan). Permeabilization was carried out in a $Ca^{2+}$-free cytosolic substitution solution (CSS; in mM: potassium methanesulfonate 100, $Na_2ATP$ 2.2, $MgCl_2$ 3.38, EGTA 10, creatine phosphate 10, Tris-maleate 20 (pH 6.8)) with 5000 U $ml^{-1}$ *Staphylococcus aureus* α-toxin for 30 min. The composition of $Ca^{2+}$ solution (activating solution) was the same as the CSS described above, except that it contained the indicated concentration of $Ca^{2+}$ buffered by

10 mM EGTA. All experiments using permeabilized tissue were performed at room temperature. In experiment to determine the $Ca^{2+}$–force relationship in the absence or presence of 300 $\mu$M theophylline, the resting tension in the relaxing solution and the maximal tension induced by 10 $\mu$M $Ca^{2+}$ were taken as 0 and 100%, respectively. In experiments to determine the relaxant effect of cAMP and cGMP on tension induced by 500 nM $Ca^{2+}$ in the absence or presence of 100 $\mu$M theophylline, the resting tension in relaxing solution and the tension induced by 500 nM $Ca^{2+}$ just before the application of theophylline and at the corresponding time point of the time-matched control were taken as 0 and 100%, respectively.

### *Solutions and drugs*

Normal PSS was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, $KH_2PO_4$ 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. High $K^+$ PSS was identical to normal PSS, except for an equimolar substitution of KCl for NaCl. $Ca^{2+}$-free PSS was produced by exclusion of $CaCl_2$ from the composition of normal PSS. PSS was bubbled with 95% $O_2$ and 5% $CO_2$, with a resulting pH of 7.4 at 37°C.

DMEM was purchased from Gibco (Grand Island, NY, U.S.A.). CCh, theophylline, α-toxin, IbTX, cAMP and cGMP were from Sigma (St Louis, MO, U.S.A.). EGTA and fura-2/AM were obtained from Dojindo (Kumamoto, Japan).

### *Data analysis*

The measured values were expressed as mean ± s.e.m. ($n$ = number of experiments using a number of different animals). Analysis of covariance was used to determine the statistical significance of the shift of the $[Ca^{2+}]_i$–tension relationship (Figure 5). Paired Student's $t$-test was used to determine the statistical significance of the effect of IbTX on the CCh-induced tension and $[Ca^{2+}]_i$ (Figure 3c), and the relaxant effect of theophylline on the CCh-induced tension and $[Ca^{2+}]_i$ in the absence or presence of IbTX (Figure 3d) and the $EC_{50}$ values of $[Ca^{2+}]_i$ in the absence or presence of 300 $\mu$M theophylline (Figure 6). Repeated measures analysis of variance and contrast analysis were used to determine the statistical significance of the effect of cAMP and cGMP in the absence or presence of 100 $\mu$M theophylline (Figure 7). For the rest of the measurements, an unpaired $t$-test was used. $P$-values less than 0.05 were considered to be significant.

## Results

### *Effects of theophylline on increases in $[Ca^{2+}]_i$ and tension induced by 40 mM $K^+$ PSS and CCh*

After recording the 100% response levels by the depolarization with 40 mM $K^+$ PSS, CCh or 40 mM $K^+$ was applied. Both CCh and 40 mM $K^+$ depolarization increased $[Ca^{2+}]_i$ and tension levels, which reached steady-state levels within 10 min. The cumulative application of theophylline (1 $\mu$M–1 mM) during the maintained phase of the contraction induced by CCh or 40 mM $K^+$ depolarization caused concentration-dependent reductions in $[Ca^{2+}]_i$ and tension (Figure 1). Comparisons were made with tension and $[Ca^{2+}]_i$ values prior to the application of theophylline. Theophylline significantly

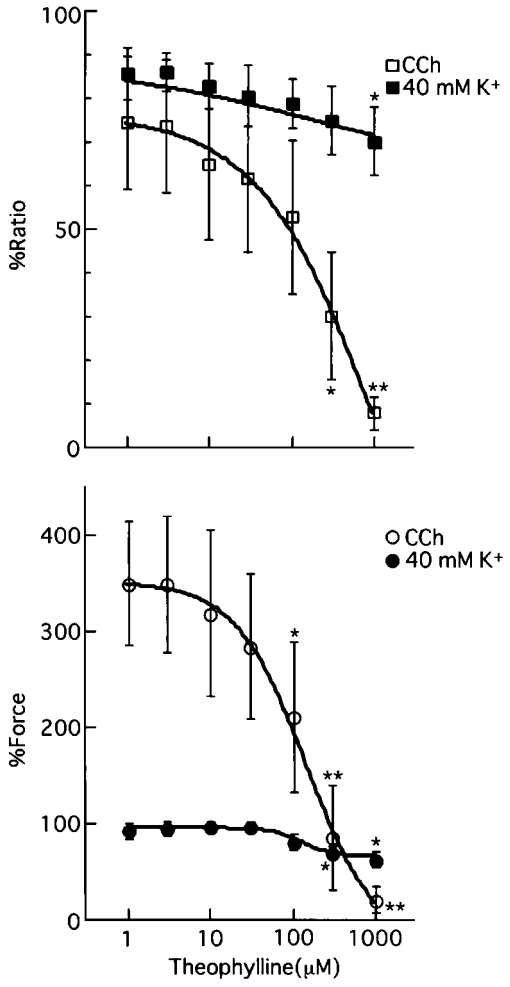


**Figure 1** The concentration-dependent effect of theophylline on the increases in $[Ca^{2+}]_i$ level and tension development induced by 40 mM $K^+$ depolarization (closed squares and closed circles, $n=4$) and 100 nM CCh (open squares and open circles, $n=4$). The fluorescence ratio and tension were expressed as a percentage by assuming the values in normal PSS (5.9 mM $K^+$ PSS) and the steady state during 40 mM $K^+$ depolarization to be 0 and 100%, respectively. The data are expressed as the means ± s.e.m. (shown as vertical bars). *$P<0.05$, compared with values just before the application of theophylline; **$P<0.01$, compared with values just before the application of theophylline (statistical significance was determined by using unpaired $t$-test).

attenuated both the increases in $[Ca^{2+}]_i$ (>300 $\mu$M theophylline) and tension (>100 $\mu$M theophylline) induced by CCh. The increases in tension and $[Ca^{2+}]_i$ produced by 40 mM $K^+$ were both inhibited by theophylline at concentrations above 300 $\mu$M and 1 mM, respectively. Application of 1 mM theophylline decreased the $[Ca^{2+}]_i$ level and tension induced by CCh to 7.89 ± 3.78 ($P<0.01$) and 20.7 ± 13.9% ($P<0.01$), respectively ($n=4$). On the other hand, application of 1 mM theophylline decreased the $[Ca^{2+}]_i$ level and tension induced by 40 mM $K^+$ depolarization to 70.2 ± 7.95 ($P<0.05$) and 62.6 ± 8.18% ($P<0.05$), respectively ($n=4$). A maximal concentration of theophylline (1 mM) did not alter the basal tension or $[Ca^{2+}]_i$ (data not shown).

A representative time course of the effect of theophylline (300 $\mu$M) on the increases in $[Ca^{2+}]_i$ and tension of porcine tracheal smooth muscle strips during contraction induced by 40 mM $K^+$ PSS is shown in Figure 2a. When theophylline

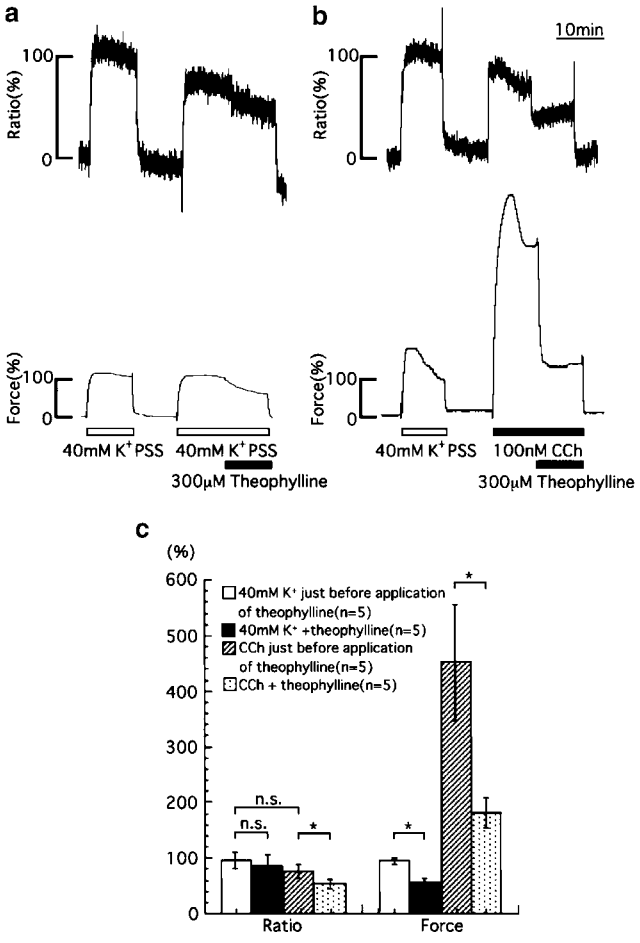


**Figure 2** Effects of 300 $\mu$M theophylline on $[Ca^{2+}]_i$ and tension of the porcine tracheal smooth muscle. Representative recordings of the effects of 300 $\mu$M theophylline on the increases in $[Ca^{2+}]_i$ and tension induced by 40 mM $K^+$ PSS (a) and 100 nM CCh (b). Theophylline was applied 10 min after the application of 40 mM $K^+$ PSS ($n=5$) or 100 nM CCh ($n=5$). (c) A summary of the results obtained from the indicated number of experiments (using a number of different animals) performed in a manner similar to those in (a, b). The data are expressed as the means$\pm$s.e.m. (shown as vertical bars). *$P<0.05$; n.s., difference not significant (statistical significance was determined by using the unpaired $t$-test).

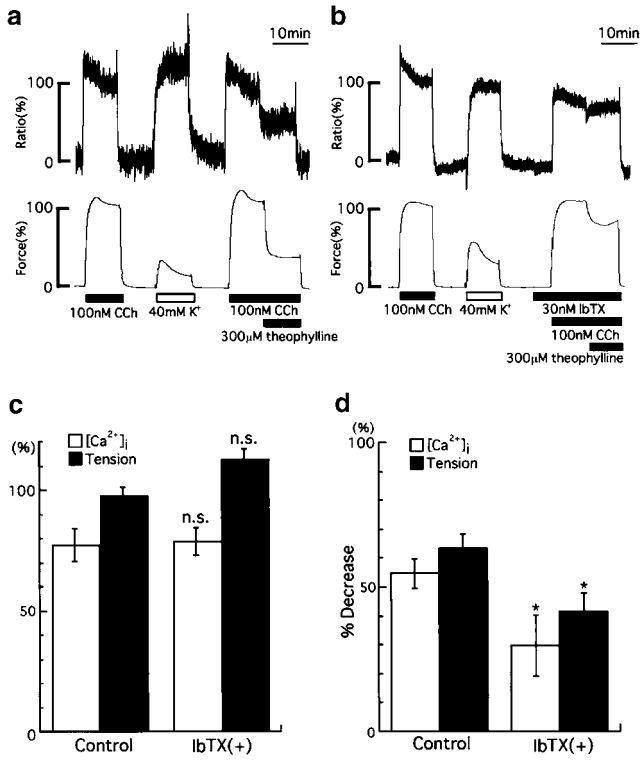


**Figure 3** Effect of theophylline on $[Ca^{2+}]_i$ and tension induced by CCh in the absence or presence of IbTX. After the responses (100%) to 100 nM CCh had been recorded, strips were incubated in normal PSS for 10 min, and then in 40 mM $K^+$ PSS for 10 min to fill intracellular $Ca^{2+}$ stores. (a) The strips were incubated with normal PSS again, and then with 100 nM CCh. At 10 min, theophylline (300 $\mu$M) was then applied during the CCh-induced contraction. (b) The recordings were obtained under similar conditions as above (a), except that 30 nM IbTX was applied 5 min before the second application of CCh. (c) A summary of the effect of IbTX on the increases in $[Ca^{2+}]_i$ and tension development induced by CCh. IbTX has no direct effect on the $[Ca^{2+}]_i$ or tension levels. (d) A summary of the percentage decreases induced by theophylline during the CCh-induced contraction in the absence or presence of IbTX. The data are expressed as the means$\pm$s.e.m. (shown as vertical bars) ($n=5$). *$P<0.05$; n.s., difference not significant (statistical significance was determined by using paired $t$-test).

(300 $\mu$M) was added 10 min after the application of 40 mM $K^+$ PSS, there was a significant reduction of tension (from 94.9$\pm$5.94 to 57.3$\pm$5.54%, $n=5$, $P<0.05$) with an insignificant decrease in $[Ca^{2+}]_i$ (from 96.3$\pm$14.7 to 86.0$\pm$18.3%, $n=5$, $P>0.05$). Figure 2b shows a representative time course of the effect of theophylline (300 $\mu$M) on the increases in $[Ca^{2+}]_i$ and tension induced by 100 nM CCh. The application of theophylline (300 $\mu$M) 10 min after the addition of 100 nM CCh caused a significant reduction of tension (from 452$\pm$104 to 181$\pm$27.1%, $n=5$, $P<0.05$), accompanied by a significant decrease in $[Ca^{2+}]_i$ (from 76.7$\pm$12.8 to 53.0$\pm$9.6%, $n=5$, $P<0.05$). Figure 2c summarizes the changes in $[Ca^{2+}]_i$ and tension induced by the applications of theophylline (300 $\mu$M) to porcine tracheal smooth muscle strips precontracted with 40 mM $K^+$ PSS or 100 nM CCh. Theophylline significantly decreased both $[Ca^{2+}]_i$ and tension when strips were precontracted by CCh, while it decreased tension without reducing $[Ca^{2+}]_i$ when strips were precontracted with 40 mM $K^+$ PSS.

### *Effects of IbTX on the theophylline-induced decreases in $[Ca^{2+}]_i$ and tension induced by CCh*

Figure 3a and b shows representative recordings of the effects of theophylline on the increases in $[Ca^{2+}]_i$ and tension induced by 100 nM CCh in the absence (a) or presence (b) of 30 nM IbTX, a BK channel blocker. When 100 nM CCh was applied, both the $[Ca^{2+}]_i$ and tension responses rapidly increased and reached plateau phases within 10 min. The values at resting and plateau phases were designated as 0 and 100%, respectively. IbTX did not directly affect the increases in $[Ca^{2+}]_i$ (77.3$\pm$6.9% in the absence and 78.7$\pm$5.8% in the presence of IbTX) and tension (97.5$\pm$3.5% in the absence and 113$\pm$4.4% in the presence of IbTX) development induced by CCh (Figure 3b and c). The application of theophylline induced sustained decreases in $[Ca^{2+}]_i$ and tension within 1 min (Figure 3a and b). In the absence of IbTX, the percent decreases in $[Ca^{2+}]_i$ and tension, calculated from the values

just before and 10 min after the application of theophylline, were 54.8±5.0 and 63.4±4.7%, respectively ($n=5$). In the presence of 30 nM IbTX, these values were inhibited significantly (29.8±10.5% for $[Ca^{2+}]_i$, 41.3±6.6% for tension, $n=5$, $P<0.05$). Figure 3d summarizes the effects of IbTX on the theophylline-induced decreases in $[Ca^{2+}]_i$ and tension during contraction induced by CCh. IbTX significantly inhibited the theophylline-induced decreases in $[Ca^{2+}]_i$ and tension.

### *Effects of theophylline on the increases in $[Ca^{2+}]_i$ and tension development induced by CCh in the absence of extracellular $Ca^{2+}$*

Figure 4a shows the representative time courses of the changes in $[Ca^{2+}]_i$ and tension development induced by 100 nM CCh in the $Ca^{2+}$-free PSS containing 2 mM EGTA. Under these conditions, basal $[Ca^{2+}]_i$ gradually declined to reach a steady state, whereas the tension remained unchanged. The application of 100 nM CCh after a 10 min incubation in $Ca^{2+}$-free PSS caused transient increases in $[Ca^{2+}]_i$ (−22.6±5.7%, $n=8$) and tension (77.5±7.4%, $n=8$). As shown in Figure 4b, pretreatment of strips with theophylline for 5 min significantly attenuated the transient increases in tension development (32.1±3.8%, $n=8$, $P<0.01$) induced by CCh, while it did not affect the transient increases in $[Ca^{2+}]_i$ (−15.9±4.2%, $n=8$, $P>0.05$). Figure 4c summarizes the changes in $[Ca^{2+}]_i$ and tension development induced by CCh in $Ca^{2+}$-free PSS containing 2 mM EGTA with or without theophylline.

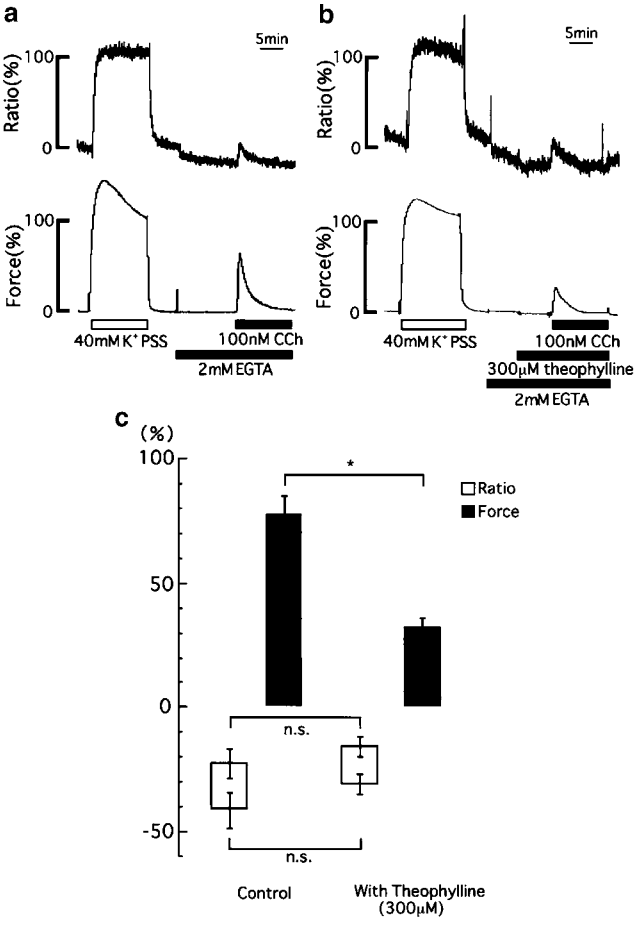


**Figure 4** Effect of theophylline on increases in $[Ca^{2+}]_i$ level and tension development induced by CCh in $Ca^{2+}$-free PSS containing 2 mM EGTA. Representative recordings show changes in $[Ca^{2+}]_i$ and tension development induced by 100 nM CCh in $Ca^{2+}$-free PSS containing 2 mM EGTA, in the absence (a) or presence of 300 $\mu$M theophylline (b). The developed tension and $[Ca^{2+}]_i$ were expressed as a percentage, assigning the values in normal PSS and the steady-state contraction induced by 40 mM $K^+$ PSS to be 0 and 100%, respectively. (c) A summary of the results obtained from experiments performed in a similar manner as in (a, b). The bottom and top of each column indicate $[Ca^{2+}]_i$ and tension levels just before and at the peak of CCh-induced contraction. The data are expressed as the means±s.e.m. (shown as vertical bars) ($n=8$). *$P<0.01$; n.s., difference not significant (statistical significance was determined by using unpaired $t$-test).

### *Effects of theophylline on the $[Ca^{2+}]_i$–force relationships*

Figure 5a shows representative recordings of the control (without theophylline) $[Ca^{2+}]_i$–force relationship of

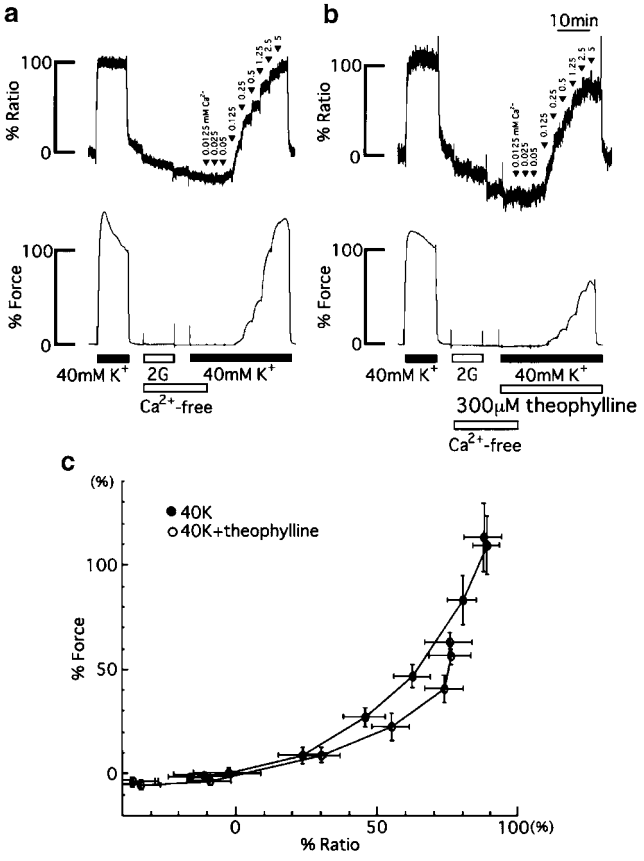


**Figure 5** Representative recordings of the changes in $[Ca^{2+}]_i$ and force induced by cumulative applications of extracellular $Ca^{2+}$ (0–5 mM) during 40 mM $K^+$ depolarization in the absence (a) and the presence of theophylline (b). The numbers noted by the arrowheads indicate the final concentration of extracellular $Ca^{2+}$ (mM) at each step. The steady-state $[Ca^{2+}]_i$–force curves (c) in the absence (closed circle) or presence of theophylline (open circle) were constructed with the data obtained from four independent experiments made in a manner similar to that in (a) and five independent experiments done as in (b). The data were obtained at the time when force reached the maximal level at each application of extracellular $Ca^{2+}$. The vertical and horizontal bars represent s.e.m.

contractions during membrane depolarization with 40 mM $K^+$ PSS. After the responses (100%) to 40 mM $K^+$ depolarization had been recorded, the strips were incubated in $Ca^{2+}$-free PSS containing 2 mM EGTA for 10 min, followed by a 5 min exposure to $Ca^{2+}$-free PSS without EGTA. The solution was then changed to a $Ca^{2+}$-free 40 mM $K^+$-depolarization solution, and extracellular $Ca^{2+}$ (0–5 mM) was cumulatively applied. Both $[Ca^{2+}]_i$ and force increased stepwise with elevations of extracellular $Ca^{2+}$ concentration. Figure 5b shows the representative recordings of responses to the cumulative application of external $Ca^{2+}$ during 40 mM $K^+$ depolarization in the presence of 300 $\mu$M theophylline. In Figure 5c, the $[Ca^{2+}]_i$–force relationships were plotted with data points obtained from the experiments carried out in a similar manner as described in Figure 5a and b. Theophylline significantly shifted the $[Ca^{2+}]_i$–force relationships to the right ($P<0.05$), indicating that theophylline induces weaker tension development for given levels of $[Ca^{2+}]_i$.

### *Effects of theophylline on the contraction of the α-toxin permeabilized strips*

To confirm the inhibitory effects of theophylline on $Ca^{2+}$ responsiveness, we applied theophylline to the α-toxin permeabilized strips of porcine tracheal smooth muscle. Figures 6a and b show representative recordings of the $Ca^{2+}$-induced contraction without (a) or with (b) 300 $\mu$M theophylline. The resting tension in the relaxing solution and the maximal tension induced by 10 $\mu$M $Ca^{2+}$ were taken as 0 and 100%, respectively. In primary experiments, we determined that 300 $\mu$M theophylline did not affect the contraction induced by 10 $\mu$M $Ca^{2+}$ (data not shown). Figure 6c summarizes the $Ca^{2+}$–force relationship obtained from experiments carried out in a similar manner as in Figure 6a and b. In the presence of 300 $\mu$M theophylline, the $EC_{50}$ value of $[Ca^{2+}]_i$ for contraction was significantly increased from $0.97\pm0.22$ ($n=5$) to $1.43\pm0.28$ $\mu$M ($n=5$, $P<0.05$).

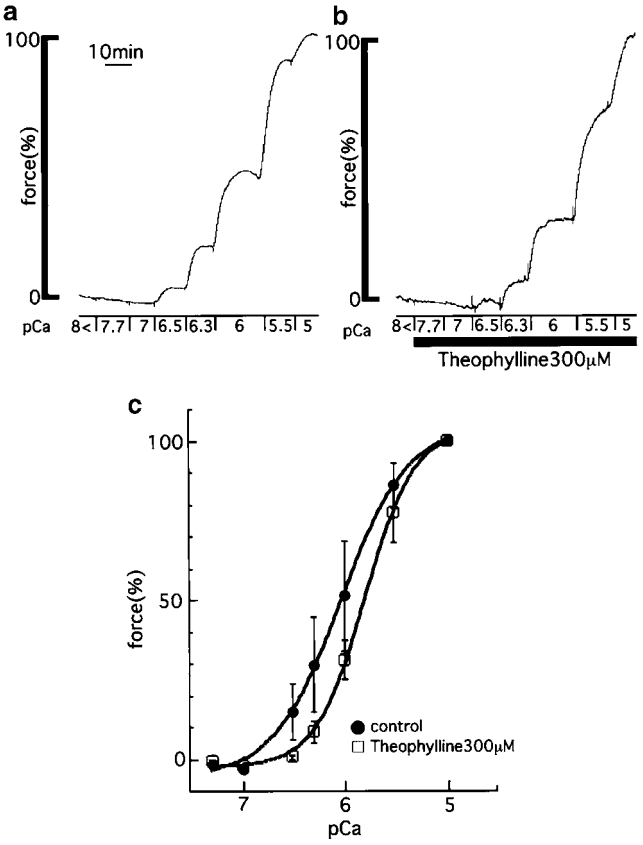


**Figure 6** Effects of theophylline on the contraction of the α-toxin permeabilized strips. (a, b) Representative recordings show the $Ca^{2+}$-induced contraction in the absence (a) or presence (b) of 300 $\mu$M theophylline. The resting tension in the relaxing solution and the maximal tension induced by 10 $\mu$M $Ca^{2+}$ were taken as 0 and 100%, respectively. Theophylline (300 $\mu$M) did not affect the contraction induced by 10 $\mu$M $Ca^{2+}$ (data not shown). (c) A summary of the results obtained from experiments performed in a similar manner as in (a, b). The data are expressed as the means ± s.e.m. (shown as vertical bars) ($n=5$).

### *Effects of theophylline on cyclic nucleotide-induced relaxation of α-toxin permeabilized strips*

Figures 7a and b show representative recordings of the effects of cAMP and cGMP on 500 nM $Ca^{2+}$-induced contraction of α-toxin permeabilized strips with or without 100 $\mu$M theophylline, respectively. In the presence of 100 $\mu$M theophylline, 1 $\mu$M cAMP significantly accelerated relaxation compared with the application of 1 $\mu$M cAMP alone. On the other hand, 100 $\mu$M theophylline did not accelerate the relaxation caused by 100 nM cGMP. The concentrations of cAMP (1 $\mu$M) and cGMP (100 nM) were chosen to induce similar extents of relaxation of the 500 nM $Ca^{2+}$-induced contraction. Figures 7c and d summarize the effect of 1 $\mu$M cAMP and 100 nM cGMP on the 500 nM $Ca^{2+}$-induced contraction of α-toxin permeabilized strips with or without 100 $\mu$M theophylline, respectively.

## Discussion

In this study, using simultaneous measurements of $[Ca^{2+}]_i$ and tension development as well as α-toxin permeabilized preparations, we determined that the major mechanisms for theophylline-induced relaxation of the porcine tracheal smooth muscle are: (1) activation of BK channels and (2) attenuation of $Ca^{2+}$ sensitivity, presumably through the action of cAMP. In addition, the data also demonstrate that theophylline minimally effects the intracellular $Ca^{2+}$ release or cGMP-mediated pathways.

Smooth muscle tone is regulated not only by $[Ca^{2+}]_i$ levels but also by the $Ca^{2+}$ sensitivity of the contractile apparatus (Somlyo *et al*., 1999). In order to evaluate the effects of a drug on $Ca^{2+}$ mobilization and $Ca^{2+}$ sensitivity, it is thus necessary to measure both $[Ca^{2+}]_i$ and the tension of intact smooth muscle strips in addition to measuring the tension of receptor-coupled permeabilized strips. In this study, we demonstrate that theophylline induces a concentration-dependent reduction in the $[Ca^{2+}]_i$ and tension induced by CCh or 40 mM $K^+$ depolarization (Figure 1). Importantly, the theophylline-induced reduction in $[Ca^{2+}]_i$ and tension induced by 40 mM $K^+$ were much weaker than those induced by CCh. To investigate this difference, we applied 300 $\mu$M theophylline to the contraction induced by CCh or 40 mM $K^+$ depolarization, since this concentration of theophylline had submaximal

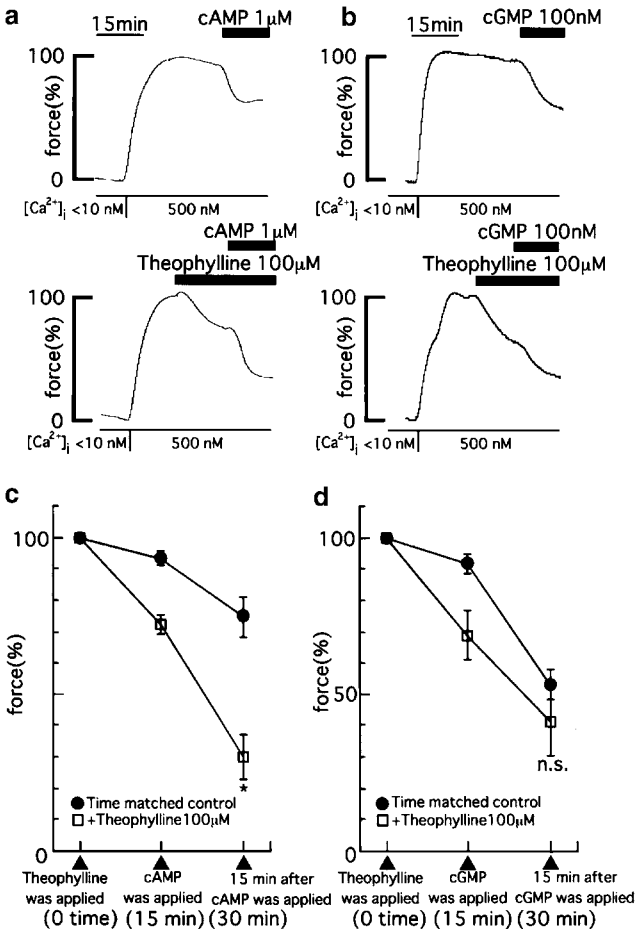


**Figure 7** Effects of theophylline on the relaxation of cyclic nucleotides on the contraction of the α-toxin permeabilized strips. (a, b) Representative recordings show the relaxant effect of 1 μM cAMP (a) and 100 nM cGMP (b) on the contraction induced by 500 nM $Ca^{2+}$ with or without 100 μM theophylline, respectively. The resting tension in the relaxing solution and the tension induced by 500 nM $Ca^{2+}$ just before the application of theophylline and at the corresponding time point of the time-matched control were taken as 0 and 100%, respectively. (c) A summary of the relaxant effect of 1 μM cAMP with or without 100 μM theophylline ($n=5$). (d) A summary of the relaxant effect of 100 nM cGMP with or without 100 μM theophylline ($n=7$). The data are expressed as the means ± s.e.m. (shown as vertical bars). $^*P<0.05$; n.s., difference not significant (statistical significance was determined by using repeated measures analysis of variance and contrast analysis).

effects on the increases in $[Ca^{2+}]_i$ and tension induced by CCh. Theophylline (300 μM) induced significant relaxation without changing $[Ca^{2+}]_i$ during the contraction induced by 40 mM $K^+$ depolarization (Figure 2a). On the other hand, 300 μM theophylline significantly decreased both $[Ca^{2+}]_i$ level and tension induced by CCh (Figure 2b). As we reported previously, CCh induced a greater contraction than $K^+$ depolarization at similar levels of $[Ca^{2+}]_i$, persuasive evidence for CCh-induced $Ca^{2+}$ sensitization (Yoshimura *et al*., 1995). We thus speculated that theophylline decreases $[Ca^{2+}]_i$ during CCh-induced contraction through its action on $K^+$ channels, because the reduction of $[Ca^{2+}]_i$ could be observed only in the presence of a physiological concentration of $K^+$. We also directly examined the possibility that theophylline decreases $Ca^{2+}$ sensitivity, since it induced relaxation without decreasing $[Ca^{2+}]_i$ levels during 40 mM $K^+$ depolarization-induced contraction. We designed experiments to confirm these speculations.

Experiments were made to identify the $K^+$ channels responsible for theophylline-induced reductions of $[Ca^{2+}]_i$. Application of IbTX, a selective inhibitor of BK channels, significantly inhibited the effects of theophylline on the increases in $[Ca^{2+}]_i$ and tension induced by CCh (Figure 3). In agreement with this finding, BK channel blockade has also been associated with the inhibitory effects of aminophylline, a complex of theophylline and ethylenediamine (Jones *et al*., 1990). Our results suggest that theophylline induces hyperpolarization and relaxation of the porcine tracheal smooth muscle as a result of opening BK channels. Since theophylline is a known nonselective PDE inhibitor, it also likely to elevate intracellular levels of cAMP and/or cGMP. Others have already reported that BK channels are activated *via* cAMP- (Kume *et al*., 1989; 1994) and cGMP- (Robertson *et al*., 1993; Stockand & Sansom, 1996) dependent protein kinases (PKA and PKG, respectively) pathways. $Ca^{2+}$-activated $K^+$ channels are subdivided into three families: small-, intermediate- and large-conductance $Ca^{2+}$-activated $K^+$ channels. Methylxanthines, including theophylline, activate the human, intermediate-conductance and $Ca^{2+}$-activated $K^+$ channels, and directly interact with the protein of the channel (Schröder *et al*., 2000). However, methylxanthines did not directly effect BK or small-conductance $Ca^{2+}$-activated $K^+$ channels, demonstrating that the effects were not secondary to a rise in intracellular $Ca^{2+}$. Our study does not determine whether theophylline directly activates BK channels. It has been reported that BK channel blockers attenuated the relaxant effects of salbutamol, isoprenaline, dibutyryl cAMP and sodium nitroprusside on CCh-contracted tracheal smooth muscle (Jones *et al*., 1990; 1993). These results support the notion that the elevation of cAMP and/or cGMP induced by theophylline activated PKA and/or PKG pathways, which subsequently open BK channels and lead to relaxation of the CCh-contracted tracheal smooth muscle.

Caffeine, a methylxanthine, induces intracellular $Ca^{2+}$ release in smooth muscle. However, theophylline, which is also methylxanthine, did not induce intracellular $Ca^{2+}$ release (Figure 4b). We also examined whether theophylline affects CCh-induced $Ca^{2+}$ release from the sarcoplasmic reticulum, since isoprenaline is known to attenuate agonist-induced intracellular $Ca^{2+}$ release in vascular smooth muscle (Ushio-Fukai *et al*., 1992). As shown in Figure 4, theophylline had no significant effect on $Ca^{2+}$ release by CCh. The theophylline-induced inhibition of contraction induced by CCh in the absence of extracellular $Ca^{2+}$ could be explained by theophylline-induced decreases in $Ca^{2+}$ sensitivity, as discussed below. It was thus concluded that theophylline has little effect on the intracellular $Ca^{2+}$ release mechanism.

We next examined the effects of theophylline on $Ca^{2+}$ sensitivity in both intact and permeabilized preparations. As shown in Figure 5, theophylline significantly shifted the $[Ca^{2+}]_i$–force curve to the right in intact strips. This observation was further confirmed in α-toxin permeabilized strips. Theophylline significantly increased the $Ca^{2+}$ $EC_{50}$ concentration and shifted the *p*Ca–tension curve to the right (Figure 6). These results were consistent with previous findings

that cAMP and/or cGMP attenuates $Ca^{2+}$ sensitivity in smooth muscle (Nishimura & van Breemen, 1989; Jones *et al*., 1999). From these results, we concluded that theophylline decreased the $Ca^{2+}$ sensitivity of the contractile apparatus of the porcine tracheal smooth muscle, presumably through the action of cAMP and/or cGMP.

The effects of theophylline on tracheal smooth muscles are probably due to its inhibitory effect on PDE, leading to increase in intracellular cAMP and/or cGMP levels. We explored the relative importance of cAMP and cGMP in theophylline-induced decreases in $Ca^{2+}$ sensitivity. As shown in Figure 7, theophylline preferentially enhanced the relaxant effect of cAMP, while it did not enhance the relaxant effect of cGMP. This finding suggests that the theophylline-induced decrease in $Ca^{2+}$ sensitivity is mainly mediated by the cAMP-PKA pathway in tracheal smooth muscle. PDE is now acknowledged to represent the activity of a large superfamily of enzymes comprised of at least 11 distinct isoenzymes. In airways, PDE4 appears to be the most important isoenzyme, based on its distribution in the airway smooth muscle and inflammatory cells (Schmidt *et al*., 1999). This isoform (PDE4) degrades cAMP and thus represents an attractive rationale for the observation that theophylline accelerates relaxation by cAMP and not cGMP.

Theophylline is frequently used as a bronchodilator, and its ability to control chronic asthma is disproportionately greater than is explainable by its relatively small degree of bronchodilator activity (Schmidt *et al*., 1999). One explanation for this discrepancy is that theophylline has any other beneficial effects on asthmatics: anti-inflammatory action and improvement in function of respiratory muscle tone (Jenne, 1995; Weinberger & Hendeled, 1996; Danialou *et al*., 1998).

In summary, this study describes some of the mechanisms that underlie the direct effects of theophylline on tracheal smooth muscle relaxation. We determine that the major mechanisms for theophylline-induced relaxation of the porcine tracheal smooth muscle include the activation of BK channels as well as the attenuation of $Ca^{2+}$ sensitivity presumably through the actions of cAMP.

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