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Relaxant effects of NKH477, a new water-soluble forskolin derivative, on guinea-pig tracheal smooth muscle: the role of Ca^{2+}-activated K^+ channels

K. Satake, [3]K. Takagi, [1]I. Kodama, [1]H. Honjo, [1]J. Toyama & [2]S. Shibata

Second Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466; [1]Departments of Circulation and Humoral Regulation, Research Institute of Environmental Medicine, Nagoya 464-01, Japan and [2]Department of Pharmacology, University of Hawaii School of Medicine, Honolulu, Hawaii, U.S.A.

1 Mechanisms underlying the bronchorelaxant action of NKH477, a newly developed water-soluble forskolin derivative, were investigated in guinea-pig isolated tracheal smooth muscle.

2 In muscles precontracted with 3 μM histamine, NKH477 (1 nM – 1 μM) caused a concentration-dependent decrease of isometric tension, resulting in a complete relaxation at 300 nM. The EC_{50} for the relaxation was 32.6 ± 4.3 nM ($n=6$).

3 In the presence of 30 or 90 nM iberiotoxin (IbTX), a selective blocker of the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel, the relaxing action of NKH477 on the histamine-induced contraction was inhibited, giving rise to a parallel shift of the concentration-response curves; the EC_{50} of NKH477 was increased to 131.4 ± 20.4 nM at 30 nM IbTX ($n=4$), and 125.3 ± 12.2 nM at 90 nM IbTX ($n=4$).

4 Pretreatment of muscles with 30 mM tetraethylammonium (TEA) caused a similar rightward shift of the concentration-response curve to NKH477 with an increase of the EC_{50} to 139.8 ± 18.4 nM ($n=5$). In contrast, the relaxing action of NKH477 was unaffected by 10 μM glibenclamide, an ATP-sensitive K^+ channel blocker, or by 100 nM apamin, a blocker of small conductance Ca^{2+}-activated K^+ channels.

5 In muscles pretreated with 1 μM nifedipine, a blocker of the voltage-dependent Ca^{2+} channel (VDC), 30 – 90 nM IbTX did not affect the relaxant effects of NKH477 on the histamine-induced contraction.

6 In muscles precontracted by a K^+-rich (40 mM) solution, NKH477 caused only minimal relaxation ($19.8 \pm 1.7\%$, $n=4$) even at the highest concentration (1 μM).

7 In experiments to measure the ratio of fura-2 fluorescence signals ($R_{340/380}$) as an index of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the application of 100 nM NKH477 or 200 nM isoprenaline to the preparation precontracted by 3 μM histamine resulted in a decrease in $[Ca^{2+}]_i$ in association with a decrease in tension. The reduction of $[Ca^{2+}]_i$ and tension by NKH477 was $47.0 \pm 5.6\%$ and $62.8 \pm 7.0\%$, respectively ($n=5$), and that with isoprenaline $60.6 \pm 7.4\%$ and $67.4 \pm 6.4\%$, respectively ($n=5$). These effects of NKH477 and isoprenaline on $[Ca^{2+}]_i$ and tension were inhibited by 30 nM IbTX. The inhibitory action of IbTX was abolished in the presence of 1 μM nifedipine.

8 These results suggest that the bronchorelaxant action of NKH477 may result, at least in part, from activation of BK_{Ca} channels, which may cause a hyperpolarization of smooth muscle cell membranes and a secondary decrease in Ca^{2+} influx through VDCs, leading to a decrease in $[Ca^{2+}]_i$.

Keywords: Airway smooth muscle; NKH477; histamine; iberiotoxin; Ca^{2+}-activated K^+ channels; voltage-dependent Ca^{2+} channels

Introduction

Bronchorelaxation in response to β-adrenoceptor stimulation is mediated, at least in part, by an increase of the intracellular concentration of adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) (Torphy & Hall, 1994). This may suggest a potential benefit of drugs, which stimulate adenylate cyclase directly like forskolin (Seamon & Daly, 1981; Muller & Baer, 1983), as an alternative method for the treatment of bronchial asthma (Tsukawaki *et al*., 1987), because their effects may not be restricted by downregulation or desensitization of β-adrenoceptors (Kume & Takagi, 1997). The poor water solubility and the low oral activity of forskolin has limited its clinical usage both as an i.v. and an oral formulation (Bauman *et al*., 1990).

NKH477, 6-(3-dimethylaminopropionyl) forskolin hydrochloride is a novel and potent water-soluble forskolin derivative (Hosono *et al*., 1992; Takeuchi *et al*., 1995). This agent, like forskolin, enhanced adenylate cyclase activity in cardiovascular tissues, resulting in an increase of cyclic AMP. NKH477 was shown to have a potent positive inotropic effect even under experimental conditions causing a reduction of cardiac β-adrenoceptor density (Takeuchi *et al*., 1995). In isolated vascular smooth muscle preparations from rat aorta or porcine coronary arteries, NKH477 attenuated the contraction induced by either high K^+ solution, noradrenaline, prostaglandin or acetylcholine in a concentration-dependent manner with a potency comparable to or greater than that of forskolin (Himeta *et al*., 1991; Shafiq *et al*., 1992; Takeuchi *et al*., 1995). However, as to the bronchorelaxant effects of NKH477, no experimental or clinical data have been published to date.

Although it is well acknowledged that an increase in the level of cyclic AMP is associated with relaxation of tracheal smooth muscle, the precise molecular events underlying the cyclic AMP-mediated relaxation are not known (Torphy & Hall, 1994). The involvement of many different mechanisms has been suggested. For instance, an increase in cyclic AMP

[3]Author for correspondence.

may reduce the affinity of myosin light chain kinase (MLCK) for the Ca^{2+}-calmodulin complex through the phosphorylation of MLCK by cyclic AMP-activated protein kinase (PKA). This would result in a decrease in the Ca^{2+}-sensitivity of the contractile elements (de Lanerolle *et al*., 1984). Alternatively, cyclic AMP may reduce $[Ca^{2+}]_i$ by enhancing the Ca^{2+} extrusion to the extracellular space via an activation of sarcolemmal Ca^{2+}-ATPase and/or an increase of Na^+/Ca^{2+} exchange secondary to an activation of the Na^+/K^+-pump (Torphy & Hall, 1994). Ca^{2+} sequestration into intracellular storage sites may also be facilitated by cyclic AMP, leading to a decrease of $[Ca^{2+}]_i$ (Torphy & Hall, 1994). Recent observations with patch clamp techniques have shown that large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are distributed abundantly in the surface of airway smooth muscle cells (McCann & Welsh, 1986; Kume *et al*., 1989), and that these channels are stimulated via cyclic AMP-dependent phosphorylation as well as by a cyclic AMP-independent, membrane-delimited signal transduction process (Kume *et al*., 1989; 1992; 1994). Activation of BK_{Ca} channels should cause membrane hyperpolarization of smooth muscle cells, and this hyperpolarization is expected to inhibit Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCs) (Small *et al*., 1993; Kaczorowski & Jones, 1995).

In the present study, we investigated the relaxant effects of NKH477 in guinea-pig isolated trachealis precontracted with histamine by measuring isometric tension and fura-2 fluorescence signals reflecting $[Ca^{2+}]_i$. In addition, the effect of blockade of BK_{Ca} channels was studied to determine their roles in the cyclic AMP-mediated bronchorelaxation.

Methods

Male guinea-pigs (250–400 g) were killed by stunning and bleeding under ether anaesthesia. Segments of the cervical trachea were excised and the tracheal ring was opened by cutting longitudinally at the cartilaginous region opposite the membranous portion. Mucosal and connective tissues were carefully removed with fine forceps under microscopic observation. Segments containing one cartilaginous ring (for isometric tension recording), and two or three cartilaginous rings (for $[Ca^{2+}]_i$ measurements) were prepared.

Isometric tension recording

The methods are essentially the same as previously described (Baba *et al*., 1986; Hiramatsu *et al*., 1994). A preparation containing a single cartilaginous ring was placed in an organ bath (1 ml volume). The strip was immersed in a modified Krebs Ringer solution of the following composition (mM): NaCl 137, $KHCO_3$ 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2 and glucose 11.8, bubbled with a mixture of 99% O_2 and 1% CO_2 (pH 7.4). The temperature of the organ bath was maintained at 37°C. After an equilibration period of about 60 min in the normal solution, 2 μM isoprenaline was applied to obtain complete relaxation, and then the passive tension was adjusted to 0.5 g in the presence of isoprenaline. When the isoprenaline was washed out, spontaneous mechanical tone developed in the normal bath solution. This spontaneous tone produced by endogenous prostaglandin was abolished by a bath application of 5 μM indomethacin during the entire period of subsequent experiments.

Histamine (3 μM) was added to the Krebs Ringer solution to induce a constant degree of tone (20 min pretreatment). This concentration of histamine was present throughout the experiments. Cumulative concentration-relaxation response to NKH477 (1 nM–1 μM) were then obtained with 3.16 fold concentration increments. Each concentration of NKH477 was allowed 15 min tissue contact. The concentration-effect curves of NKH477 were constructed in the absence (control) or presence of the following modifying agents: 30 or 90 nM iberiotoxin (IbTX), 30 mM tetraethylammonium (TEA), 10 μM glibenclamide (GC), 100 nM apamin, 1 μM nifedipine, and a combination of 1 μM nifedipine plus 30 or 90 nM IbTX. Nifedipine was allowed to equilibrate with the tissue for 60 min before the histamine application. All other modifying agents were allowed to equilibrate with the tissue for at least 20 min before the histamine application. In some experiments the concentration-response curve to NKH477 (1 nM–1 μM) was obtained in the muscle precontracted with K^+-rich (40 mM) solution (20 min pretreatment), where NaCl was replaced with an equimolar concentration of KCl. At the end of each experiment, the preparation was exposed to Ca^{2+}-free solution to define the level of complete relaxation (0% contraction). The Ca^{2+}-free solution was prepared by replacing 2.4 mM $CaCl_2$ in the normal solution with 2.2 mM NaCl and 0.2 mM EGTA. Relaxation by NKH477 was expressed as a percentage of the maximal relaxation induced by the Ca^{2+}-free solution.

Fura-2 loading and cytosolic Ca^{2+} measurements

Segments containing two or three cartilage rings were placed horizontally in a small chamber (0.6 ml volume). One end of the segment was fixed to the chamber and the other end was connected to a force-displacement transducer to monitor isometric tension. The chamber was perfused with the normal solution having the same composition as described before. Muscle strips were exposed to 10 μM acetoxymethyl ester of fura-2 (fura-2/AM) for 6 h at room temperature (22–24°C). The noncytotoxic detergent, pluronic F-127 (0.01%), was added to increase the solubility of fura-2/AM. After the loading, the chamber was perfused with the normal solution at 37°C for at least 60 min to wash out the extracellular fura-2/AM before the measurements. Indomethacin 5 μM was then added throughout the subsequent period of experiments to avoid the development of spontaneous tone.

The method for measurement of $[Ca^{2+}]_i$ was similar to that described by Ozaki *et al*. (1987). The mucosal side of the muscle strips was exposed to the excitation light, and the light emitted from the strip was collected by a photomultiplier. The isometric contractile force and the fura-2 fluorescence of the strips were measured simultaneously, by use of a displacement transducer and a spectrofluorometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). The fluorescence of fura-2-loaded tissue with a 340-nm excitation light was four to five times greater than the fluorescence of fura-2-unloaded tissue. The absolute value of $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura-2 for Ca^{2+} in smooth muscle cytoplasm may be different from that obtained *in vitro*. Therefore, $R_{340/380}$ was used as a relative indicator of $[Ca^{2+}]_i$.

Histamine (3 μM) was applied to the muscle to induce a steady-state contraction (15–20 min pretreatment). NKH477 (100 nM) or isoprenaline (200 nM) was then applied for 5 min. The effects of NKH477 and isoprenaline on the muscle precontracted by histamine were examined in the absence (control) or presence of 30 nM IbTX, 1 μM nifedipine, and a combination of 1 μM nifedipine plus 30 nM IbTX. IbTX and nifedipine were allowed to equilibrate with the tissue for 10 min and 60 min, respectively, before the application of

histamine. The basal tension before histamine application was regarded as the maximal relaxation level.

Drugs used

The drugs used were NKH477 (Nippon Kayaku Co., Ltd., Tokyo, Japan), iberiotoxin (Peptide Institute, Inc, Osaka, Japan), tetraethylammonium, glibenclamide, histamine, apamin, nifedipine and isoprenaline, pluronic F-127 (Sigma Chemical Co., St. Louis, MO, U.S.A.), fura-2/AM (Dojin Laboratories, Kumamoto, Japan). Glibenclamide (10 mM) and nifedipine (10 mM) were dissolved in dimethyl sulphoxide (DMSO) and ethanol, respectively. The final DMSO and ethanol concentrations did not exceed 0.5% and 0.1%, respectively. Neither solvent had significant effects on the muscle tension and the fluorescence ratio.

Statistical analysis of results

Data are expressed as means (±s.e.means) with number of preparations used (n). Student's unpaired t test was used to evaluate the statistical significance of differences between means. Values of $P<0.05$ were considered to be significant.

Results

Relaxant action of NKH477 in the tracheal smooth muscle precontracted by histamine

The cumulative application of NKH477 (1 nM – 1 μM) to the muscle precontracted by histamine (3 μM) resulted in a concentration-dependent decrease in tension, and full (100%) relaxation was obtained at 300 nM (Figure 1). The 50% effective concentration (EC_{50}) of NKH477 was 32.6±4.3 nM ($n=6$) (Table 1).

In order to examine the involvement of BK_{Ca} channels in the relaxant action of NKH477, the effect of pretreatment with iberiotoxin (IbTX), a selective inhibitor of BK_{Ca} channels (Galvez *et al*., 1990; Kaczorowski & Jones, 1995), was investigated. Application of IbTX (30 nM) had no significant effects on the contraction induced by 3 μM histamine. However, in the presence of 30 nM IbTX the relaxant action of NKH477 in the muscle precontracted with histamine was inhibited, and the concentration-response curves to NKH477 were shifted to the right with no change in the maximal response (Figure 1a). Application of the higher concentration (90 nM) of IbTX resulted in an increase of the maximal contraction induced by 3 μM histamine by 16.1±3.7% ($n=4$). The concentration-response curves to NKH477 in the presence of 90 nM IbTX showed a similar parallel shift to the right, as observed with 30 nM IbTX. The EC_{50} of NKH477 in the presence of IbTX 30 nM (131.4±20.3 nM, $n=4$) and 90 nM (125.3±12.2 nM, $n=4$) were significantly higher than the value in the absence of IbTX by 4.0 and 3.8 times, respectively (Table 1).

The effects of 30 mM tetraethylammonium (TEA), and a non-specific K^+ channel blocker, were analogous to those of 90 nM IbTX; the maximal contraction induced by 3 μM histamine was increased by 16.3±1.3% ($n=5$), and the concentration-response curves to NKH477 were shifted in parallel to the right, giving rise to an increase of the EC_{50} to 139.8±18.4 nM ($n=4$) (Figure 1b, Table 1).

The effect of glibenclamide, an ATP-sensitive K^+ (K_{ATP}) channel blocker (Schmid-Antomarchi *et al*., 1987; Sturgess *et al*., 1988), or apamin, a small-conductance K^+ (SK_{Ca}) channel blocker (Cook & Quast, 1990) on the relaxant action of NKH477 was also examined. Neither glibenclamide (10 μM) nor apamin (100 nM) affected the baseline tone, and the contraction induced by 3 μM histamine. The concentration-response curves to NKH477 in the presence of glibenclamide or apamin were virtually unchanged from those obtained in the absence of these compounds (Table 1).

Effects of nifedipine on the antagonism of IbTX to the relaxant action of NKH477

The effects of nifedipine, a blocker of voltage-dependent Ca^{2+} channels (VDCs) were examined in order to test the possible involvement of VDCs in the antagonism of IbTX to the relaxant action of NKH477. Although a single treatment of tracheal smooth muscle with 1 μM nifedipine for 60 min caused no significant changes in the baseline tone, the maximal amplitude of contraction induced by 3 μM histamine was reduced by 15.8±2.7% ($n=8$). The concentration-response curves to NKH477 in muscle precontracted by 3 μM histamine were shifted in parallel to the right when compared with those

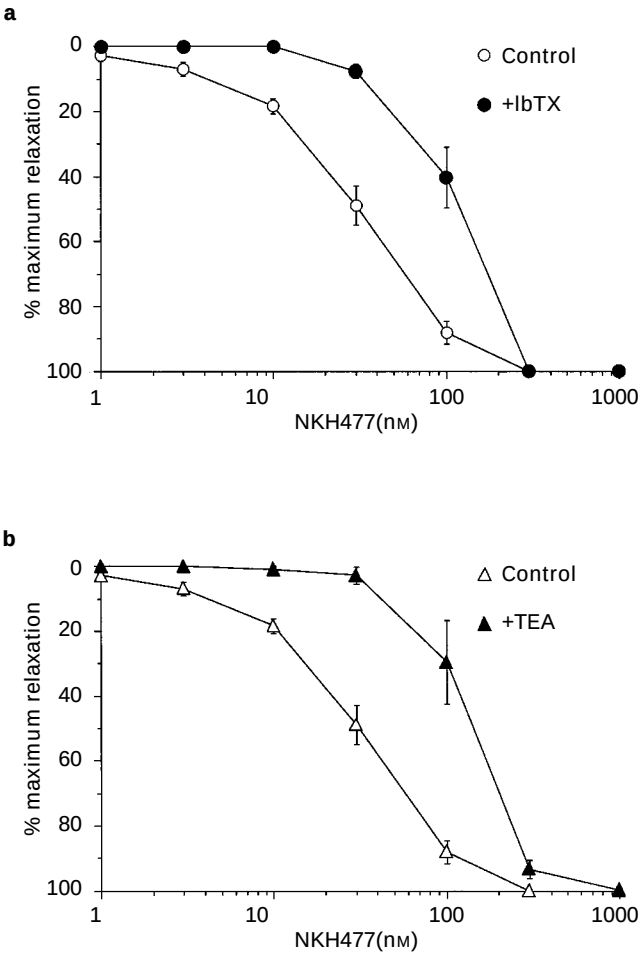

Figure 1 Effects of iberiotoxin (IbTX) and tetraethylammonium (TEA) on the concentration-response curves to NKH477. NKH477 (1 nM – 1 μM) was applied cumulatively to guinea-pig isolated trachealis precontracted with 3 μM histamine in the absence and presence of 30 nM IbTX (a) or 30 mM TEA (b). IbTX or TEA was applied 20 – 30 min before the histamine application. Ordinates: relaxation expressed as % of the maximal relaxation induced by Ca^{2+}-free solution. Abscissae: concentration of NKH477 on a logarithmic scale. (a) Concentration-response curves obtained in the absence of IbTX (control, $n=6$) and in the presence of 30 nM IbTX ($n=4$). (b) Concentration-response curves obtained in the absence of TEA (control, $n=6$) and in the presence of 30 mM TEA ($n=5$). Data points are the means and vertical lines show s.e.mean.

Table 1 Effects of tetraethylammonium (TEA), iberiotoxin (IbTX), glibenclamide, apamin, and nifedipine on relaxation concentration-response curves to NKH477

Condition	EC_{50} (nM)		
Control	32.6±4.3		
TEA (30 mM)	139.8±18.4*		
Iberiotoxin (30 nM)	131.4±20.4*	NS	
Iberiotoxin (90 nM)	125.3±12.2*		
Glibenclamide (10 μM)	40.6±7.2		
Apamin (100 nM)	36.0±9.4		
Nifedipine (1 μM)	56.5±4.0*	NS	NS
Nifedipine (1 μM)+ Iberiotoxin (30 nM)	55.3±5.1*		
Nifedipine (1 μM)+ Iberiotoxin (90 nM)	70.0±14.3*		

Concentration producing 50% of the maximal reversal of 3 μM histamine tone (EC_{50}) was obtained from the relaxation concentration-response curves to NKH477 under control conditions and in the presence of 30 mM TEA, 30 nM IbTX, 90 nM IbTX, 10 μM glibenclamide, 100 nM apamin, 1 μM nifedipine, 1 μM nifedipine plus 30 nM IbTX or 1 μM nifedipine plus 90 nM IbTX. Values are the mean±s.e.mean ($n=4-6$). *Significantly different from control at $P<0.05$. NS: no significant difference between the two values.

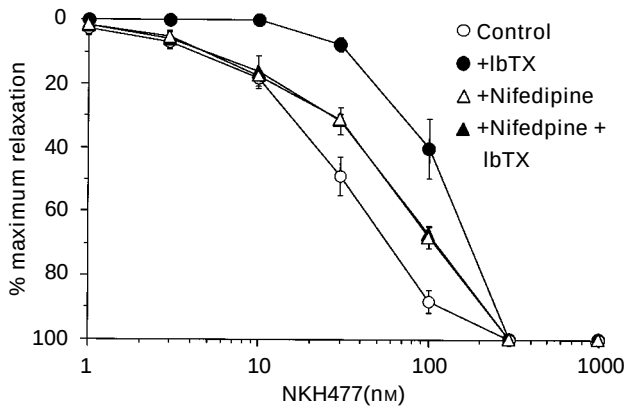

Figure 2 Effects of nifedipine on the concentration-response curves to NKH477. NKH477 (1 nM–1 μM) was applied cumulatively to guinea-pig isolated trachealis precontracted with 3 μM histamine in the absence and presence of iberiotoxin (IbTX) and/or nifedipine. Nifedipine (1 μM) was applied 60 min before the histamine application. IbTX (30 nM) was applied 20–30 min before the histamine application. Ordinate scale and abscissa scale are the same as in Figure 1. The concentration-response curves were obtained from the tissues untreated with either IbTX or nifedipine (control, $n=6$), from the tissues pretreated with 30 nM IbTX alone ($n=4$), from the tissues pretreated with 1 μM nifedipine plus 30 nM IbTX ($n=4$). Data points are the means and vertical lines show s.e.means.

in the absence of nifedipine (Figure 2). The EC_{50} of NKH477 in the presence of nifedipine was 1.7 fold higher than the value in the absence of nifedipine (Table 1). In the presence of 1 μM nifedipine, IbTX (30 or 90 nM) did not affect the concentration-response curves to NKH477 (Figure 2); there was no significant difference in the EC_{50} of NKH477 between the preparations with and without IbTX (Table 1). Thus, the antagonism of IbTX to the bronchorelaxant action of NKH477 was inhibited by nifedipine.

Relaxant effects of NKH477 on contraction induced by high K^+ solution

The relaxant effects of NKH477 on high K^+-induced contraction (40 mM K^+) were investigated in four preparations. The amplitude of the sustained contraction induced by the high K^+ solution was comparable with that induced by 3 μM histamine. The cumulative application of NKH477 (1 nM–1 μM) caused only minimal relaxation with an $EC_{50}>1$ μM. The average relaxation caused by 1 μM NKH477 was 19.8±1.7% ($n=4$). An elevation of the extracellular K^+ concentration leads to a reduction of outward K^+ currents by shifting the K^+ equilibrium potential (E_K). The relaxant action of NKH477 through activation of K^+ channels would therefore be minimized in such high K^+ medium.

Effects of NKH477 and isoprenaline on $[Ca^{2+}]_i$ in histamine-stimulated tracheal smooth muscle

The changes in $[Ca^{2+}]_i$ and tension induced by NKH477 following histamine stimulation were measured simultaneously from the tracheal smooth muscle loaded with fura-2/AM. Representative records are shown in Figure 3. The application of 3 μM histamine caused a rapid increase in $R_{340/380}$ associated with an increase in tension, and these changes reached a steady state within 10 min. The additional application of 100 nM NKH477 for 5 min resulted in a decrease in $R_{340/380}$, and a parallel decrease in tension (Figure 3a). The effects of NKH477 on both $R_{340/380}$ and tension were reversed when the compound was washed out. Figure 3b shows an experiment in the presence of IbTX. The application of 30 nM IbTX had no direct effect on basal levels of $R_{340/380}$ and tension. The response of $R_{340/380}$ and tension to histamine (3 μM) were also unaffected by IbTX. However, in the presence of IbTX 100 nM NKH477 caused no appreciable changes in $R_{340/380}$ or in tension. The results obtained in eleven preparations are summarized in Figure 4. The average decreases in $R_{340/380}$ (6.5±1.2%) and in relaxations (9.3±2.3%) in response to 100 nM NKH477 in the presence of 30 nM IbTX ($n=7$) were significantly less than the corresponding values (47.0±5.6% for $R_{340/380}$; 62.8±7.0% for tension) in the absence of IbTX ($n=5$).

Similar experiments were carried out in muscles pretreated with nifedipine. The application of 1 μM nifedipine for 60 min had no direct effect on $R_{340/380}$ and tension. The effects of NKH477 in the preparations pretreated with 1 μM nifedipine are summarized in Figure 4. The average decreases of $R_{340/380}$ and relaxation in response to 100 nM NKH477 in the absence of IbTX were 15.3±1.7% and 46.9±3.2%, respectively ($n=6$). These values are significantly less than those in experiments without nifedipine. The average decreases of $R_{340/380}$ and relaxation in response to 100 nM NKH477 in the presence of IbTX were 18.3±1.2% and 43.0±3.3%, respectively ($n=6$). There were no significant differences in the NKH477-induced reduction in $R_{340/380}$ and relaxation between the two groups with and without IbTX. Thus, the antagonism of IbTX to the NKH477 action was inhibited by nifedipine.

We also examined the effects of isoprenaline on $[Ca^{2+}]_i$ and their modification by IbTX and nifedipine. The application of 200 nM isoprenaline for 5 min to the muscle precontracted with 3 μM histamine in the absence of the modifying agents (control) resulted in parallel decrease in $R_{340/380}$ and in tension (60.6±7.4% and 67.4±6.4%, respectively, $n=5$). In the presence of 30 nM IbTX, the decreases in $R_{340/380}$ and tension in response to 200 nM isoprenaline were reduced significantly to 12.8±3.3% and 10.8±6.7%, respectively ($n=4$, $P<0.05$). When the muscle had been preincubated with 1 μM nifedipine, the decreases in $R_{340/380}$ and tension in response to 200 nM isoprenaline were 45.0±7.2% and 44.5±8.3%, respectively, in the absence of IbTX ($n=4$), whereas they were 40.8±4.4%

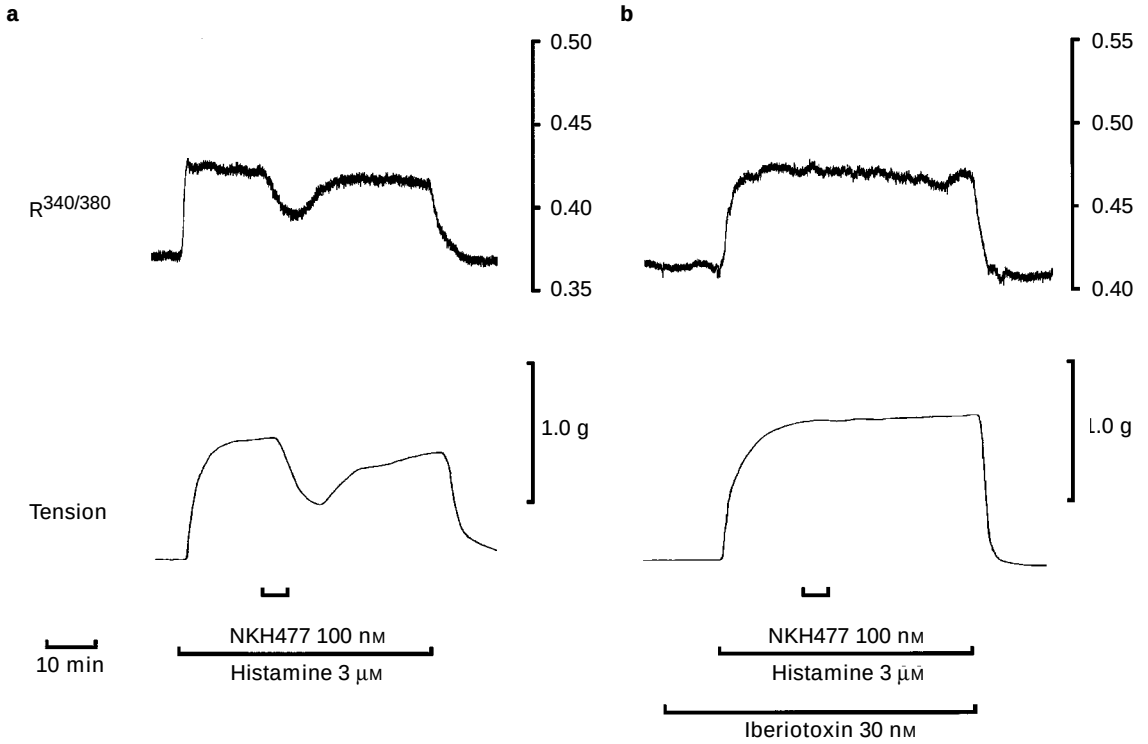

Figure 3 Simultaneous recordings of tension and the fura-2 fluorescence signal reflecting $[Ca^{2+}]_i$ during application of histamine and NKH477 in the absence and presence of iberiotoxin (IbTX). (a) NKH477 was applied for 5 min after the maximal contraction induced by 3 μM histamine in the absence of IbTX. (b) NKH477 was applied for 5 min after the maximal contraction induced by 3 μM histamine in the muscle that had been exposed to 30 nM IbTX, 10 min before the histamine application.

and $41.5 \pm 3.3\%$, respectively, in the presence of 30 nM IbTX ($n=4$); there were no significant differences between these values in the absence and presence of IbTX. Thus, as with NKH477, the antagonism by IbTX of the isoprenaline actions on $[Ca^{2+}]_i$ and tension was attenuated by nifedipine.

Figure 5 shows the relationship between the decrease in $R_{340/380}$ and the decrease in tension (relaxation) induced by 100 nM NKH477 (a) and by 200 nM isoprenaline (b) in all the preparations tested ($n=24$ for NKH477, $n=17$ for isoprenaline). There was a significant linear relationship between the two parameters in the experiments with either NKH477 or isoprenaline. The relationship can be expressed by the following equations: (a) $y=0.91x+19.9$ ($r=0.66$, $P<0.01$); (b) $y=1.11x-3.1$ ($r=0.97$, $P<0.01$), where y represents the percentage relaxation and x represents the percentage decrease in $R_{340/380}$. These results indicate that the bronchorelaxant action of NKH477 is, like isoprenaline, associated with a decrease in $[Ca^{2+}]_i$ and the decrease in $[Ca^{2+}]_i$ is antagonized by IbTX only when VDCs are available.

Discussion

Bronchorelaxant action of NKH477

The present study has revealed that NKH477 has a concentration-dependent inhibitory effect against the histamine-induced contraction of guinea-pig tracheal smooth muscle. The EC_{50} for the relaxant action (32.6 nM) is comparable to the value for its positive inotropic effect in guinea-pig papillary muscle ($\sim$30 nM) (Takeuchi *et al*., 1995). NKH477 in a similar concentration range was also shown to relax vascular smooth muscles precontracted by a variety of spasmogens *in vitro* (Himeta *et al*., 1991; Shafiq *et al*., 1992; Ito *et al*., 1993). The pD_2 values (the concentration producing a 50% increase in the maximal effect induced by 12.5 mM $CaCl_2$) of NKH477 in rat isolated thoracic aorta after contraction induced by noradrenaline (1 μM) or prostaglandin $F_{2\alpha}$ (30 μM) were 7.80 and 7.25, respectively (Himeta *et al*., 1991). The IC_{50} of NKH477 in porcine isolated coronary artery, after a contraction had been induced by acetylcholine (10 μM), was 70 nM (Shafiq *et al*., 1992). These findings suggest that NKH477 may have a potent bronchorelaxant action at doses showing positive inotropic and vasodilating effects. It was found by previous investigators that the EC_{50} of forskolin in relaxing the guinea-pig tracheal smooth muscle precontracted by cholinoceptor agonists or leukotrienes was in the range 94–740 nM (Tsukawaki *et al*., 1987; Hiramatsu *et al*., 1994). The bronchodilating potency of NKH477 may, therefore, be higher than that of forskolin.

The role of Ca^{2+}-activated K^+ channels

In the present study, we used IbTX as an inhibitor of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, since it is more selective than charybdotoxin (ChTX) (Galvez *et al*., 1990; Kaczorowski & Jones, 1995). In the presence of 30 or 90 nM IbTX, the relaxing action of NKH477 on the histamine-induced contraction of guinea-pig tracheal smooth muscle was inhibited, giving rise to a parallel shift of the concentration-response curves; the EC_{50} of NKH477 was increased by 3.8–4.0 fold, but the maximal relaxation induced by NKH477 was unaffected by the treatment with IbTX. Pretreatment of the muscle with 30 mM TEA, a non-specific K^+ channel blocker, also caused a parallel rightward shift of the concentration-response curves to NKH477 by 4.3 fold. However, pretreatment of the muscle with 10 μM glibenclamide or 100 nM apamin did not affect the concentration-response curves to NKH477.

Hiramatsu *et al.* (1994) showed that the concentration-response curves to forskolin in carbachol-stimulated guinea-pig tracheal smooth muscle were shifted in parallel to the right by 2.5 fold in the presence of 100 nM ChTX, but unaffected by pretreatment with 10 μM glibenclamide or 100 nM apamin. Our observations are consistent with those results, suggesting that BK_{Ca} channels, but not K_{ATP} nor SK_{Ca} channels, are involved in the bronchodilating action of forskolin and NKH477.

The relaxation responses of bronchial smooth muscle by other agents which increase cyclic AMP are also antagonized by ChTX or IbTX. Jones *et al.* (1990; 1993) showed that the concentration-response curves to isoprenaline and salbutamol (a β_2-selective agonist) in guinea-pig trachealis precontracted

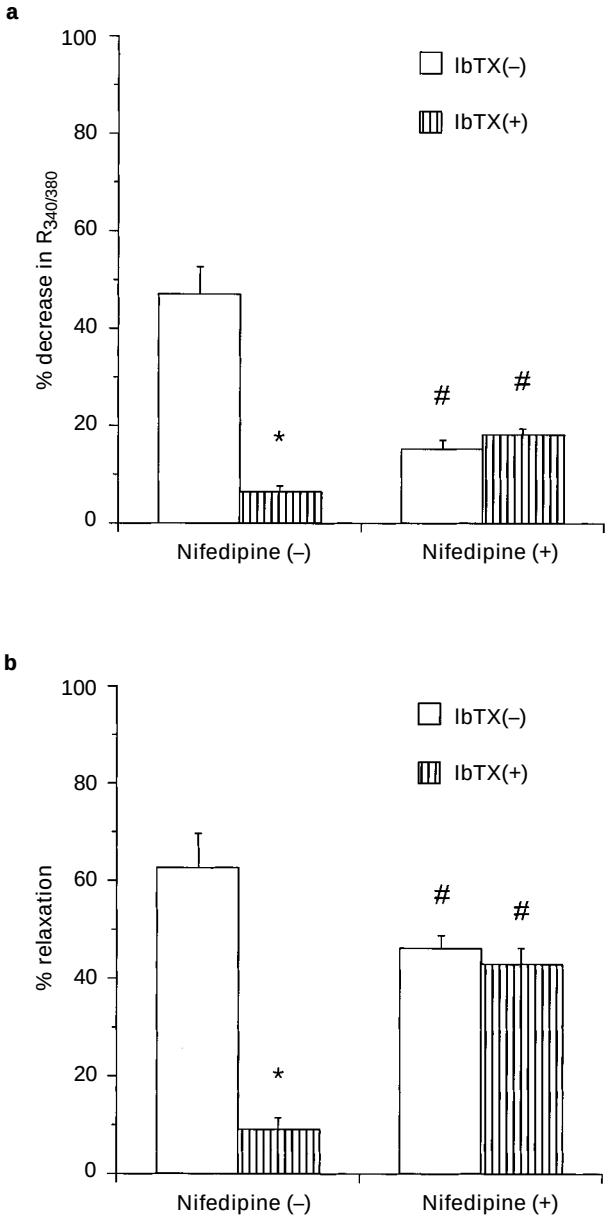

Figure 4 Effects of nifedipine-pretreatment on the NKH477-induced decreases in $R_{340/380}$ and relaxation in guinea-pig isolated trachealis precontracted with histamine. NKH477 (100 nM) was applied 5 min after the maximal contraction induced by 3 μM histamine in the absence and presence of 30 nM iberiotoxin (IbTX). IbTX was applied 10 min before the histamine. Pretreatment with 1 μM nifedipine was for 60 min before the addition of histamine. Ordinates: percentage decreases in $R_{340/380}$ (a) and percentage relaxation (with reference to resting tension before the histamine application) (b). Open columns: the responses to NKH477 in the absence of IbTX in tissues without nifedipine-pretreatment ($n=5$), and those in tissues pretreated with nifedipine ($n=6$). Hatched columns: the responses to NKH477 in the presence of IbTX in tissues without nifedipine pretreatment ($n=7$) and those in tissues pretreated with nifedipine ($n=6$). Data presented are means ± s.e.means. *Significantly different from the corresponding values in the absence of IbTX ($P<0.05$). # Significantly different from the corresponding values without nifedipine-pretreatment ($P<0.05$).

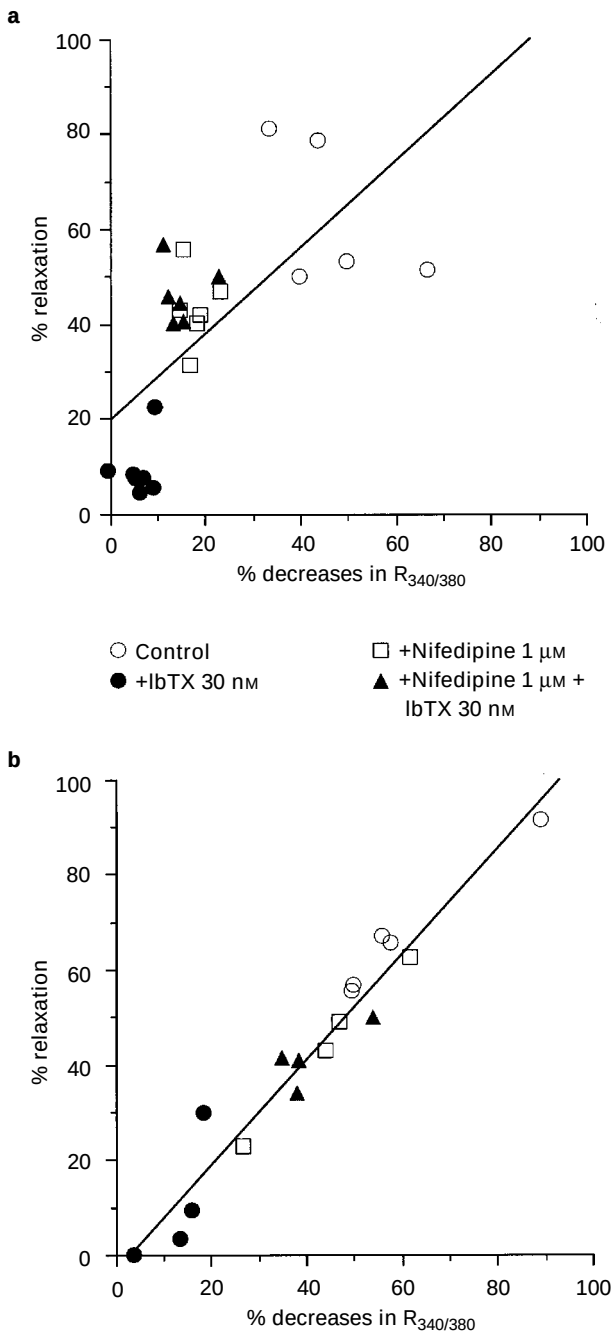

Figure 5 Relationship between the decrease in $R_{340/380}$ and the relaxation induced by 100 nM NKH477 (a) or 200 nM isoprenaline (b) in histamine-stimulated guinea-pig trachealis. Ordinates: percentage relaxation (with reference to tension before the addition of histamine). Abscissae: percentage decreases in $R_{340/380}$ of fura-2 signals. Data were obtained from 24 tissues with NKH477 (the same experiments shown in Figure 4) and 17 tissues for isoprenaline in the absence of the modifying agents (control) and in the presence of 30 nM IbTX, 1 μM nifedipine or 1 μM nifedipine plus 30 nM IbTX. Regression lines drawn from the data points are expressed by the following equations: (a) $y=0.91x+19.9$ ($r=0.66$, $P<0.01$); (b) $y=1.11x-3.1$ ($r=0.97$, $P<0.01$), where y represents the percentage relaxation and x represents the percentage decrease in $R_{340/380}$.

with carbachol were shifted to the right (13 to 40 fold) in the presence of ChTX (60–180 nM) or IbTX (20–180 nM) in association with a substantial decrease of the maximal relaxation (non-competitive antagonism). However, in the same series of experiments, ChTX was shown to cause parallel rightward shifts of the concentration-response curves to aminophylline (3–8 fold) and dibutyryl cyclic AMP (5–7 fold), with no appreciable changes in the maximal relaxation (apparently competitive antagonism). The non-competitive antagonism of ChTX (60–180 nM) or IbTX (50 nM) to the relaxation by isoprenaline and salbutamol was also observed by Laurent *et al.* (1993) and Huang *et al.* (1993), respectively. In contrast apparently competitive antagonism by ChTX (100 nM) of the relaxation induced by isoprenaline was observed by Murray *et al.* (1991) and Hiramatsu *et al.* (1994). There is no clear interpretation for the different modes of antagonism of the BK_{Ca} channel blockers with these agents. It could be due to different experimental conditions, or variable intrinsic effects of ChTX and IbTX on the basal tone of trachealis.

In porcine single tracheal smooth muscle cell experiments (Hiramatsu *et al.*, 1994) the extracellular application of 1 μM forskolin to cell-attached patches was shown to cause about 4 fold increases in the open probability of the BK_{Ca} channel, without affecting its unitary current amplitude. Kume *et al.* (1994) demonstrated in inside-out patches of porcine tracheal smooth muscle cells that the application of cyclic AMP-dependent protein kinase (PKA) results in a concentration-dependent augmentation of BK_{Ca} channel activity, through an increase of the open probability, whereas the direct application of cyclic AMP did not enhance the BK_{Ca} channel activity. NKH477 may activate, like forskolin, the BK_{Ca} channels probably through their phosphorylation mediated by PKA.

The potency of the antagonism by IbTX of the NKH477-induced bronchorelaxation ($\sim$4 fold shift of the concentration-response curve) is comparable to those obtained for ChTX or IbTX of forskolin (Hiramatsu *et al.*, 1994), aminophylline and dibutyryl cyclic AMP responses (Jones *et al.*, 1990), but much less than those found for ChTX or IbTX of responses to β-agonists (Jones *et al.*, 1990; 1993; Huang *et al.*, 1993; Hiramatsu *et al.*, 1994). Such differences could be attributed to the dual signal transduction pathways of BK_{Ca} channel stimulation by β-agonists: (i) direct regulation by a stimulating guanine nucleotide binding (G) protein of adenylate cyclase, G_s (membrane-delimited pathway); and (ii) indirect activation of adenylate cyclase, leading to cyclic AMP-dependent phosphorylation (Kume *et al.*, 1989; 1992). Kume *et al.* (1994) demonstrated in patch clamp studies that the dual BK_{Ca} channel stimulating pathways work independently, and that the membrane-delimited pathway is more efficient than PKA. β-Agonists are therefore considered to be more potent in activating the BK_{Ca} channels than other cyclic AMP elevating agents.

Involvement of voltage-dependent Ca^{2+} channels (VDCs)

In guinea-pig tracheal smooth muscles pretreated with 1 μM nifedipine, the concentration-response curves to NKH477 against the histamine-induced contraction were shifted to the right by 1.7 fold. More importantly, the antagonism by 30–90 nM IbTX of the relaxation by NKH477 was attenuated in the presence of 1 μM nifedipine. These observations can be interpreted as the involvement of VDCs in the NKH477-induced bronchorelaxation. Huang *et al.* (1993) showed that the antagonism of salbutamol provided either by ChTX or by IbTX in acetylcholine-contracted guinea-pig tracheal smooth muscle could be inhibited by nifedipine (0.3 μM), $CdCl_2$ (0.1 mM) or by reducing the concentration of Ca^{2+} in the Krebs solution. They therefore suggested that ChTX and IbTX, by closing BK_{Ca} channels, may cause cellular depolarization or inhibit the BK_{Ca} channel-dependent hyperpolarization, and hence promote the influx of Ca^{2+} through VDCs, giving rise to a kind of functional antagonism. This suggestion receives some support from the observations by Murray *et al.* (1991) and Isaac *et al.* (1996) in guinea-pig tracheal smooth muscle, that IbTX (100 nM) and ChTX (100 nM) each caused increases in mechanical tone that were accompanied by the conversion of the spontaneous slow waves into spike-like regenerative action potentials, leading to an increase in Ca^{2+} influx through VDCs. It was also found that nifedipine (1 μM) prevented the effects of IbTX (100 nM) and ChTX (100 nM) to increase the spontaneous tone or to antagonize the relaxing action of isoprenaline in guinea-pig trachealis (Cook *et al.*, 1995; Isaac *et al.*, 1996).

Our experiments, in which fura-2 signals were measured, revealed that tension and $R_{340/380}$, which reflects $[Ca^{2+}]_i$, always change in parallel in response to NKH477. In the preparations not treated with nifedipine, the reduction of $[Ca^{2+}]_i$ and the relaxation induced by NKH477 (100 nM) were both attenuated in the presence of IbTX (30 nM). The extent of the $[Ca^{2+}]_i$ reduction and that of relaxation in response to NKH477 (100 nM) in the preparations pretreated with 1 μM nifedipine were both significantly less than those in the experiments without nifedipine. Moreover, the reversal of the effect of NKH477 on $[Ca^{2+}]_i$ and tension by IbTX was abolished in muscles pretreated with nifedipine. Comparable results were obtained in the experiments with isoprenaline (Figure 5b). Thus, the reduction of $[Ca^{2+}]_i$ and the relaxation induced by isoprenaline (200 nM) were attenuated in parallel in the presence of IbTX (30 nM); this IbTX action to reverse the isoprenaline-induced changes in $[Ca^{2+}]_i$ and tension was abolished by pretreatment of the muscle with nifedipine (1 μM).

The present results on $[Ca^{2+}]_i$ provide new evidence to support the hypothesis of functional antagonism (Huang *et al.*, 1993). NKH477 is expected to cause hyperpolarization of tracheal smooth muscle cell membrane, as shown for isoprenaline, forskolin and other agents that increase cyclic AMP (Allen *et al.*, 1985; Honda *et al.*, 1986), most likely due to activation of BK_{Ca} channels. This would result in a decrease in Ca^{2+} influx through VDCs, leading to a decrease in $[Ca^{2+}]_i$. BK_{Ca} channels are known to be activated not only by G_s and cyclic AMP but also by cyclic GMP (Kaczorowski & Jones, 1995). Recently, Mikawa *et al.* (1997) found that the relaxation of guinea-pig trachealis induced by atrial natriuretic peptide (ANP) or 8-bromocyclic GMP was antagonized by IbTX (30 nM), and the antagonism was inhibited in the presence of nifedipine (1 μM). Moreover, the modification of relaxation by IbTX was associated with parallel changes in $[Ca^{2+}]_i$ measured by fura-2 signals; the decrease in $[Ca^{2+}]_i$ in response to ANP or 8-bromocyclic GMP was inhibited by IbTX. These observations seem to suggest that a common mechanism (a decrease in Ca^{2+} influx through VDCs secondary to a hyperpolarization elicited by activation of BK_{Ca} channels) is involved in both cyclic AMP and cyclic GMP-dependent bronchorelaxation.

An increased Ca^{2+} influx through VDCs secondary to a depolarization induced by blockade of BK_{Ca} channels is expected to inhibit the bronchodilation by other relaxants (Small *et al.*, 1993). However, ChTX and IbTX failed to inhibit the airway smooth muscle relaxant activity of cromakalim (BRL-34915) (Jones *et al.*, 1990; 1993; Murray *et al.*, 1991) and

pinacidil (Jones *et al*., 1990). One possible interpretation for this failure is 'accentuated antagonism' of the relaxants provided by the BK_{Ca} channel blockers. In our fura-2 signal experiments, IbTX (30 nM) did not affect the basal levels of $R_{340/380}$ and tension. The response of $R_{340/380}$ and tension to histamine (3 μM) was also unaffected by IbTX (30 nM). These findings suggest that selective blockade of BK_{Ca} channels can effect Ca^{2+} influx only when the channels are activated by substances such as cyclic AMP, G_s or cyclic GMP.

Conclusion

NKH477 has a potent bronchorelaxant action. This effect may result at least in part from activation of BK_{Ca} channels, which causes hyperpolarization of smooth muscle cell membranes and a secondary decrease in Ca^{2+} influx through VDCs. Nevertheless, we cannot rule out other mechanisms responsible for the bronchorelaxation by NKH477, since the shift of the concentration-response curves to NKH477 in the presence of IbTX was relatively small ($\sim$4 fold), and at concentrations $\geqslant$300 nM, the compound still caused a full relaxation of the histamine-contracted preparations even in the presence of IbTX. Activation of PKA by cyclic AMP is known to result in the phosphorylation of a variety of specific proteins, that in turn trigger a variety of biochemical processes leading to cellular relaxation (Torphy & Hall, 1994). Such processes may include a reduction of the Ca^{2+} sensitivity of contractile proteins, a promotion of Ca^{2+} extrusion from the cell and an enhancement of Ca^{2+} sequestration into the intracellular stores or a reduction in the Ca^{2+} release from the stores. The hyperpolarizaton induced by K^+ channel activation could also lead to relaxation by mechanisms other than the inhibition of Ca^{2+} influx via VDCs (Tomita & Kume, 1994). Further and more extensive experimental studies are required to elucidate these mechanisms.

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References

ALLEN, S.L., BEECH, D.J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1985). Electrophysiological and other aspects of the relaxant action of isoprenaline in guinea-pig isolated trachealis. *Br. J. Pharmacol.,* **86,** 843–854.

BABA, K., SATAKE, T., TAKAGI, K. & TOMITA, T. (1986). Effects of verapamil on the response of the guinea-pig tracheal muscle to carbachol. *Br. J. Pharmacol.,* **88,** 441–449.

BAUMAN, G., FELIX, S., SATTELBERGER, U. & KLEIN, G. (1990). Cardiovascular effects of forskolin (HL 362) in patients with idiopathic congestive cardiomyopathy – a comparative study with dobutamine and sodium nitroprusside. *J. Cardiovasc. Pharmacol.,* **16,** 93–100.

COOK, N.S. & QUAST, U. (1990). Potassium channel pharmacology. In *Potassium Channels: Structure, Classification, Function and Therapeutic Potential*. ed. Cook, N.S. pp. 181–255, Chichester: Ellis Horwood, Ltd.

COOK, S.J., ARCHER, K., MARTIN, A., BUCHEIT, K.H., FOZARD, J.R., MULLER, T., MILLER, A.J., ELLIOTT, K.R.F., FOSTER, R.W. & SMALL, R.C. (1995). Further analysis of the mechanisms underlying the tracheal relaxant action of SCA40. *Br. J. Pharmacol.,* **114,** 143–151.

DE LANEROLLE, P., NISHIKAWA, M., YOST, A. & ADELSTEIN, R.S. (1984). Increased phosphorylation of myosin light chain kinase after an increase in cyclic AMP in intact smooth muscle. *Science,* **223,** 1415–1417.

GALVEZ, A., GIMENEZ-GALLEGO, G., REUBEN, J.P., ROY-CONTANCIN, L., FEIGENBAUM, P., KACZOROWSKI, G.J. & GARCIA, M.L. (1990). Purification and characterizaton of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion *Buthus tamulus*. *J. Biol. Chem.,* **265,** 11083–11090.

HIMETA, M., HOSONO, M., FUJIHARA, R. & NAKAMURA, K. (1991). Characterization of the vasodilator effects of NKH477, a novel forskolin derivative, in isolated rat and dog arteries. *Jpn. J. Pharmacol. (Suppl I),* **55,** 171.

HIRAMATSU, T., KUME, H., KOTLIKOFF, M.I. & TAKAGI, K. (1994). Role of calcium-activated potassium channels in the relaxation of tracheal smooth muscles by forskolin. *Clin. Exp. Pharmacol. Physiol.,* **21,** 367–375.

HONDA, K., SATAKE, T., TAKAGI, K. & TOMITA, T. (1986). Effects of relaxants on electrical and mechanical activities in the guinea-pig tracheal muscle. *Br. J. Pharmacol.,* **87,** 665–671.

HOSONO, M., TAKAHIRA, T., FUJITA, A., FUJIWARA, R., ISHIZUKA, O., TATEE, T. & NAKAMURA, K. (1992). Cardiovascular and adenylate cyclase stimulant properties of NKH477, a novel water-soluble forskolin derivative. *J. Cardiovasc. Pharmacol.,* **19,** 625–634.

HUANG, J.C., GARCIA, M.L., REUBEN, J.P. & KACZOROWSKI, G.J. (1993). Inhibition of β-adrenoceptor agonist relaxation of airway smooth muscle by Ca^{2+}-activated K^+ channel blockers. *Eur. J. Pharmacol.,* **235,** 37–43.

ISAAC, L., MCARDLE, S., MILLER, N.M., FOSTER, R.W. & SMALL, R.C. (1996). Effects of some K^+ channel inhibitors on the electrical behaviour of guinea-pig isolated trachealis and on its response to spasmogenic drugs. *Br. J. Pharmacol.,* **117,** 1653–1662.

ITO, S., SUZUKI, S. & ITOH, T. (1993). Effects of a water-soluble forskolin derivative (NKH477) and a membrane-permeable cyclic AMP analogue on noradrenaline-induced Ca^{2+} mobilization in smooth muscle of rabbit mesenteric artery. *Br. J. Pharmacol.,* **110,** 1117–1125.

JONES, T.R., CHARETTE, L., GARCIA, M.L. & KACZOROWSKI, G.J. (1990). Selective inhibition of relaxation of guinea-pig trachea by charybdotoxin, a potent Ca^{2+}-activated K^+ channel inhibitor. *J. Pharmacol. Exp. Ther.,* **255,** 697–706.

JONES, T.R., CHARETTE, L., GARCIA, M.L. & KACZOROWSKI, G.J. (1993). Interaction of iberiotoxin with β-adrenoceptor agonists and sodium nitroprusside on guinea pig trachea. *J. Appl. Physiol.,* **74,** 1879–1884.

KACZOROWSKI, G.J. & JONES, T.R. (1995). High conductance calcium-activated potassium channels. In *Airways Smooth Muscle: Peptide receptors, Ion Channels and Signal Transduction*. ed. Raeburn, D. & Giembycz, M.A. pp. 169–198, Basel: Birkhäuser Verlag.

KUME, H., GRAZIANO, M.P. & KOTLIKOFF, M.I. (1992). Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 11051–11055.

KUME, H., HALL, I., WASHABAU, R.J., TAKAGI, K. & KOTLIKOFF, M.I. (1994) β-Adrenergic agonists regulate K_{Ca} channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. *J. Clin. Invest.,* **93,** 371–379.

KUME, H. & TAKAGI, K. (1997). Inhibitory effects of G_s on desensitization of β-adrenergic receptors in tracheal smooth muscle. *Am. J. Physiol.,* **273,** L556–L564.

KUME, H., TAKAI, A., TOKUNO, H. & TOMITA, T. (1989). Regulation of Ca^{2+}-dependent K^+-channel activity in tracheal myocytes by phosphorylation. *Nature,* **341,** 152–154.

LAURENT, F., MICHEL, A., BONNET, P.A., CHAPAT, J.P. & BOUCARD, M. (1993). Evaluation of the relaxant effects of SCA40, a novel charybdotoxin-sensitive potassium channel opener, in guinea-pig isolated trachealis. *Br. J. Pharmacol.,* **108,** 622–626.

McCANN, J.D. & WELSH, M.J. (1986). Calcium-activated potassium channels in canine airway smooth muscle. *J. Physiol.,* **372,** 113–127.

MIKAWA, K., KUME, H. & TAKAGI, K. (1997). Effects of BK_{Ca} channels on the reduction of cytosolic Ca^{2+} in cGMP-induced relaxation of guinea-pig trachea. *Clin. Exp. Pharmacol. Physiol.,* **24,** 175–181.

MULLER, M.J. & BAER, H.P. (1983). Relaxant effects of forskolin in smooth muscle. Role of cyclic AMP. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **322,** 78–82.

MURRAY, M.A., BERRY, J.L., COOK, S.J., FOSTER, R.W., GREEN, K.A. & SMALL, R.C. (1991). Guinea-pig isolated trachealis: the effects of charybdotoxin on mechanical activity, membrane potential changes and the activity of plasmalemmal K^+-channels. *Br. J. Pharmacol.,* **103,** 1814–1818.

OZAKI H., SATO, K., SATOH, T. & KARAKI, H. (1987). Simultaneous recordings of calcium signals and mechanical activity using fluorescent dye fura 2 in isolated strips of vascular smooth muscle. *Jpn. J. Pharmacol.,* **45,** 429–433.

SCHMID-ANTOMARCHI, H., DE WEILLE, J., FOSSET, M. & LAZDUNSKI, M. (1987). The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel in insulin-secreting cells. *J. Biol. Chem.,* **262,** 15840–15844.

SEAMON, K.B. & DALY, J.W. (1981). Forskolin: a unique diterpene activator of cyclic AMP-generating systems. *J. Cyclic Nucleotide Res.,* **4,** 201–224.

SHAFIQ, J., SUZUKI, S., ITOH, T. & KURIYAMA, H. (1992). Mechanisms of vasodilation induced by NKH477, a water-soluble forskolin derivative, in smooth muscle of the porcine coronary artery. *Circ. Res.,* **71,** 70–81.

SMALL, R.C., CHIU, P., COOK, S.J., FOSTER, R.W. & ISAAC, L. (1993). β-adrenoceptor agonists in bronchial asthma: role of K^+-channel opening in mediating their bronchodilator effects. *Clin. Exp. Allergy,* **23,** 802–811.

STURGESS, N.C., KOZLOWSKI, R.Z., CARRINGTON, C.A., HALES, C.N. & ASHFORD, M.L.J. (1988). Effects of sulphonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. *Br. J. Pharmacol.,* **95,** 83–94.

TAKEUCHI, M., TAKAOKA, H., HATA, K., MORI, M., YAMAKAWA, H., YAMAGUCHI, K. & YOKOYAMA, M. (1995). NKH477: a new inotropic agent. *Cardiovasc. Drug Rev.,* **13,** 339–352.

TOMITA, T. & KUME, H. (1994). Electrophysiology of potassium channels in airway smooth muscle. In *Airways Smooth Muscle: Development and Regulation of Contractility*. ed. Raeburn, D. & Giembycz, M.A. pp 163–184, Basel: Birkhäuser Verlag.

TORPHY, T.J. & HALL, I.P. (1994). Cyclic AMP and the control of airways smooth muscle tone. In *Airways Smooth Muscle: Biochemical Control of Contraction and Relaxation*. ed. Raeburn, D. & Giembycz, M.A. pp. 215–232, Basel: Birkhäuser Verlag.

TSUKAWAKI, M., SUZUKI, K., SUZUKI, R., TAKAGI, K. & SATAKE, T. (1987). Relaxant effects of forskolin on guinea pig tracheal smooth muscle. *Lung,* **165,** 225–237.

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