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Short communication

cAMP-independent mechanism is significantly involved in β_2-adrenoceptor-mediated tracheal relaxation

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Abstract

The role of cAMP in the β_2-adrenoceptor-mediated relaxation in response to salbutamol was examined in guinea pig tracheal smooth muscle. The concentration-dependent salbutamol-induced relaxation was antagonized in a competitive fashion by a β_2-selective adrenoceptor antagonist, butoxamine, with a pA_2 value of 6.90. Salbutamol (10 μM) elevated the tracheal smooth muscle cAMP content by about fivefold, a response which was significantly inhibited by an adenylyl cyclase inhibitor, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ 22,536, 100 μM). However, the salbutamol-elicited relaxation was not diminished by SQ 22,536 (100 μM). These results provide evidence for the first time that a cAMP-independent mechanism(s) is involved in β_2-adrenoceptor-mediated tracheal smooth muscle relaxation in the guinea pig.

Keywords: Airway smooth muscle; β_2-Adrenoceptor; cAMP; (Guinea pig); Salbutamol; SQ 22,536

1. Introduction

Airway smooth muscles relax in response to the stimulation of β-adrenoceptors. The receptor subtype for triggering this relaxation of airway smooth muscle in response to β-adrenoceptor agonists is mainly the β_2-type (Lands et al., 1967; Nagatomo and Koike, 2000). As in the case of various other β-adrenoceptor-mediated cellular responses, stimulation of β_2-adrenoceptors with catecholamines or with selective agonists elevates the tissue cAMP content in tracheal smooth muscle (Ellis et al., 1995; Hoiting et al., 1996; Jones et al., 1990; McGrogan et al., 1995; Nakagawa et al., 1986). Furthermore, direct adenylyl cyclase activators, such as forskolin, phosphodiesterase inhibitors, membrane-permeable cAMP analogs, and the heterotrimeric G_s-protein activator, cholera toxin (CTX), produce a potent relaxation and an elevation of the cAMP content in tracheal smooth muscle (Devillier et al., 2001; Hoiting et al., 1996; Jones et al., 1990; McGrogan et al., 1995; Nakagawa et al., 1986; Tanaka et al., 2003; Tsukawaki et al., 1987; Watanabe et al., 1976). Therefore, increased levels of cAMP, via G_s-protein-coupled adenylyl cyclase, are recognized to be largely responsible for the β_2-adrenoceptor-mediated relaxation of tracheal smooth muscle (Johnson, 1998; Torphy, 1994; Torphy and Hall, 1994). However, we have recently shown that the relaxation of gastrointestinal smooth muscles mediated by a nonconventional type of β-adrenoceptor (β_3-adrenoceptor) can be produced independently of the increase in tissue cAMP levels (Horinouchi and Koike, 2002). It still remains unknown whether this cAMP-independent mechanism(s) only underlies β_3-adrenoceptor-mediated relaxation, or whether this mechanism is also involved in the relaxation mediated by non-β_3-type β-adrenoceptors. The present study was thus carried out using salbutamol, a selective β_2-adrenoceptor agonist (Brittain et al., 1968; Waldeck, 2002), to determine whether β_2-adrenoceptor-mediated relaxation of tracheal smooth muscle is capable of being elicited independently of an increase in tissue cAMP level.

2. Materials and methods

Male or female Hartley guinea pigs weighing 350–600 g (Saitama Experimental Animals, Saitama, Japan) were used in the present study. Guinea pigs were housed in rooms in which temperature (20–22 °C) and relative air humidity (50 ± 5%) were strictly regulated. Food and water were

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available ad libitum to all animals. This study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences [accredited by The Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan].

2.1. Mechanical responses

Guinea pigs were killed by cervical dislocation and exsanguinated from the common carotid or external iliac artery. Tracheal tissues were carefully isolated and immersed in Ringer–Locke solution (in mM: NaCl, 154.0; KCl, 5.6; $CaCl_2$, 2.2; $MgCl_2$, 2.1; $NaHCO_3$, 5.9; and glucose, 2.8) bubbled with 95% O_2–5% CO_2 mixture. The tracheal tissues were cleaned of unnecessary adipose and connective tissues under a dissecting microscope. Subsequently, tracheal cartilage containing smooth muscles were cut into about 2-mm-long pieces. In this series of experiments, the intimal surface of tracheal tissue was gently rubbed with moistened filter paper to remove tracheal epithelium as much as possible. Preparations were suspended with stainless steel hooks (outer diameter, 200 μm) in a 5-ml organ bath (UC-5; UFER Medical Instrument, Kyoto, Japan) containing Ringer–Locke solution, which was maintained at 36 ± 1 °C and bubbled with the O_2–CO_2 mixture. Tension changes were isometrically recorded with a force displacement transducer (T7-8-240; Orientec, Tokyo, Japan) connected to an amplifier (high-gain DC amplifier: model, AD 632J; Nihon Kohden, Tokyo, Japan).

Relaxant effects of salbutamol were examined as follows. In this tracheal tissue, when the preparation was preloaded with an initial tension of 2.0 g, tension developed spontaneously. During the tension development due to stretch, the bath solution was renewed every 20 min for 60 min. This spontaneous tension development lasted for several hours without an appreciable decline. Sixty minutes after the initial tension increase, muscle was contracted with histamine (10 μM) for 15 min, which was subsequently washed out. After this procedure, the tracheal preparation was incubated for another 60 min with renewal of bath solution every 20 min, and was contracted again with 10 μM histamine. When the active tension obtained with histamine and the initial muscle stretch reached a steady-state level about 30 min after the application of histamine, salbutamol was cumulatively applied to the bath medium until a maximum response was obtained. At the end of each experiment, papaverine (100 μM) was applied to the bath medium to obtain the maximum relaxant response. Butoxamine (0.3–3 μM) or ICI-118,551 (10–100 nM) was applied to the bath medium simultaneously with histamine, i.e., 30 min before cumulative application of salbutamol. When the effects of SQ 22,536 [9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine] (Turcato and Clapp, 1999) were examined, this adenylyl cyclase inhibitor (100 μM) was added to the bath medium simultaneously with histamine application. Thereafter, salbutamol was cumulatively applied to the bath medium 20 min after the application of histamine in the absence and presence of SQ 22,536.

2.2. Determination of tissue cAMP content

After removal of unnecessary adipose and connective tissue, smooth muscle bundles were carefully isolated from tracheal tissue and then cut into segments about 8 mm in length. Each muscle bundle was incubated in an organ bath containing Ringer–Locke solution (5 ml), which was continuously gassed with 95% O_2–5% CO_2 and maintained at 36 ± 1 °C. After a 60-min incubation, preparations were exposed to salbutamol (10 μM) for 5 min. When SQ 22,536 (100 μM) was used, it was applied to the bath medium 20 min before stimulation with salbutamol. At the end of the protocol, preparations were rapidly frozen in liquid N_2 to terminate the reaction and homogenized in 6% trichloroacetic acid solution, and then centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant fraction and the tissue pellets were used for the measurement of cAMP and protein content, respectively. The cAMP in the supernatant was extracted with water-saturated ether for four times to remove TCA and then lyophilized. cAMP content was measured using an enzyme immunoassay (EIA) system (cAMP Biotrak EIA system; Amersham Biosciences, Buckinghamshire, UK). Tissue pellets were dissolved in 1 ml of 1 M NaOH for protein determination (Lowry et al., 1951). The cAMP content is expressed in picomoles (pmol) per milligram of sample protein. In these measurements, tracheal smooth muscle preparations were not exposed to phosphodiesterase inhibitors as reported previously (Tanaka et al., 2003; Turcato and Clapp, 1999; Yamaki et al., 2001) because the inhibitors could elevate cAMP to unphysiologically high levels, and thus could elevate cGMP levels, both of which may complicate data interpretation (Turcato and Clapp, 1999).

2.3. Drugs

Drugs used in the present study were as follows: salbutamol (albuterol; α-[(*t*-butylamino)methyl]-4-hydroxy-*m*-xylene- α,α′ -diol) hemisulfate, butoxamine (α-[1-(*t*-butylamino)ethyl]-2,5-dimethoxybenzyl alcohol) hydrochloride, histamine dihydrochloride, papaverine hydrochloride, indomethacin (Sigma-Aldrich, St. Louis, MO, USA); ICI-118,551 ((±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) hydrochloride, SQ 22,536 (9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine) (Sigma-RBI, St. Louis, MO, USA). All other chemicals used were of analytical grade. Indomethacin was dissolved in pure ethanol at a concentration of 10 mM. Distilled water was used to dissolve and dilute all other drugs. All drugs are expressed in molar concentrations (M) in the bathing medium.

2.4. Data analysis

To construct concentration–response relationships for salbutamol, the percent relaxant response was calculated considering the tension level before application of salbutamol (spontaneous tone plus histamine-elicited tension) as 0% and the maximum relaxation obtained after application of 100 μM papaverine as 100%. Data were plotted as a function of salbutamol concentration and fitted to the equation:

$$E = E_{max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H})$$

where E is % relaxation at a given salbutamol concentration, E_{max} is the maximum relaxation, A is the concentration of salbutamol, n_H is the slope function and EC_{50} is the effective salbutamol concentration that produces a 50% response. Curve fitting was carried out using GraphPad Prism™ (Version 4.00) (GraphPad Software, San Diego, CA, USA). The EC_{50} values were converted to logarithmic values (pD_2, $-\log EC_{50}$) for statistical analysis. The competitive antagonistic potencies of butoxamine and ICI-118,551 are expressed as pA_2 values and were calculated according to the method originally reported by Arunlakshana and Schild (1959).

Data are presented as mean values ± S.E.M. or mean values with 95% confidence intervals in parentheses and n refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad Prism™ by unpaired t-test, unpaired t-test with Welch's correction if necessary, and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A P value less than 0.05 was considered statistically significant.

3. Results

In guinea pig tracheal smooth muscle, salbutamol evoked a potent relaxation in a concentration-dependent manner with a pD_2 value of 7.66 ± 0.10 (n = 4) (Fig. 1A, open circles). The relaxant response to salbutamol was competitively antagonized by butoxamine, and thus the concentration–response curves for salbutamol were shifted to the right in a parallel fashion with increasing concentrations of butoxamine (Fig. 1A, open circles vs. filled symbols). Schild regression analysis carried out for butoxamine against salbutamol gave a pA_2 value of 6.90 (95% confidence intervals: 6.72–7.16, n = 12) and a straight line with a slope of 0.98, which was not significantly different from unity (0.78–1.19, n = 12) (Fig. 1B). The pA_2 value for butoxamine against salbutamol was not changed by the treatment with indomethacin; the value in the presence of 3 μM indomethacin was 6.66 (6.47–6.98) [slope = 1.20: 0.84–1.56] (n = 12 for each).

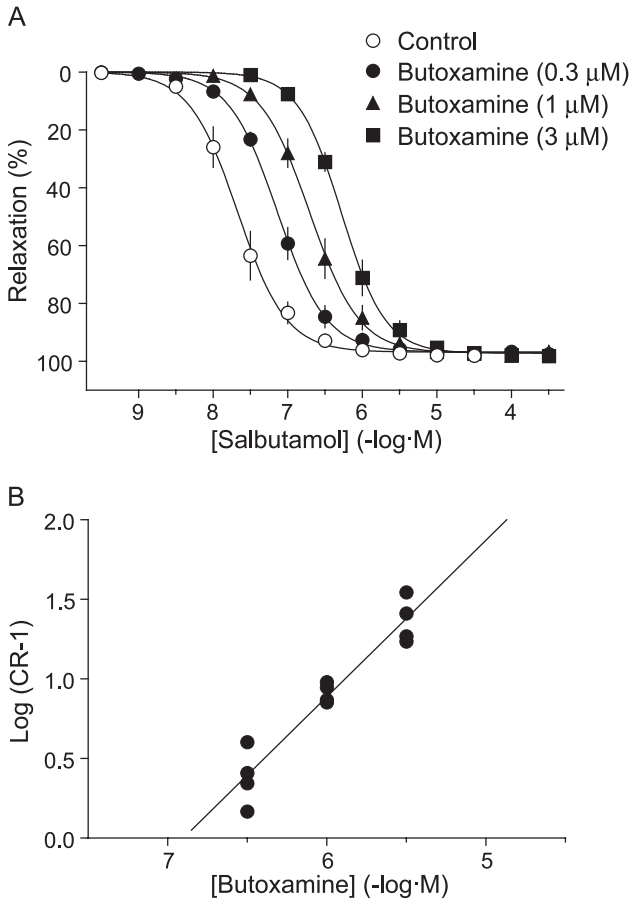

Fig. 1. Salbutamol relaxes guinea pig tracheal smooth muscle through mediation of the β_2-type of adrenoceptor. (A) Concentration–response relationships for salbutamol-elicited relaxation in the absence and presence of butoxamine. Tracheal smooth muscle relaxation was calculated with respect to the tension before administration of salbutamol (0% relaxation) and the maximally relaxed tension attained with 100 μM papaverine (100% relaxation). Data are mean values ± S.E.M. for four experiments. The maximum relaxation in response to salbutamol was control: 98.0 ± 0.8%; 0.3 μM butoxamine, 98.2 ± 0.9%; 1 μM butoxamine, 98.2 ± 0.9%; 3 μM butoxamine, 98.5 ± 0.6% (n = 4 for each). (B) The Schild plot analysis for competitive antagonism by butoxamine against salbutamol. The data analyzed (n = 12 points) are from experiments shown in panel A.

The salbutamol-induced relaxation was also competitively antagonized by ICI-118,551. The pA_2 value for ICI-118,551 given by the Schild regression analysis was 8.84 (8.56–9.28) [slope = 1.13: 0.85–1.40] (n = 9 for each).

Fig. 2A shows the increase in tissue cAMP content in response to salbutamol and its inhibition by SQ 22,536. Salbutamol (10 μM) elevated the tissue cAMP content from 4.7 ± 0.7 pmol/mg protein to 21.5 ± 3.6 pmol/mg protein (n = 12 for each, P < 0.01), producing a 4.6-fold increase. In contrast, the cAMP content was not significantly elevated by salbutamol (10 μM) in the presence of SQ 22,536 (100 μM). However, SQ 22,536 (100 μM) did not diminish the relaxant response to salbutamol (Fig. 2B); pD_2 values for salbutamol and the maximum relaxant responses (E_{max}) were 7.53 ± 0.05 and 96.3 ± 1.3% (n = 4 for each) in the absence of SQ 22,536, and 7.66 ± 0.03 and 98.8 ± 0.3%

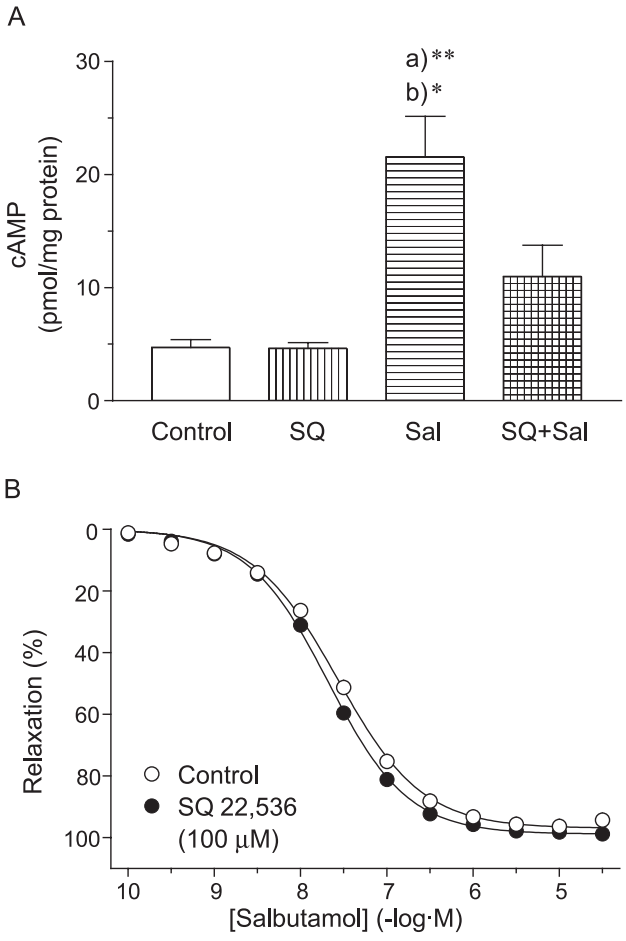

Fig. 2. cAMP-independent mechanism(s) is substantially involved in the salbutamol-induced relaxant response of guinea pig tracheal smooth muscle. (A) Basal cAMP content (control) was not affected by SQ 22,536 (100 μM, SQ). Salbutamol (10 μM, Sal) increased the tissue cAMP content, which was significantly inhibited by SQ 22,536. Data are mean values ± S.E.M. of 8–12 experiments. (a)**$P<0.01$, significant differences from control (basal level) and SQ 22,536 (100 μM) treatment; (b)*$P<0.05$, significant differences from SQ 22,536 (100 μM) plus salbutamol (10 μM) treatment (one-way ANOVA with Tukey's multiple comparison test). (B) Concentration–response relationships for salbutamol-induced relaxation in the absence and presence of the adenylyl cyclase inhibitor SQ 22,536 (100 μM). SQ 22,536 did not diminish the salbutamol-induced relaxation. Tracheal smooth muscle relaxation is expressed as percent inhibition of the muscle contraction induced by histamine (10 μM) plus spontaneous tone. Data are mean values ± S.E.M. of four experiments.

($n=4$ for each) in the presence of 100 μM SQ 22,536. These pD_2 and E_{max} values were not significantly different ($P>0.05$) in the absence and presence of SQ 22,536. SQ 22,536 (100 μM) also did not affect the salbutamol-induced relaxation in the presence of indomethacin (3 μM) ($n=4–5$ for each experiment).

4. Discussion

The present studies show that salbutamol is capable of relaxing guinea pig tracheal smooth muscle when the activity of adenylyl cyclase is inhibited, and thus when the cAMP content is not increased. This finding indicates that a mechanism(s) independent of cAMP is involved in the salbutamol-induced relaxation of tracheal smooth muscle, and provides evidence that a cAMP-independent mechanism(s) is involved in the β_2-adrenoceptor-mediated smooth muscle relaxant response.

In the present study, we showed that salbutamol, a selective agonist for β_2-adrenoceptor (Brittain et al., 1968; Waldeck, 2002), caused a concentration-dependent and full relaxation of guinea pig tracheal smooth muscle. The salbutamol-induced relaxation is mediated through β_2-type of adrenoceptors because the relaxant response was competitively antagonized by butoxamine with a pA_2 value of 6.90, which is in agreement with the pA_2 value for this antagonist vs. salbutamol in guinea pig taenia caecum (6.68) (Koike et al., 1997). The main role of the β_2-type of adrenoceptor in the salbutamol-induced relaxation is supported by the competitive antagonism observed with another β_2-selective adrenoceptor antagonist, ICI-118,551; its pA_2 value (8.84) was consistent with the values obtained in guinea pig uterus (9.26) (Bilski et al., 1983) and in guinea pig tracheal muscle against salbutamol (9.07) (Brandt and Meyer, 1987).

The main finding of this study is that the β_2-adrenoceptor-mediated relaxation in response to salbutamol was not diminished when the activity of adenylyl cyclase was suppressed by its inhibitor (SQ 22,536). This result indicates that in addition to a cAMP-dependent mechanism(s), a mechanism(s) independent of cAMP elevation [cAMP-independent mechanism(s)] is also relevant in the β_2-adrenoceptor-mediated relaxation of tracheal smooth muscle. This finding is unexpected because, so far, the elevation of intracellular cAMP content, which subsequently activates cAMP-dependent mechanism(s) [protein kinase A (PKA)-dependent cellular event(s)], has been generally thought to be primarily responsible for β_2-adrenoceptor-mediated relaxation of this smooth muscle (Johnson, 1998; Torphy, 1994). Similar to our present finding, β_2-adrenoceptor-induced inhibition of histamine-stimulated phosphoinositide turnover in airway smooth muscle has been suggested to be independent of cAMP accumulation (Chilvers et al., 1997). However, we have to stress that we are not ruling out the involvement of cAMP-dependent mechanism(s) in the β_2-adrenoceptor-mediated relaxant response of tracheal smooth muscle because salbutamol significantly elevated the tissue cAMP content (Fig. 2A). Indeed, the cyclic AMP-dependent mechanism(s) exists and operates in guinea pig tracheal smooth muscle as shown by the following: (1) isoprenaline-induced relaxation is potentiated by a selective inhibitor of cAMP-specific phosphodiesterase (our unpublished observation); (2) a membrane-permeable cAMP analog (8-bromo-cAMP) produces a strong relaxation (Tanaka et al., 2003); and (3) the direct adenylyl cyclase activator forskolin and the G_s-protein activator CTX both increase the tissue cAMP content and cause smooth muscle relaxation (Tanaka

et al., 2003). However, at present, it seems practically impossible to determine the extent of the involvement of cAMP-dependent and -independent mechanism(s) in the whole β_2-adrenoceptor-mediated relaxant response since these two mechanisms are likely to be tightly coupled functionally through G_s (Tanaka et al., 2003).

In the present study, SQ 22,536 (100 μM) significantly suppressed the cAMP-elevating action of salbutamol and there were no significant differences in tissue cAMP levels between basal and SQ 22,536 plus salbutamol. However, even in the presence of 100 μM SQ 22,536, salbutamol (10 μM) elevated the tissue cAMP content by about twofold, although the change was insignificant: from 4.6 ± 0.5 pmol/mg protein ($n = 10$) to 11.0 ± 2.8 pmol/mg protein ($n = 8$) ($P > 0.05$, Tukey's multiple comparison test) (Fig. 2A). Therefore, the possibility cannot be completely ruled out that this increase in cAMP content is responsible for the salbutamol-induced relaxation in the presence of SQ 22,536 (Fig. 2B). However, the involvement of a cAMP-independent mechanism is substantial in β_2-adrenoceptor-mediated tracheal relaxation, as suggested by the following evidence: (1) Forskolin (10 μM), which evokes almost complete relaxation of this smooth muscle, elevates the tissue cAMP content by about 300-fold vs. basal (Tanaka et al., 2003). However, the elevation of the tissue cAMP content induced by salbutamol (10 μM), which also evokes complete relaxation, was only fivefold (Fig. 2A). If we assume that the cAMP-dependent route is the sole mechanism to trigger β_2-adrenoceptor-mediated relaxation, this huge difference in the extent of cAMP elevation between forskolin and salbutamol cannot be explained. (2) CTX (5 μg/ml), an activator of G_s-protein, elevates the tracheal tissue cAMP content by about 30-fold vs. basal. The CTX-induced elevation of cAMP content is also significantly suppressed by 100 μM SQ 22,536. However, 5 μg/ml CTX evokes a full relaxation, but, this relaxant response to CTX is not affected by 100 μM SQ 22,536 (Tanaka et al., 2003). These findings also support the contribution of a cAMP-independent mechanism in β_2-adrenoceptor-mediated tracheal relaxation. (3) The contribution of the cAMP-independent relaxant mechanism is also substantial in β_3-adrenoceptor-mediated gastrointestinal smooth muscle relaxation (Horinouchi and Koike, 2002) and IP receptor-mediated vascular smooth muscle relaxation (Turcato and Clapp, 1999; Yamaki et al., 2001).

We have already shown that the involvement of the cAMP-independent mechanism(s) is substantial in β_3-adrenoceptor-mediated relaxation of gastrointestinal smooth muscles (Horinouchi and Koike, 2002). Because our present study indicates that the cAMP-independent mechanism(s) is also involved in the smooth muscle relaxation mediated through β_2-adrenoceptors, this mechanism(s) may not be restricted to the nonconventional type of β (β_3)-adrenoceptor-mediated relaxant response. Furthermore, the cAMP-independent mechanism(s) is also involved in the prostacyclin receptor (IP receptor)-mediated relaxation of vascular smooth muscles (Turcato and Clapp, 1999; Yamaki et al., 2001). Therefore, this mechanism(s) may have a substantial regulatory function in G_s-protein-coupled receptor-mediated smooth muscle relaxation. Because in the case of β_3-adrenoceptor or IP receptor-mediated relaxation, activation of some types of K^+ channels (K_v channel and/or MaxiK channel) can partly account for the cAMP-independent mechanism(s) (Horinouchi et al., 2003; Yamaki et al., 2001), a similar mechanism(s) (K^+ channel activation) might also underlie the β_2-adrenoceptor mediated tracheal relaxation (Johnson, 1998; Jones et al., 1990; Kume et al., 1994; Torphy, 1994; Torphy and Hall, 1994). However, the detailed cellular events by which stimulation of β_2-adrenoceptors evokes smooth muscle relaxation independently of tissue cAMP elevation should be established in the future.

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