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Original Article

Relaxing action of adrenergic β_2-agonists on guinea-pig skinned tracheal muscle

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Abstract

Although adrenergic β_2-agonist-induced smooth muscle relaxation has been attributed to increased intracellular cyclic AMP (cAMP), a relaxation response has been observed at low β_2-agonist concentrations that do not cause increased cAMP. To elucidate the mechanism of tracheal muscle relaxation induced by low concentrations of β_2-agonists, we used a guinea-pig skinned tracheal smooth muscle preparation to examine the effects on the contractile protein system. The isotonic contraction of β-escin-treated skinned tracheal muscle from guinea-pig was measured. When the intracellular Ca^{2+} concentration was maintained at 1 μmol/L in the presence of guanosine 5′-triphosphate (GTP; 100 μmol/L), neither isoproterenol (10 nmol/L) nor salbutamol (60 nmol/L) affected Ca^{2+} sensitivity, but a significant decrease in Ca^{2+} sensitivity was observed in the presence of okadaic acid (1 μmol/L). The decrease in Ca^{2+} sensitivity was a slow response and was blocked by pretreatment with propranolol (1 μmol/L). Forskolin (1 μmol/L) did not affect Ca^{2+} sensitivity. These results suggest that adrenergic β_2-agonists may activate protein phosphatase through an unknown pathway involving the β_2-receptor, which enhances dephosphorylation of the myosin light chain and/or thin filament proteins, resulting in relaxation of the tracheal smooth muscle.

Key words: adrenergic β_2-agonists, β-escin, guinea-pig, okadaic acid, skinned tracheal muscle.

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Introduction

Adrenergic β_2-agonists are fast-acting and potent bronchodilators used widely as a first-choice inhalant for asthma attacks even if the patient is receiving corticosteroids. However, some mechanisms of the bronchodilating effect of β_2-agonists remain unclear.

Interestingly, the magnitude of the increase of intracellular cyclic AMP (cAMP) in smooth muscle during β_2-agonist administration does not reflect the degree of the relaxing response,[1,2] although many studies have reported a correlation between the increase in cAMP and the relaxing action.[3–6] The latter studies that reported a cAMP increase used drug concentrations that exceeded the maximum response in the relaxation curve.

What triggers the expression of the relaxing action of adrenergic β_2-agonists? A decrease in intracellular Ca^{2+} level has been reported during tracheal smooth muscle relaxation.[7,8] Several mechanisms are involved in this decreased Ca^{2+} level, and they can be broadly divided into the inhibition of Ca^{2+} influx,[8–11] increase of Ca^{2+} uptake to the store site[8,12] and the enhancement of Ca^{2+} efflux.[13,14]

To investigate the mechanisms of cAMP-independent tracheal muscular relaxation, we used adrenergic β_2-agonists at the concentrations that induced maximum relaxation without a cAMP increase. We examined the relaxing actions of isoproterenol (ISO) and salbutamol (Salb) using skinned preparations of guinea-pig tracheal muscle, which were devoid of plasma membrane functions such as ion channels and membrane excitability.

Methods

Preparation of tracheal skinned muscle

A female Hartley strain guinea-pig (bodyweight 200–250 g) was decapitated at the cervical vertebrae and

exsanguinated under pentobarbital sodium (30 mg/ kg, i.p.) anesthesia. The trachea was removed and immediately immersed in a normal external solution (NES; 150 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L calcium methanesulfonate, 1 mmol/L magnesium methanesulfonate, 10 mmol/L glucose, and 5 mmol/L *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), pH 7.4).

Skinned muscle was prepared by a modified method of Watanabe and Nakano.[15] The isolated trachea was cut open along the center of the cartilages in NES at room temperature. The two ends were fixed on a resin support. The mucosal and serosal layers were removed carefully to expose the tracheal smooth muscle layer. Minute muscle bundle samples measuring 200 μm in width and 1 mm in length were prepared. Two stainless steel hooks (approximately 100 μm in thickness) were passed through the cartilage on both ends of a muscle sample. The hook on one end was stabilized, and the other hook was connected by a silk thread to an isotonic transducer (AS2103, Sanei). The change in the length of the muscle bundle sample was measured as an index of contraction. A minute sample loaded with 0.2 *g* of force was equilibrated in NES for 30 min at 25°C. After confirming the contractibility using 100 μmol/L acetylcholine, the sample was incubated for 60–90 min in a cytoplasm substitution solution (CSS) containing Ca^{2+} (3 μmol/L) and β-escin (50 μmol/L), and the resulting muscle bundle was used as the skinned muscle. The CSS contained 200 mmol/L potassium methanesulfonate, 1.5 mmol/L magnesium methanesulfonate, 3.5 mmol/L MgATP, 10 mmol/L creatine phosphate and 20 mmol/L PIPES (pH 7.0). The CSS used to contract skinned strips was dissolved in 10 mmol/L *O,O*'-bis(2-aminoethyl) ethylene-glycol *N,N,N',N'*- tetraacetic acid (EGTA). To obtain the various Ca^{2+} concentrations used in the experiment, the Ca^{2+} concentration in the CSS was adjusted by changing the ratio of EGTA/Ca-EGTA. The dissociation constant of Ca-EGTA was assumed to be $10^{6.4}$ M^{-1}. This constant was used to calculate the Ca^{2+} concentration in the CSS.[15,16]

Measurement of isotonic contraction of skinned muscle

A β-escin-treated skinned muscle was washed in Ca^{2+}-free CSS and treated with A23187 (10 μmol/L) for 20 min to remove the Ca^{2+} in the sarcoplasmic reticulum. After the contraction of the muscle had reached a plateau at a fixed Ca^{2+} concentration (1 μmol/L), the muscle was transferred to a well (420 μL) containing specific concentrations of test substances and the response was recorded. The contraction induced by a substance was determined by setting the length of the muscle sample in Ca^{2+}-free medium at 0, and assigning the contraction of the sample induced by guanosine 5'-triphosphate (GTP) or okadaic acid (OA) in the presence of 1 μmol/L Ca^{2+} as 100%. The change in muscle length induced by a substance was expressed as a percentage. All experiments were conducted at 25°C using CSS supplemented with calmodulin (1 μmol/L).

Determination of intracellular cAMP levels

The intracellular cAMP level in the smooth muscle of the intact tracheal tissue after ISO, Salb or forskolin (Fors) treatment was measured by an enzyme immunoassay.

An intact tracheal muscle strip (1 mm × 7 mm) with cartilage removed was loaded with 0.5 *g* of force. After incubating in 5 mL Krebs-HEPES solution (110.5 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 1 mmol/L NaH_2PO_4, 11.5 mmol/L glucose and 24.5 mmol/L HEPES; pH 7.4) supplemented with 3-isobutyl-1-methylxanthine (0.1 mmol/L) for 30 min at 37°C under 100% O_2, the test substance was added and allowed to react for 16 min. The reaction was then stopped by adding ice-cold trichloroacetic acid (12%; w/v). The sample was homogenized in a glass homogenizer. The homogenate was centrifuged at 2000 *g*, 4°C for 15 min. The supernatant was extracted by ether, and the aqueous layer was dried under N_2 gas at 60°C. The cAMP concentration in the resulting sample was measured using a cAMP enzyme-immunoassay system (Amersham). The protein concentration in the sample was measured according to Lowry's method.[17]

Statistical analyses

The data are expressed as mean ± SEM. After performing a test for equal variance, the differences of the mean values were analyzed using a one-tailed unpaired *t*-test.

Chemicals

The concentrations of chemicals are expressed in molar concentration of the active substance. The following chemicals were used: A23187 (Calbiochem, San Diego, CA, USA); acetylcholine hydrochloride (Daiichi Seiyaku Co Ltd, Tokyo, Japan); adenosine 3',5'-cyclic monophosphate (cAMP; Sigma Chemical Co., St Louis, MO, USA); adenosine 5'-triphosphoric acid disodium salt (ATP; Wako Pure Chemical Industries, Osaka, Japan);

β-escin (Sigma); calmodulin (Sigma); cAMP enzyme-immunoassay system (Amersham Co., Tokyo, Japan); *O,O'*-bis (2-aminoethyl) ethylene-glycol-*N,N,N',N'*-tetra-acetic acid (EGTA; Wako Pure Chemical Industries); forskolin (Wako Pure Chemical Industries); HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Wako Pure Chemical Industries); 3-isobutyl-1-methylxanthine (Aldrich Chemical Co. Inc., Milwaukee, WI, USA); piperazine-1, 4-bis(2-ethanesulfonic acid) (PIPES; Wako Pure Chemicals); ± isoproterenol hydrochloride (Sigma); salbutamol sulfate (Wako Pure Chemical Industries, Osaka, Japan); okadaic acid ammonium (RBI, Natick, MA, USA).

RESULTS

Changes in intracellular cAMP level induced by adrenergic β_2-agonists or Fors

The concentrations of β_2-agonists and Fors used in the experiments were the concentrations that produced maximum relaxation of guinea-pig isolated tracheal muscle strips in an organ bath determined in a preliminary experiment (data not shown). No differences in cAMP levels were observed between the untreated control preparation and the preparation treated with ISO (10 nmol/L) or Salb (60 nmol/L), but a significant increase in cAMP level was observed in the preparation treated by Fors (1 μmol/L) (Fig. 1).

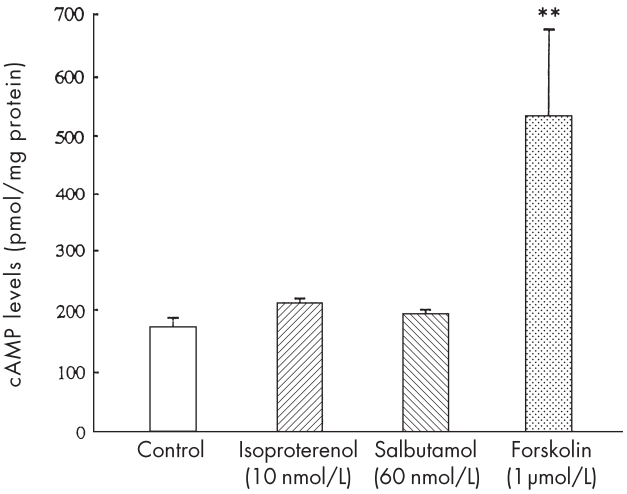

Fig. 1 Intracellular cAMP levels after adrenergic β_2-agonists and forskolin administration. Intracellular cAMP levels were measured by the cAMP enzyme immunoassay system. After 30 min of isobutyl-methylxanthine (0.1 mmol/L) treatment, the preparations from intact tracheal muscle were treated for 16 min with isoproterenol (10 nmol/L), salbutamol (60 nmol/L) or forskolin (1 μmol/L) at concentrations that caused maximum relaxation. Only the Fors group showed significant increase ($P < 0.01$) in cAMP levels compared with the control group. Points show the mean values ± SEM ($n = 3$–12).

Ca^{2+} sensitivity in skinned tracheal muscle

In the skinned tracheal muscle obtained by β-escin treatment, contraction increased with increases in Ca^{2+} concentration in CSS (Fig. 2). Contraction was observed at an intracellular Ca^{2+} concentration of 0.3 μmol/L and a peak response was reached at 10 μmol/L, with an ED_{50} value of 1.26 μmol/L.

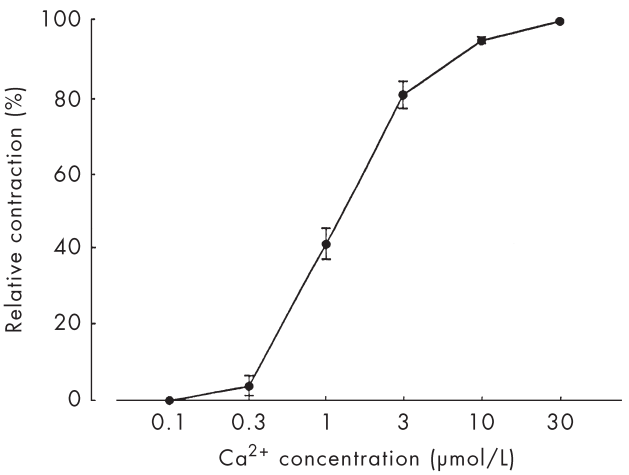

Fig. 2 Relationship between relative contraction and Ca^{2+} concentration in guinea-pig skinned tracheal muscle preparations. Skinned muscle preparations showed contraction depending on the Ca^{2+} concentration in the solution. Points show the mean values ± SEM ($n = 5$).

Effect of adrenergic β_2-agonists on skinned muscle in the presence of GTP

With the intracellular Ca^{2+} concentration maintained at 1 μmol/L, the skinned muscle was treated with a β_2-agonist for 90 min in the presence of GTP (100 μmol/L). Treatment with ISO (10 nmol/L) or Salb (60 nmol/L) did not affect the Ca^{2+} sensitivity of the skinned muscle (Fig. 3). However, when ISO (10 nmol/L) was added in the presence of OA (1 μmol/L), a phosphatase inhibitor, relaxation occurred slowly, and approximately 20% relaxation from the maximum contraction level was observed after 30 min (Fig. 4). The same relaxation effect was also observed with Salb (60 nmol/L) treatment.

The ISO- or Salb-induced relaxation was reproducible, and the relaxation effect increased with increases in

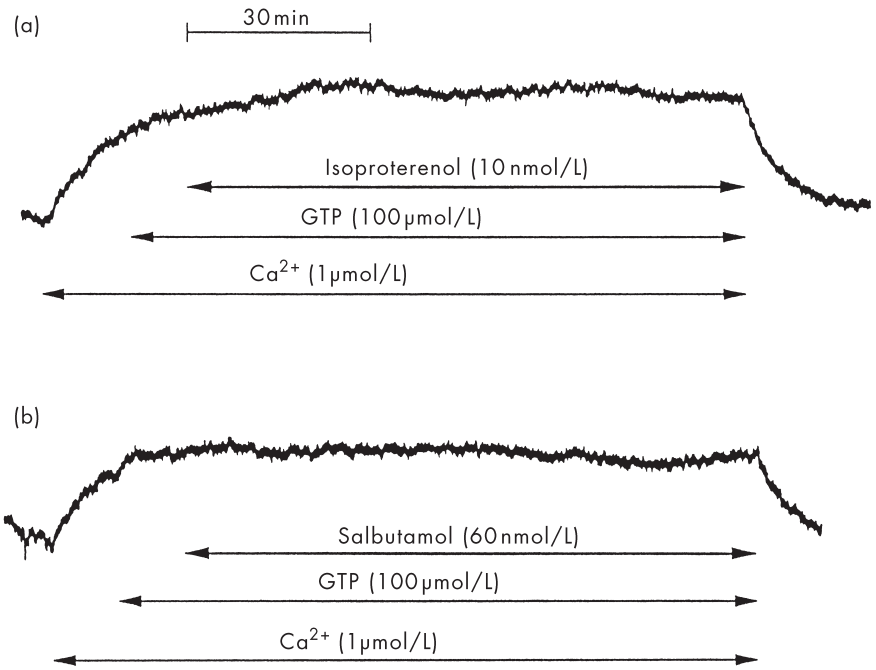

Fig. 3 Effect of adrenergic β_2 agonists on skinned preparations in the presence of guanosine 5′-triphosphate (GTP). The preparations were treated with each β_2 agonist for 90 min in the presence of 1 μmol/L Ca^{2+} and 100 μmol/L GTP. Isoproterenol (a) or salbutamol (b) did not affect contraction in the skinned muscle preparations.

ISO concentration and treatment duration (Fig. 5). At an ISO concentration of 1 nmol/L, significant relaxation was observed after 30 min compared to the OA control, whereas at an ISO concentration of 10 nmol/L, significant relaxation was observed after 10 min. At a Salb concentration of 60 nmol/L, significant relaxation was observed after10 min.

The ISO- or Salb-induced relaxation in skinned muscle in the presence of OA was significantly inhibited by pretreatment with propranolol (1 μmol/L) for 10 min (Fig. 6).

Effect of Fors or cAMP on skinned muscle

The addition of Fors (1 μmol/L), an adenylate cyclase activator, had no effect on the Ca^{2+} sensitivity of the skinned muscle, regardless of the presence or absence of OA (Fig. 7). No relaxation response was observed when a high concentration (100 μmol/L) of cAMP was added.

Discussion

We examined the mechanism of tracheal muscle relaxation due to adrenergic β_2-agonists using guinea-pig skinned tracheal muscle obtained by β-escin treatment.

Various reports have provided evidence that the fast and strong tracheal relaxation induced by adrenergic β_2-agonists requires a decrease of intracellular Ca^{2+} concentration in smooth muscle.[7,8] However, the mechanism of the intracellular Ca^{2+} decrease is still being debated, in part because the action mechanisms of β_2-agonists that cause a decreased Ca^{2+} level involve complicated processes in the membrane such as ion channels,[8–11] Ca^{2+} pumping[13,14] and Ca^{2+} uptake into sarcoplasmic reticulum.[8,12] Therefore, these mechanisms cannot be studied in a simple system. In the present study, we targeted only the action of β_2-agonists on the contractile protein system by using skinned muscle to exclude the various ion-channel functions, electrical functions of the membrane and even sarcoplasmic reticular functions, and by maintaining a constant intracellular Ca^{2+} concentration.

Skinned-sample preparations have various experimental advantages. β-Escin treatment results in small openings in the cellular membrane. The intracellular ionic composition, especially the intracellular Ca^{2+} concentration, can be set free through exchange with the cytoplasmic substitution solution. Since there is no influx or efflux of ions through various ion channels, only the contraction/relaxation responses caused by intracellular proteins with large molecule sizes can be examined. In addition, in the sarcoplasmic reticulum, the Ca^{2+} that acts as the intracellular Ca^{2+} store was depleted by A23187 treatment, thereby excluding contraction/relaxation mechanisms that involve the sarcoplasmic reticulum. These were the experimental conditions in the present study.

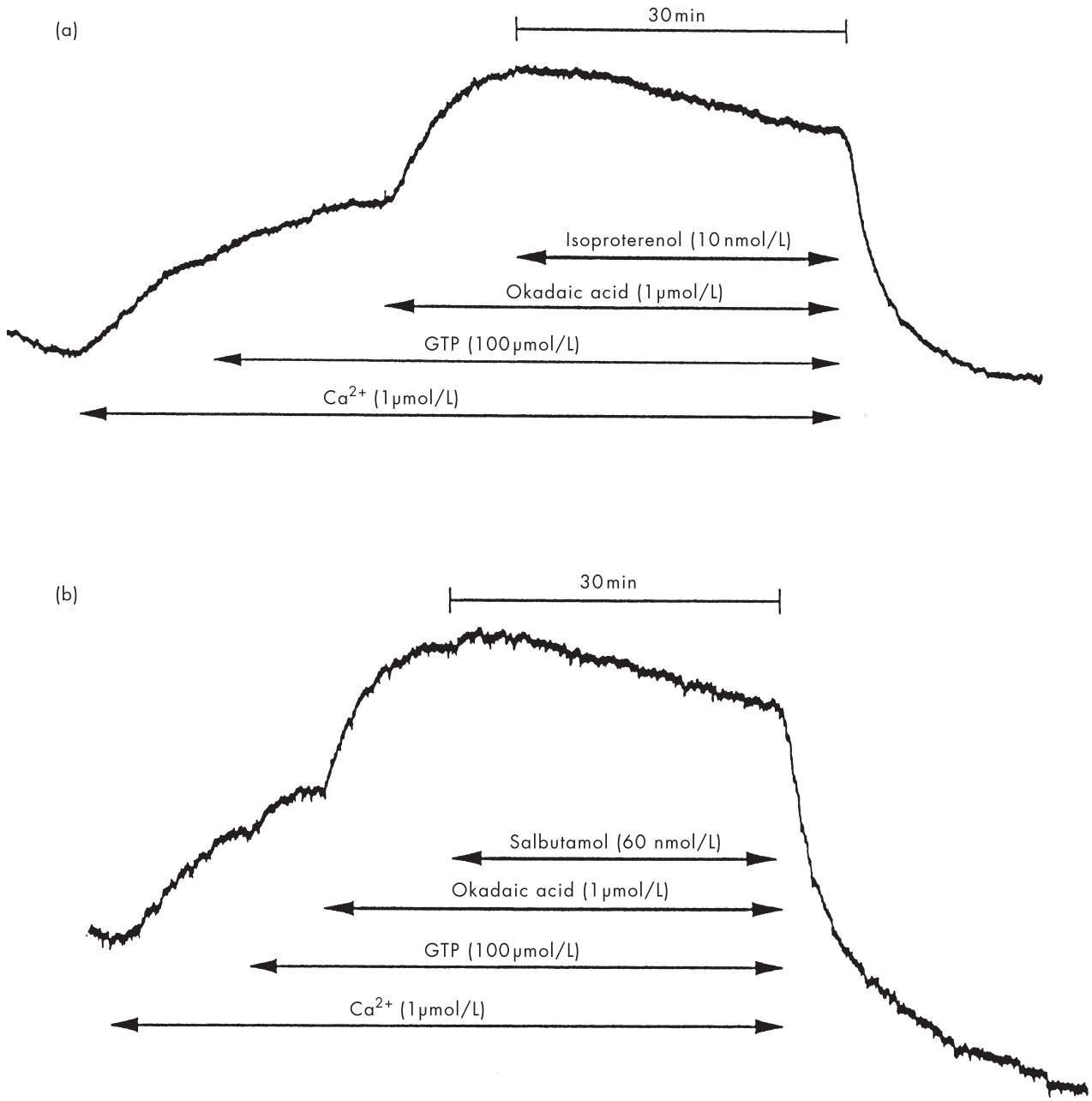

Fig. 4 Effect of adrenergic β_2 agonists in the presence of okadaic acid. Okadaic acid (OA; 1 μmol/L) administration in the presence of GTP (100 μmol/L) increased contraction. The OA-induced contraction was slowly relaxed by isoproterenol (a) or salbutamol (b) treatment.

We measured the isotonic contraction in skinned preparations. Contraction in skinned muscle preparations is conventionally determined by placing the sample horizontally in a measuring device and then recording the isometric tension.[18–20] In our experiment, isotonic contraction was measured in the vertical direction. A muscle preparation experimentally loaded with a constant weight is similar to the physiological conditions in the body before excision, and relaxation can be measured accurately.

Isotonic recordings showed that contraction responses of the skinned tracheal muscle depended on the intracellular Ca^{2+} concentration (Fig. 2). This contraction response is caused by the formation of myosin-actin

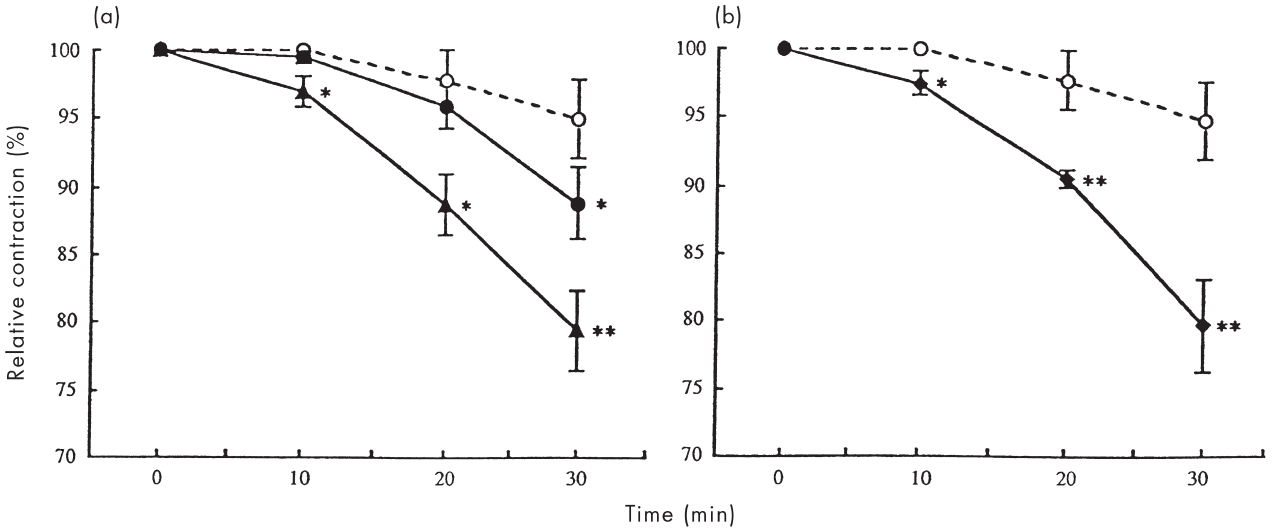

Fig. 5 Relaxation effect of adrenergic β_2 agonists in the presence of 1 μmol/L okadaic acid (OA). The relaxation effects of isoproterenol (a) or salbutamol (b) in skinned muscle preparations were concentration and time dependent. *$P < 0.05$, **$P < 0.01$ compared with the corresponding point in OA alone (○). (●), OA and 1 nmol/L isoproterenol; (▲), OA and 10 nmol/L isoproterenol; (◆), OA and 60 nmol/L salbutamol. Points show the mean values ± SEM ($n = 4$).

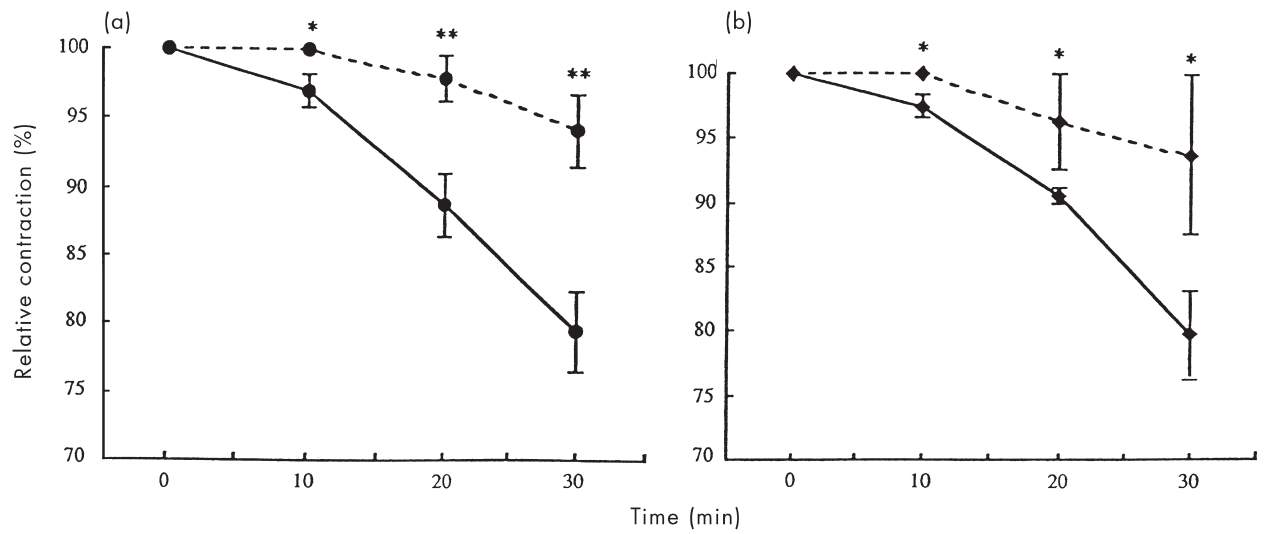

Fig. 6 Effect of propranolol on the relaxation effect of adrenergic β_2 agonists. The relaxation effect induced by 10 nmol/L isoproterenol (a; –●–) or 60 nmol/L salbutamol (b; –◆–) in skinned muscle preparations was significantly suppressed by pretreatment with 1 μmol/L propranolol (--●--, --◆--). *$P < 0.05$, **$P < 0.01$ compared with the corresponding point for the agonist alone. Points show the mean values ± SEM ($n = 3$–4).

cross bridges as a result of the activation of myosin light chain kinase (MLCK) through the formation of the Ca^{2+}-calmodulin complex, which depends on the concentration of Ca^{2+}. Contraction was observed in our preparations at an intracellular Ca^{2+} concentration of 0.3 μmol/L.

With the intracellular Ca^{2+} concentration maintained at 1 μmol/L, the Ca^{2+}-induced contraction was enhanced by the addition of GTP (Fig. 3). A similar result was obtained by the addition of GTPγS (data not shown). These results suggest that GTP-binding protein activation is associated with the increase in Ca^{2+} sensitivity. In other

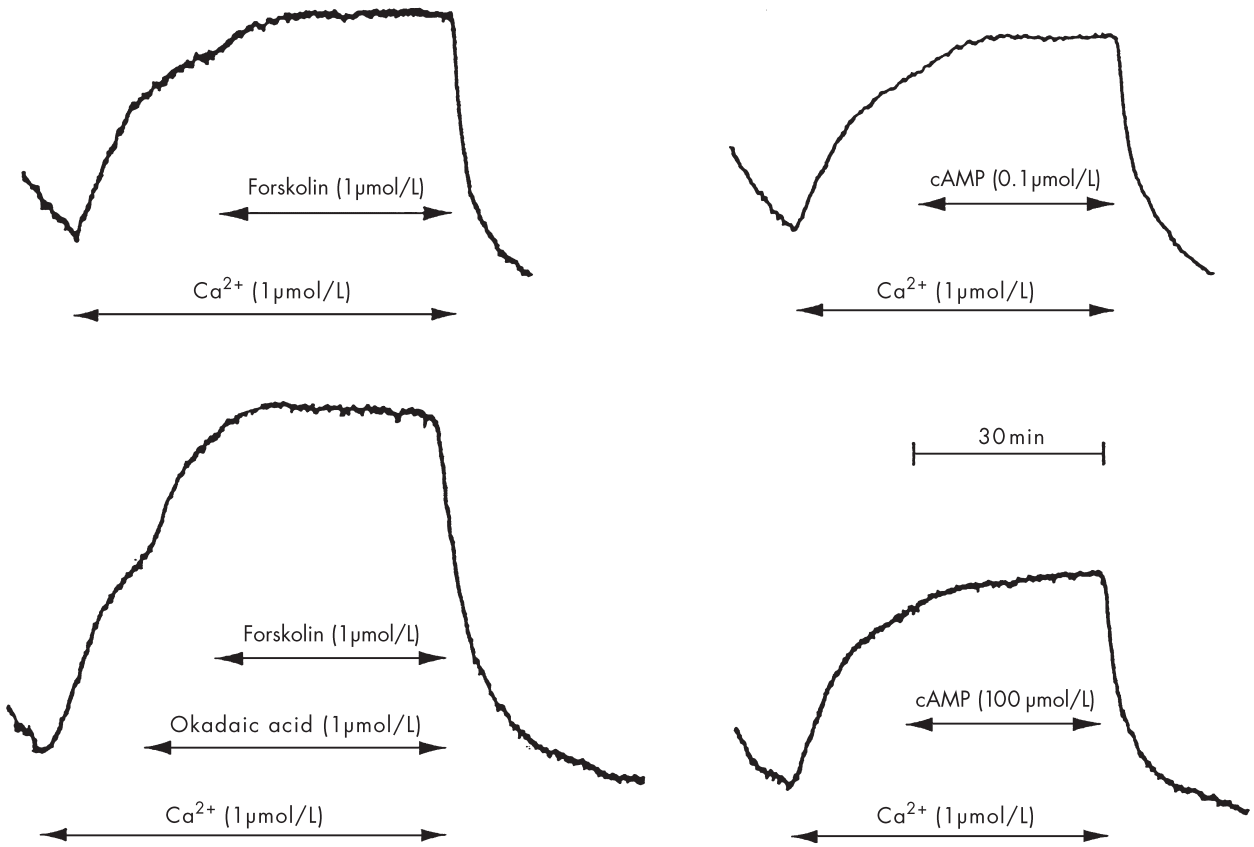

Fig. 7 Effect of forskolin and cAMP on skinned muscle preparations. The Ca^{2+} sensitivity of guinea-pig skinned tracheal muscle preparations was not affected by the addition of forskolin (1 μmol/L) or cAMP (0.1 or 100 μmol/L).

studies, the addition of GTP or GTPγS to skinned smooth muscle increased the Ca^{2+} sensitivity of the muscles.[21,22]

In the presence of 1 μmol/L Ca^{2+} and 100 μmol/L GTP, neither ISO (10 nmol/L) nor Salb (60 nmol/L) affected the Ca^{2+} sensitivity (Fig. 3). However, both β_2-agonists significantly inhibited the contraction induced by OA (1 μmol/L) (Figs. 4,5). Considering the reports that OA does not affect MLCK or phosphodiesterase of guinea-pig taenia coli[23] and that 1 μmol/L OA almost completely and reversibly inhibits myosin light chain phosphatase (MLCP),[24] the contraction induced by OA in our skinned tracheal muscle may be caused mainly by the inhibition of dephosphorylation of the phosphorylated myosin light chain. However, one cannot exclude the possibility that the contraction may be the result of the effect of OA, a non-specific phosphatase inhibitor, on the actin side that interacts with phosphorylated myosin to form cross bridges. The reason is that calponin and caldesmon, which are thin filament regulatory proteins associated with actin filaments, are present in the skinned tracheal muscle. Phosphorylation of these proteins has been reported to activate actomyosin ATPase and contract tracheal muscle.[25] Adrenergic β_2-agonist-induced relaxation was observed in the presence of OA but not in its absence (Figs 3,4). These results suggest that adrenergic β_2-agonists act on the OA targets: MLCP and other protein phosphatases. Their activation causes relaxation. The mechanisms by which the activities of these phosphatases are enhanced are unknown, and require further studies.

In a preliminary experiment, we confirmed that the relaxation in isolated tracheal muscle induced by 10 nmol/L of ISO was inhibited by 1 μmol/L propranolol. The findings of complete inhibition of β_2-agonist-induced relaxation in skinned muscles by propranolol (Fig. 6) and the lack of a relaxation effect for Fors (Fig. 7) suggest that activation of MLCP and other protein phosphatases by β_2-agonists is not related to the intracellular cAMP increase, but may be mediated by β_2-receptors in the membrane.

In the present study, β_2-agonists were used at concentrations that did not increase the intracellular cAMP level (Fig. 1). At these concentrations, β_2-agonists inhibited OA-induced contraction (Figs 4,5). In addition, as shown in Fig. 7, the Ca^{2+} sensitivity of the skinned muscle was not affected by the addition of Fors that activates adenylate cyclase and increases the cAMP level (Fig. 1), and was also not affected by cAMP at a concentration sufficient to activate cAMP-dependent protein kinase (PKA). Other studies have reported that skinned smooth muscle preparations obtained by α-toxin treatment were relaxed by Fors and cAMP,[19,26] which is different from our results. In our skinned preparation PKA efflux caused by β-escin treatment may conceivably cause the difference in findings. Savineau and Marthan[18] used a β-escin concentration two-fold higher than that used in the present study and reported inhibition of Ca^{2+}-induced contraction by cAMP. Therefore, we may exclude the possibility of PKA efflux from our skinned preparations. Contrary to the result of Savineau and Marthan, cAMP and Fors did not affect Ca^{2+} sensitivity in our skinned preparations. The cause of this discrepancy is unknown, but may be due to differences in response between human and guinea-pig tracheal muscle, or it may be associated with partial PKA efflux or other factors. Even if it is hypothesized that the differences in the findings are due to PKA efflux from our preparations, there is still a possibility that slow inhibition of OA-induced contraction by adrenergic β_2-agonists is due to phosphatase activation by unknown pathways different from that mediated by PKA. These results suggest that the mechanism of tracheal smooth muscle relaxation induced by β_2-agonists is not associated with adenylate cyclase at a given concentration of Ca^{2+}, but involves a pathway that inhibits responses of the contractile protein system.

Rapid tracheal smooth muscle relaxation induced by low concentrations of adrenergic β_2-agonists, which is considered useful experimentally or clinically, may be a response caused by a rapid decrease of intracellular Ca^{2+} due to signal transduction through the receptor-G-protein. The present study suggests that low concentrations of β_2-agonists not only lower intracellular Ca^{2+} concentration, but also activate protein phosphatase via β_2-receptors and G-protein. The functional role of protein phosphatase activation by β_2-agonists is unknown, but it may have a modulating role in maintaining and augmenting the β_2-agonist-induced relaxation response that depends on lowered intracellular Ca^{2+} concentration.

Further studies are required to examine the mechanism of relaxation by protein phosphatase activation induced by adrenergic β_2-agonists.

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