β-Adrenergic Agonists Regulate K_{Ca} Channels in Airway Smooth Muscle by cAMP-dependent and -independent Mechanisms

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Abstract

Stimulation of calcium-activated potassium (K_{Ca}) channels in airway smooth muscle cells by phosphorylation-dependent and membrane-delimited, G protein actions has been reported (Kume, H. A. Takai, H. Tokuno, and T. Tomita. 1989. *Nature [Lond.]*. 341:152–154; Kume, H., M. P. Graziano, and M. I. Kotlikoff. 1992. *Proc. Natl. Acad. Sci. USA*. 89:11051–11055). We show that β-adrenergic receptor/channel coupling is not affected by inhibition of endogenous ATP, and that activation of K_{Ca} channels is stimulated by both α_S and cAMP-dependent protein kinase (PKA). PKA stimulated channel activity in a dose-dependent fashion with an EC_{50} of 0.12 U/ml and maximum stimulation of 7.38±2.04-fold. Application of α_S to patches near maximally stimulated by PKA significantly increased channel activity to 15.1±3.65-fold above baseline, providing further evidence for dual regulatory mechanisms and suggesting that the stimulatory actions are independent. Analysis of channel open-time kinetics indicated that isoproterenol and α_S stimulation of channel activity primarily increased the proportion of longer duration events, whereas PKA stimulation had little effect on the proportion of short and long duration events, but resulted in a significant increase in the duration of the long open-state. cAMP formation during equivalent relaxation of precontracted muscle strips by isoproterenol and forskolin resulted in significantly less cAMP formation by isoproterenol than by forskolin, suggesting that the degree of activation of PKA is not the only determinant of tissue relaxation. We conclude that β-adrenergic stimulation of K_{Ca} channel activity and relaxation of tone in airway smooth muscle occurs, in part, by means independent of cyclic AMP formation. (*J. Clin. Invest.* 1994. 93:371–379.) Key words: potassium channels • bronchodilation • ion channels • membrane transduction • G proteins • β-adrenergic receptors

Introduction

β_2-adrenergic agonists are widely used clinically as bronchodilators, and are the principal bronchodilator agents used in the treatment of asthma. Although the precise target proteins mediating β-adrenergic smooth muscle relaxation are not well established, one prominent action of these and other smooth muscle relaxant hormones is the opening of potassium channels (1–7), and an attendant cellular hyperpolarization (5, 8–11). Previous experiments have demonstrated distinct cellular pathways by which β-adrenergic receptor agonists stimulate large-conductance, calcium-activated potassium (K_{Ca}) channels in airway smooth muscle cells (1, 2). These channels are densely distributed in the cell membrane of airway smooth muscle cells (1, 2, 12–17) and have recently been implicated as important target proteins for β-adrenergic relaxation (18, 19). The generally accepted transduction mechanism by which β-adrenergic receptors are linked to cellular actions is through the G protein-dependent stimulation of adenylyl cyclase, an associated rise in cAMP, and the subsequent activation of cAMP-dependent protein kinase A (PKA)[1] as identified in (20, 21). Such coupling has been demonstrated with respect to the regulation of K_{Ca} channels, since channel activity is stimulated by forskolin, and by exogenous A kinase in inside-out patches (1, 22). However, the recent demonstration of membrane-delimited, phosphorylation-independent coupling of G_S-linked membrane receptors to ion channels (2, 23, 24) suggests that β-adrenergic transduction pathways exist that are independent of cAMP formation. We have previously demonstrated that β-adrenergic agonists stimulate K_{Ca} channels in membrane patches from airway smooth muscle cells, and that this stimulation is mimicked by the GTPγS-activated α subunit of G_S (α_S) as noted in (2). α_S effects are independent of phosphorylation, since they can be demonstrated in the presence of competitive inhibitors of ATP, and are not altered by inhibitors of A kinase. The relative importance of these discrete stimulatory pathways in physiologic receptor–channel coupling, however, remains unclear. Here we examine β-adrenergic receptor–K_{Ca} channel coupling via phosphorylation-dependent and membrane-delimited pathways, and explore the relationship between tissue relaxation and cAMP formation. We provide evidence that the membrane-delimited, G protein coupling between receptor and channel is a functionally important one, that the actions of the dual stimulatory pathways occur independently, and that the relaxant actions of β-agonists on airway tissue do not correlate with cAMP formation.

Methods

Cell dissociation and electrophysiology. Tracheal smooth muscle cells from porcine and ferret were enzymatically dissociated as previously described (25). No differences in channel activity or modulation were observed between species. The solution for cell dispersion contained collagenase (300 U/ml, type D; Boehringer Mannheim Biochemicals, Indianapolis, IN), elastase (8 U/ml, ESFF; Worthington Biochemical Corp., Freehold, NJ), soybean trypsin inhibitor (1 mg/ml, Sigma Chemical Co., St. Louis, MO), and EGTA (1.8 mM; Sigma Chemical

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1. *Abbreviations used in this paper:* PKA, cAMP-dependent protein kinase A.

Co.). After gentle agitation with the enzyme solution for 30 min, single smooth muscle cells were obtained. Single-channel currents were recorded in outside-out and inside-out patches, using standard patch clamp techniques (26), as previously described (15). Current records were digitized at 1 or 5 kHz, and filtered at 200 Hz or 1 KHz (−3 dB), respectively. Open-state probability and kinetics analysis were performed using previously described software routines provided by M. Nelson (University of Vermont). Channel open-state probability (P_o) is described as the mean open-state probability for all channels times the number of channels in the patch (nP_o), because the total number of channels in any given patch (n) was not determined. Open-time analysis was performed on data digitized at 5 kHz and filtered at 1 kHz using half-crossing analysis (27). Records were not corrected for missed events, but open times of less than 0.4 ms were excluded from open-time fits to minimize the effect of missed openings on estimates of mean lifetimes. For dose–response experiments in which basal channel activity was modulated by addition of drug, we express the change in channel activity as percent stimulation, where the increase in nP_o above basal is divided by the maximum stimulation observed. We also report the fold increase in channel activity, which is the value of nP_o in the experimental condition divided by the value determined for the identical time period immediately before drug addition. The voltage dependence of nP_o was determined by fitting the data to a generalized Boltzmann equation of the form: $nP_o = nP_{o_{max}}/(1 + e^{((V-V_{50})/k)})$, where $nP_{o_{max}}$ is the maximum attainable open-state probability, V_{50} is the potential at which nP_o is half maximal, k is the slope factor, and V is the holding potential. Dose-dependent stimulation of channel activity was analyzed by fitting the data to a saturation isotherm equation of the form: Percent maximal stimulation $= A + \frac{100 - A}{1 + (10^c/10^x)^D}$, where A is the resting activity as a percent of maximum, C is the EC_{50} value, D is the Hill coefficient, and X is the drug concentration. Fits were obtained by least squares method using either SigmaPlot (Jandel Scientific, Corte Madera, CA) or InPlot (GraphPAD Software for Science, San Diego, CA).

Recording conditions were designed to simulate physiological ionic concentrations with ~ 100 nM free Ca^{++} at the cytoplasmic patch surface. The internal solution (cytosolic patch surface) was (in mM): 126 KCl, 5 NaCl, 1 $MgCl_2$, 2.5 EGTA, and 10 HEPES adjusted to pH 7.2 with KOH; $CaCl_2$ was added to adjust free calcium to 0.1 μM (28). The external solution (extracellular patch surface) was: 125 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 Hepes adjusted to pH 7.4 with NaOH.

Recombinant α_S (45-kD form) was kindly provided by Dr. M. P. Graziano, Department of Pharmacology and Biochemistry, Merck Sharp & Dohme Research Laboratories, Rahway, NJ (29). Isoproterenol, ATP (magnesium salt), GTP, 5′-adenylylimidodiphosphate (AMP-PNP), forskolin, and methacholine chloride were obtained from Sigma Chemical Co. The catalytic subunit of cAMP-dependent protein kinase was also obtained from Sigma Chemical Co. (purified from bovine heart, P2645) and was used from frozen aliquots stored at −70°C.

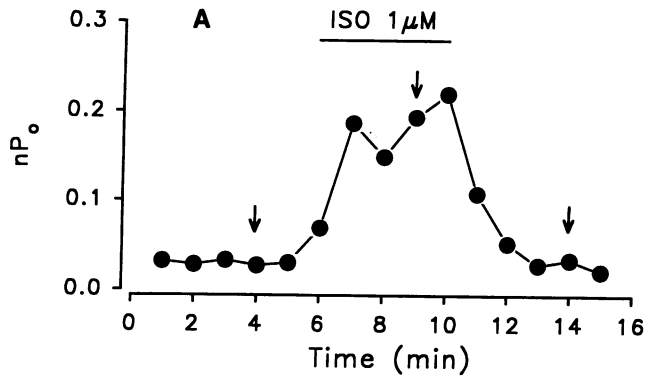
Measurement of tension and cAMP. Tracheal muscle strips were dissected from the extrathoracic tracheae of horses undergoing euthanasia. The tracheal mucosa was dissected free, and muscle strips (~ 1 × 10 mm) were prepared by dissecting parallel to the longitudinal axis of the muscle. Strips were transferred to 10-ml muscle baths filled with warm (37°C), oxygenated Hepes buffer solution and attached at one end to isometric force transducers (Harvard Apparatus, South Natick, MA). Force output of the muscle strips was recorded on a strip chart recorder (Gould Inc., Glen Barnier, MD). After a 45-min equilibration period, the length for maximal active force development (L_o) was determined by increasing the length of each muscle strip by 1-mm increments until the maximal active contractile response to methacholine (10 μM) was achieved. Strips were then stimulated with metacholine (10 μM); at maximum steady-state tension, muscle strips were treated with isoproterenol (1 μM) or forskolin (50 μM), which had previously been shown to induce equivalent degrees of relaxation (60%). When complete relaxation was achieved, muscles were snap frozen with liquid N_2 and stored at −70°C for determination of cAMP content. Strips were also frozen for assays of cAMP content under conditions of basal tone. For measurement of cAMP, one ml of 6% trichloroacetic acid (4°C) was added to each frozen tissue strip in a glass homogenizing tube. Tissues were homogenized at maximum speed for 30 s using a tissuemizer (Tekmar Co., Cincinnati, OH) the homogenate transferred to 1.5-ml microcentrifuge tubes, and precipitated protein removed by centrifugation for 15 min at 3000 *g*. The samples were then transferred to 15-ml glass tubes and trichloroacetic acid removed by four successive extractions with a fivefold excess volume of water-saturated ether. The samples were vacuum dried and then resuspended in assay buffer. cAMP was finally determined using a commercially available Scintillation Proximity Assay kit (Amersham Corp., Arlington Heights, IL) and is expressed as pmol/mg dry wt tissue. Statistical comparisons of cAMP content was made by one-way ANOVA, using the Student–Newman–Keuls test. All data are expressed as mean±SEM.

Results

Isoproterenol stimulates K_{Ca} channels independent of phosphorylation. We have previously demonstrated that β-adrenergic agents activate K_{Ca} channels in outside-out patches (2). Although we thought it unlikely that cAMP-dependent PKA-mediated phosphorylation could occur in these experiments, we first examined whether receptor–channel coupling could occur in the presence of the competitive ATP inhibitor, AMP-PNP. Fig. 1 *A* shows an experiment in which channel open-state probability was stimulated by 1 μM isoproterenol in the presence of GTP (100 μM) and AMP-PNP (1 mM), and returned to control level within 5 min after drug washout. Mean open-state probability (nP_o) over 5 min for each experimental condition (control, β-agonist, and 5 min after washout) were 0.030, 0.177, and 0.028, respectively. As shown in Fig. 1 *B*, stimulation results in an increase in channel activity, without an alteration in current amplitude. In three similar experiments addition of isoproterenol produced a 4.62±0.9-fold stimulation of channel activity, which is roughly equivalent to the level of channel stimulation previously reported in outside-out experiments in the absence of ATP, but without AMP-PNP (2).

Consistent with a membrane-delimited, G protein-dependent coupling mechanism, application of guanine nucleotides stimulated channel activity in inside-out patches exposed to isoproterenol. Fig. 2 *A* shows an experiment in which K_{Ca} channel activity was examined before and after addition of GTP (100 μM) to a patch in which the pipette (extracellular membrane surface) solution contained isoproterenol (1 μM). Addition of the guanine nucleotide led to a marked increase in channel activity; mean nP_o before and after addition of GTP was 0.014 and 0.043, respectively. Stimulation of channel activity resulted in an apparent shift in the relationship between voltage and open-state probability by 10–15 mV (Fig. 2 *B*).

Dose-dependent stimulation of K_{Ca} channels by cAMP-dependent PKA. Stimulation of K_{Ca} channels by the catalytic subunit of cAMP-dependent PKA was examined in inside-out patches. Patches were perfused with solutions containing successively higher concentrations of kinase, in the presence of constant ATP (500 μM), in order to determine the dose-dependence of kinase regulation. Fig. 3 *A* shows a typical experiment in which K_{Ca} channel activity was progressively stimulated by this cumulative dose–response protocol. The concentration–response relationship between PKA and channel open-state probability determined from five patches is shown in Fig. 3 *B;* the data are well fit by a simple saturation isotherm



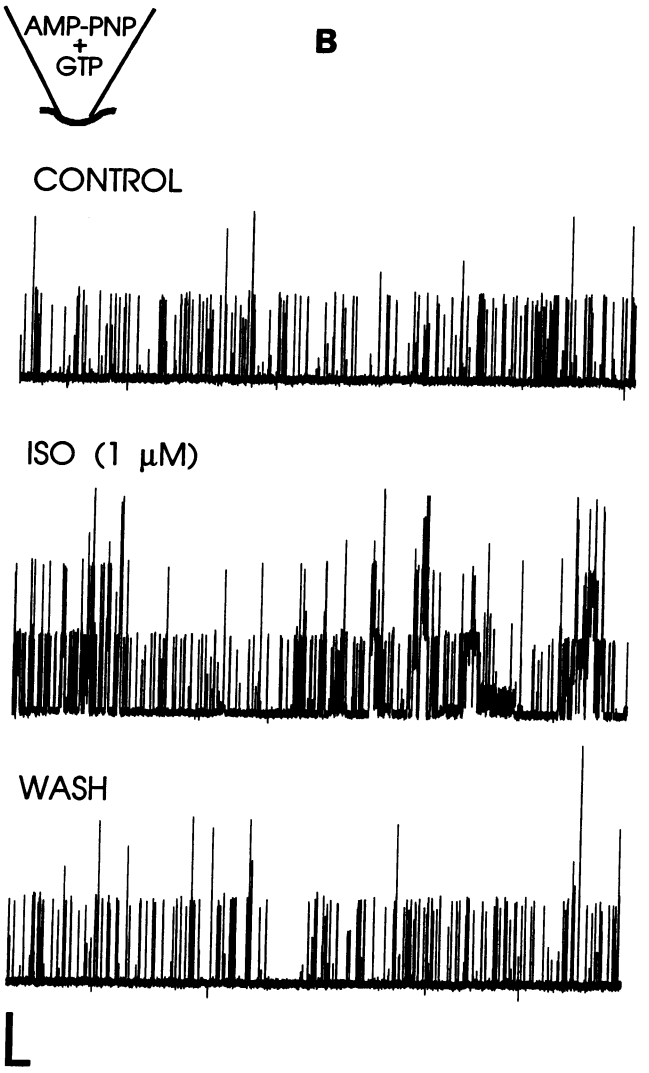


Figure 1. Isoproterenol stimulates K_{Ca} channels in outside-out patches independent of phosphorylation. Patches were exposed to GTP (100 μM) and AMP-PNP (1 mM) at the cytosolic (pipette) surface and held at 0 mV. (*A*) Continuous nP_o vs. time for an experiment showing the increase in K_{Ca} channel activity after perfusion with isoproterenol (*ISO*, 1 μM), and the recovery of control channel activity following drug washout. Data points shown are averaged nP_o for 1-min intervals. Arrows indicate start of data shown in *B*. (*B*) K_{Ca} channel currents recorded before, during, and after stimulation of channel activity with isoproterenol. Traces show ~ 1 min of data from each experimental condition at the indicated points in *A*. Calibration bars, 3 pA and 4 s; ferret cell 0910B1.

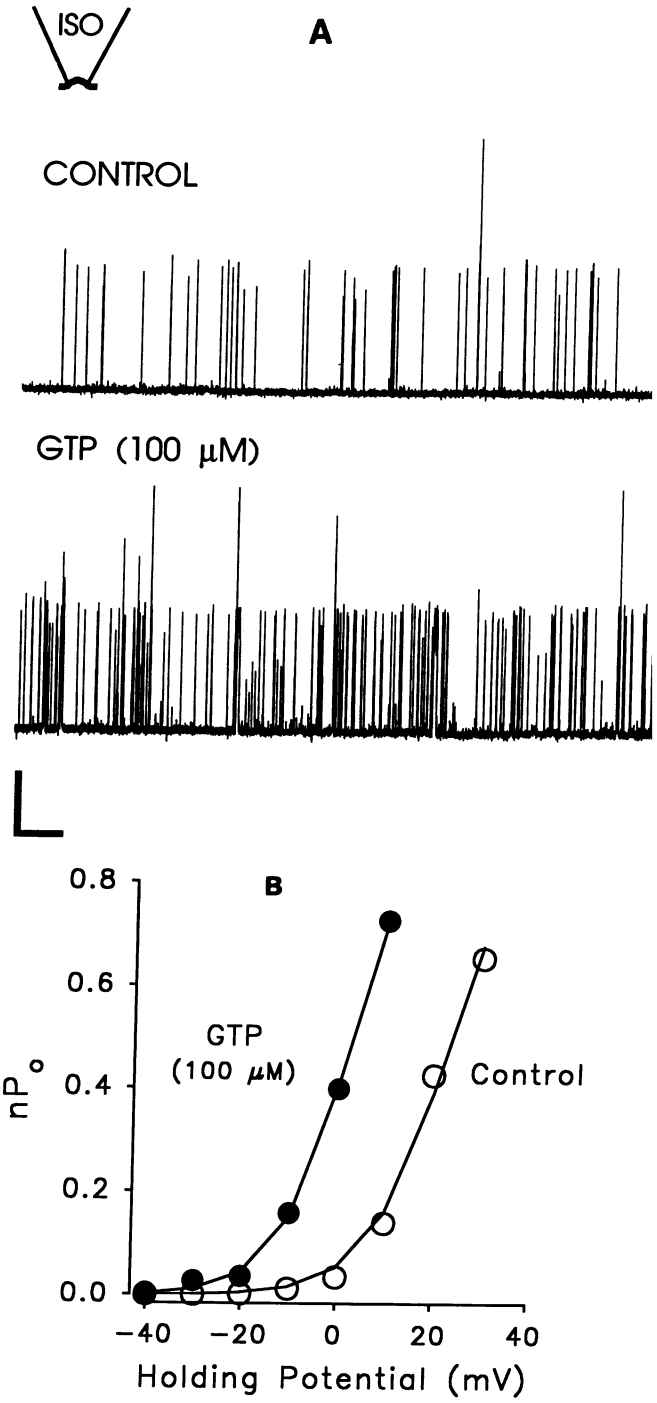


Figure 2. β-adrenergic stimulation of K_{Ca} channels is guanine nucleotide dependent. (*A*) Record of outward K_{Ca} currents before and after addition of GTP (100 μM) to inside-out patches in the presence of isoproterenol (1 μM) at the extracellular surface. Traces show 30 s of data before and immediately after addition of drug; holding potential 0 mV. Calibration bars, 3 pA and 2 s. (*B*) Relationship between open-state probability and membrane potential for the patch shown in *A*. At all membrane potentials GTP increased channel activity. Solid lines show the Boltzmann fit to the data and demonstrate the substantial left-shift following GTP exposure. Porcine cell 0808A.

with an EC_{50} of 0.12 U PKA/ml. The maximum level of channel stimulation by PKA was observed at either 0.5 (two experiments) or 5.0 (three experiments) U/ml. At peak effect, the

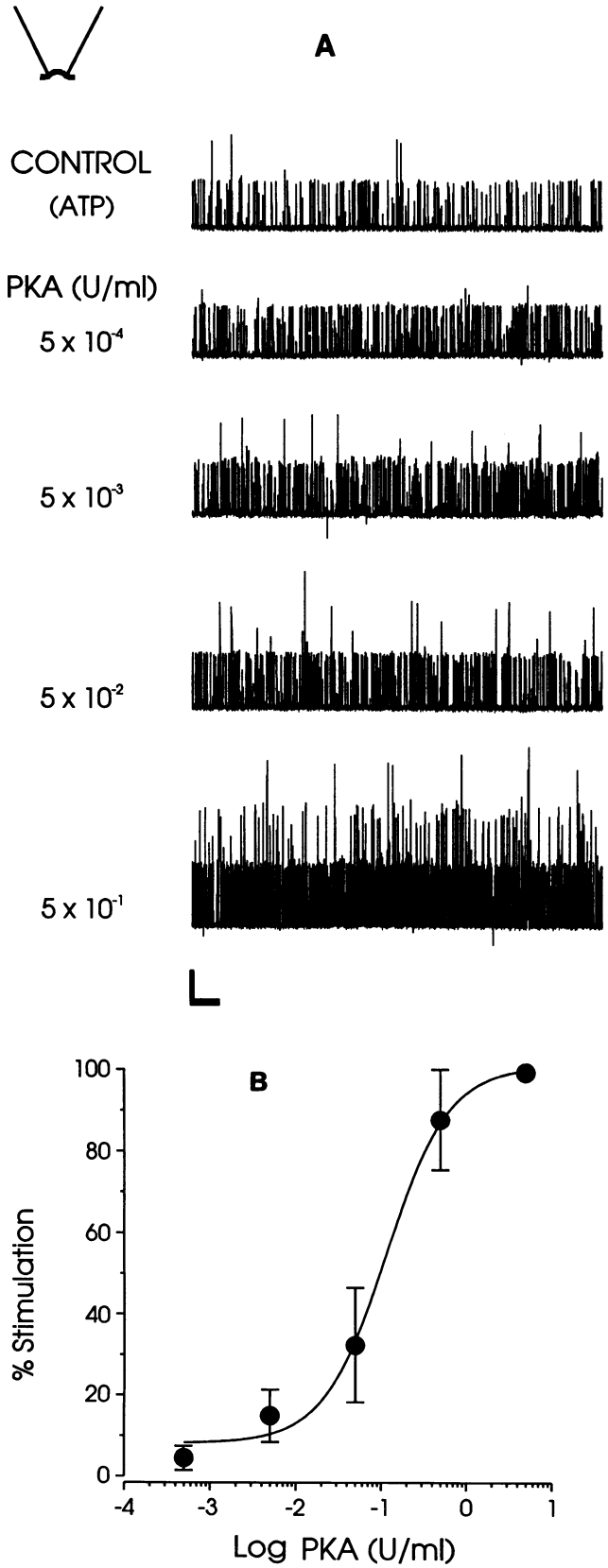


Figure 3. Concentration dependence of K_{Ca} stimulation by cAMP-dependent protein kinase. (*A*) Single-channel recordings of K_{Ca} channel activity modulated by cumulative application of the catalytic subunit of PKA in an inside-out patch; ATP (500 μM) is present throughout. Records show 2 min of continuous data at 0-mV holding potential. Calibration bars, 3 pA and 8 s. Ferret cell JUN11A1. (*B*) Dose–response relationship between the catalytic subunit of PKA and stimulation of K_{Ca} channel activity from five inside-out patches as in *A*. The solid line represents a fit of the data to a saturation isotherm, the parameters of the fit were: 8.2%, 0.12 U/ml, and 1.2 for the baseline activity, EC_{50}, and Hill slope, respectively, with an r^2 value of 0.998.

mean stimulation was 7.38±2.04-fold, which was substantially less than the 15.6-fold stimulation previously observed for 1 nM α_S (2), using identical experimental conditions.

*Simultaneous effects of PKA and α_S*GTPγS.* To examine the dual pathway of β-receptor/channel coupling, inside-out patches were stimulated to near maximum with PKA (0.5 U/ml). This dose was chosen since it provided near maximal stimulation in all patches (Fig. 3 *B*) and a stable stimulation of channel activity over time. Following incubation with PKA for 5 min, recombinant α_S*GTPγS (1 nM) was added. As shown in Fig. 4 *A*, K_{Ca} channels were potently stimulated by addition of α_S protein after stimulation by the near maximally effective concentration of PKA. In five similar experiments, application of 1 nM α_S protein stimulated channel activity after near maximal kinase stimulation. In these experiments, PKA produced a 6.00±0.85-fold stimulation, and addition of α_S a 15.1±3.65-fold increase over baseline channel activity (Fig. 4 *B*). The fold stimulation produced during the condition of combined PKA and α_S was more than twice as great as the maximal fold stimulation that could be produced by PKA alone in experiments in which a full dose–response was performed (7.38-fold), suggesting that PKA and α_S actions on the channel occur independently. The stimulation of channel activity by PKA and α_S occurred over a range of membrane potentials, resulting in an apparent leftward shift in the relationship between nP_o and membrane potential. A representative experiment of three similar IV determinations is shown in Fig. 4 *C;* at each potential, application of α_S (1 nM) augmented the apparent leftward shift in the relation between open-state probability produced by PKA (0.5 U/ml). The mean shift in V_{50} for PKA was 12.2±4.3 mV, and a further 15.4±3.8 mV when α_S was added. Although the degree to which α_S stimulated channel activity in the presence of PKA was in some cases equivalent to the degree of stimulation previously observed by α_S alone (2), we were not able to determine whether the effects were strictly additive. Also, since each patch contained more channels than could be reliably estimated, we could not discriminate between a shift in the voltage dependence of channel activity, and an increase in the maximum nP_o in a given patch.

The effect of β-agonist, α_S, and PKA on kinetics of K_{Ca} channels. We next quantitatively examined the effects of stimulation of channel activity by isoproterenol (outside-out patches) and by PKA and α_S (inside-out patches) at the level of channel open-time kinetics. K_{Ca} open-times were well fit by the sum of two exponentials of mean duration τ_1 and τ_2, similar to previous reports (13–15, 30). Typical open-time histograms before and after exposure of an outside-out patch to isoproterenol are shown in Fig. 5, and the mean values of open-time kinetics from six similar experiments are summarized in Table I. The time constant of the short mean time was not affected by application of isoproterenol, whereas the proportion of long openings was significantly increased from 43.9±4.50% to 66.3±5.30% ($P < 0.01$). Although isoproterenol increased τ_2

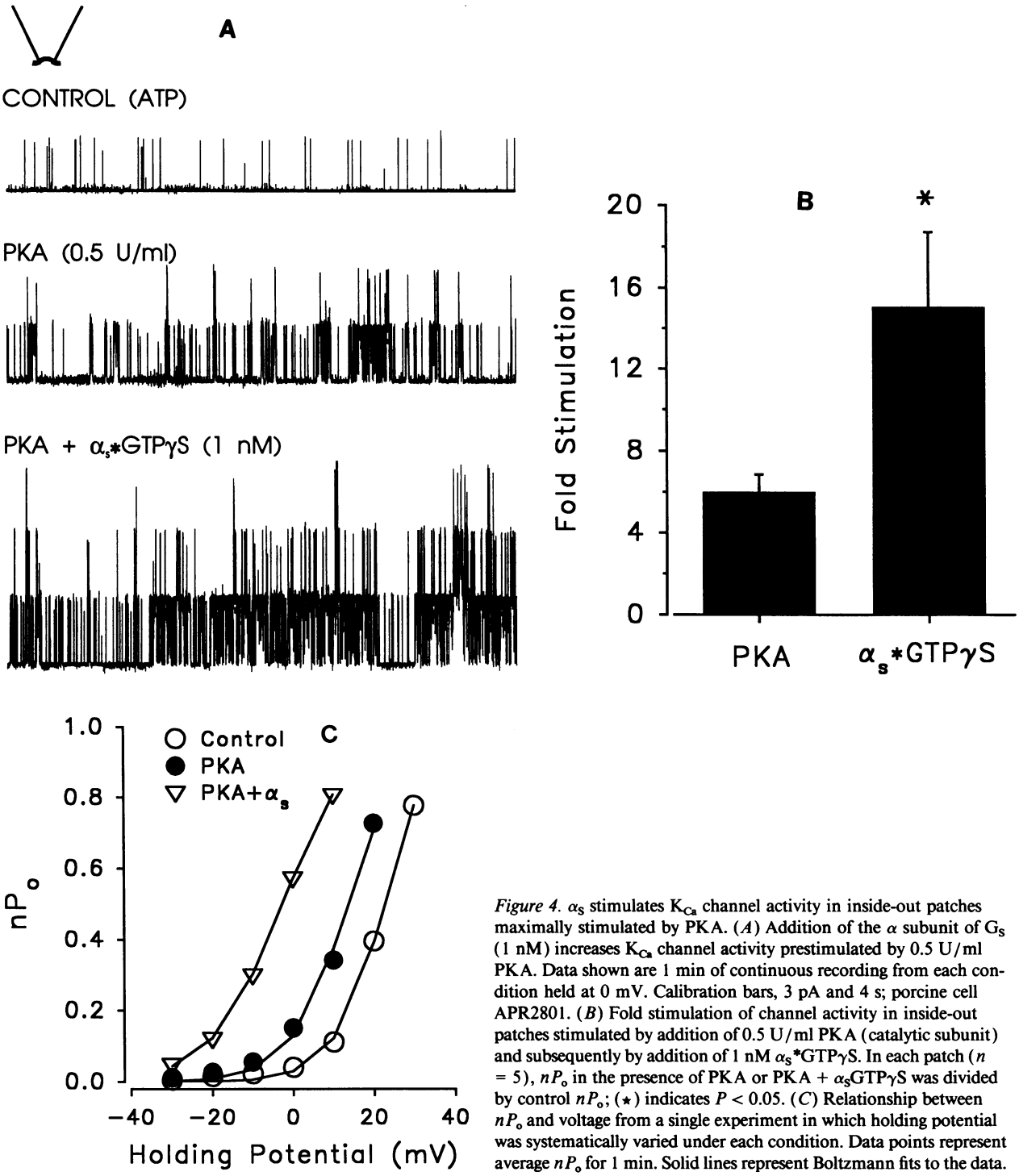


Figure 4. α_S stimulates K_{Ca} channel activity in inside-out patches maximally stimulated by PKA. (*A*) Addition of the α subunit of G_S (1 nM) increases K_{Ca} channel activity prestimulated by 0.5 U/ml PKA. Data shown are 1 min of continuous recording from each condition held at 0 mV. Calibration bars, 3 pA and 4 s; porcine cell APR2801. (*B*) Fold stimulation of channel activity in inside-out patches stimulated by addition of 0.5 U/ml PKA (catalytic subunit) and subsequently by addition of 1 nM α_S*GTPγS. In each patch (n = 5), nP_o in the presence of PKA or PKA + α_SGTPγS was divided by control nP_o; (⋆) indicates $P < 0.05$. (*C*) Relationship between nP_o and voltage from a single experiment in which holding potential was systematically varied under each condition. Data points represent average nP_o for 1 min. Solid lines represent Boltzmann fits to the data.

by 43%, this increase was not significant. Table II shows the mean values of open-time kinetics from ten inside-out patches exposed to α_S (100 pM). The effect of α_S on open-time kinetics was remarkably similar to that produced by isoproterenol on open-time kinetics, α_S did not alter the mean lifetimes, but increased the proportion of long open-time events from 43.0±4.69% to 67.5±2.24% ($P < 0.01$). By contrast, exposure of six inside-out patches to PKA (0.5 U/ml) did not produce a marked increase in the probability that a given channel opening would be of long duration (Table III). Rather, the major kinetic effect was on open-state time constants, resulting in an increase in the mean duration of the long openings, τ_2, from 10.3±1.49 ms to 14.9±.786 ms ($P < .01$). Thus the effect of cAMP-dependent protein kinase on channel kinetics was distinct from that of α_S, consistent with distinct or independent modulatory effects at the channel protein.

Relaxation of airway smooth muscle does not correlate with [cAMP]. If β-adrenergic receptor binding stimulates K_{Ca} channel activity by cAMP-independent as well as cAMP-dependent mechanisms, and if channel stimulation is an important mech-

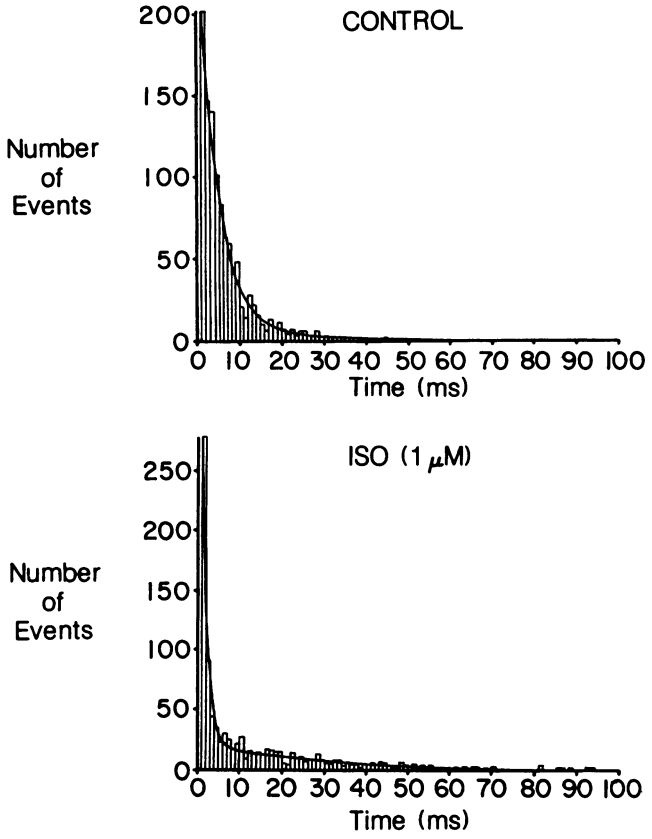


Figure 5. Isoproterenol alters K_{Ca} open-state kinetics. Open-time histograms from an outside-out patch before and after exposure to isoproterenol (1 μM). Under control conditions the open-state lifetimes in this experiment are well fit by two open-states of mean lifetime 3.9 and 15.2 ms (*solid lines*), with 36% of the openings of longer duration. After isoproterenol exposure, the mean lifetimes were 2.0 and 13.9 ms, with 74% of the openings being of longer duration. Data from 5 min of continuous recording in each condition.

anism of relaxation, one would predict that cellular levels of cAMP after hormonal relaxation would be lower than those achieved during equivalent relaxations produced by bypassing the hormone receptor and directly stimulating adenylyl cyclase. This hypothesis was tested by measuring cAMP formation in precontracted airway smooth muscle strips. In pilot experiments, the concentrations of isoproterenol and forskolin required to produce 60% relaxation after a precontraction to 10 μM methacholine were determined to be 1 and 50 μM, respectively. In a series of experiments, precontracted strips were exposed to these respective doses of either isoproterenol or forskolin and then rapidly frozen after full relaxation had been achieved. cAMP content was determined in these strips as well as control strips frozen at resting tension. At equivalent levels of relaxation (61.5±3.7% for ISO, 59.3±3.9% for forskolin; tension before relaxation not significantly different), cAMP content was increased significantly more under conditions of relaxation by forskolin, than isoproterenol ($P < 0.025$). Isoproterenol produced a 2.1-fold increase in cAMP, whereas forskolin produced a 4.3-fold increase (Fig. 6). Thus the ability of isoproterenol and forskolin to relax airway smooth muscle was not correlated with the degree to which they stimulated total

Table I. Alterations in Open-State Kinetics and Lifetime Distributions Observed for Outside-Out Patches

	Control	Isoproterenol
nP_o	0.055±0.018	0.257±0.075*
τ_1 (ms)	2.4±.48	1.9±.09
Percent of openings	57.0±4.07	33.7±5.30‡
τ_2 (ms)	11.5±1.34	16.5±2.25
Percent of openings	43.9±4.50	66.3±5.30‡

Data from outside-out patches activated by isoproterenol. All data were well fit by a model with open-states of short (τ_1) or long (τ_2) mean duration. The percent of short duration or long duration openings, as determined from the area of the individual exponentials, is shown for each condition. Mean values of open time kinetics from six outside-out patches perfused with isoproterenol (1 μM) indicate isoproterenol exposure resulted in a significant increase in the percentage of long duration openings, as well as an increase in the long open-state lifetime that was not significant. * $P < 0.05$, ‡ $P < 0.01$.

cellular cAMP, consistent with cellular mechanisms of relaxation provoked by β-adrenergic stimulation that are independent of the formation of cAMP.

Discussion

β-adrenergic agonists are widely used clinically as bronchodilators and are potent inhibitors of airway smooth muscle contraction. The molecular basis for adrenoreceptor-coupled smooth muscle relaxation is thought to result from the G protein-dependent coupling of β-adrenergic receptors to adenylyl cyclase, a rise in cellular cAMP levels, and stimulation of cAMP-dependent protein kinase (20). However, the demonstration that G_S subunits can stimulate calcium channels in heart cells (23, 24, 31) and potassium channels in smooth muscle cells (2), suggests that other membrane proteins may be direct targets for the regulatory actions of β-receptors, and that functional coupling to membrane ion channels may occur through cAMP-independent mechanisms. Such coupling to K_{Ca} channels is

Table II. Alterations in Open-State Kinetics and Lifetime Distributions Observed for Inside-Out Patches

	Control	α_s
nP_o	0.040±0.0063	0.170±0.025*
τ_1 (ms)	2.2±0.33	2.4±0.48
Percent of openings	57.0±4.75	32.5±2.24*
τ_2 (ms)	14.5±1.82	15.0±2.47
Percent of openings	43.0±4.69	67.5±2.24*

Data from inside-out patches at 0 mV activated by α_s. All data were well fit by a model with open states of short (τ_1) or long (τ_2) mean duration. The percent of short duration or long duration openings, as determined from the area of the individual exponentials, is shown for each condition. Mean values of open time kinetics from ten inside-out patches exposed to α_s (100 pM). α_s markedly increased the proportion of long duration events, with no significant effect on open times. * $P < 0.01$.

Table III. Alterations in Open-State Kinetics and Lifetime Distributions Observed for Inside-Out Patches

	Control	PKA
nP_o	0.034±0.0094	0.18±0.043*
τ_1 (ms)	2.1±0.41	1.8±0.22
Percent of openings	46.2±4.36	42.1±3.73
τ_2 (ms)	10.3±1.49	14.9±0.786*
Percent of openings	53.8±4.36	57.9±3.73

Data from inside-out patches at 0 mV activated by PKA. All data were well fit by a model with open states of short (τ_1) or long (τ_2) mean duration. The percent of short duration or long duration openings, as determined from the area of the individual exponentials, is shown for each condition. Mean open-time kinetics from six inside-out patches perfused with the catalytic subunit of cAMP-dependent protein kinase (0.5 U/ml). PKA activation of K_{Ca} channels was characterized by an increase in the mean duration of τ_2, with no significant effect on τ_1 or the proportion of long-duration events. * $P < 0.01$.

likely to be an important component of the bronchodilation produced by β-adrenergic agonists, since charybdotoxin, a specific peptidyl inhibitor of K_{Ca} channels in this tissue (14, 16), markedly inhibits the ability of nonselective and β_2-selective agonists to relax airway smooth muscle (18, 19). In human airways, 10 nM charybdotoxin significantly altered the EC_{50} for isoproterenol, whereas inhibitors of other potassium channels were without effect (19). Thus, regulation of K_{Ca} channels by cellular mechanisms distinct from those associated with a rise in cAMP could play an important role in the bronchodilation produced by β-adrenergic agonists.

Application of the β-adrenergic agonist isoproterenol (1 μM) to the extracellular surface of outside-out patches, exposed to GTP (100 μM) and AMP-PNP (1 mM) at the intracellular surface, opened single K_{Ca} channels (Fig. 1). This experiment suggests that the membrane-delimited and phosphorylation-independent coupling between hormone receptor and ion channel is a physiologically relevant one. While application of exogenous G protein subunits or active kinase to the cytoplasmic surface of an inside-out patch may not reflect cellular conditions, the demonstration of receptor–effector coupling using physiologic drug concentrations provides strong evidence for the relevance of this coupling mechanism. Moreover, addition of GTP (100 μM) in the presence of isoproterenol at the extracellular surface increased channel activity and shifted the voltage–nP_o relation curve to the left by 10–20 mV, an effect similar to that produced by addition of α_S to inside-put patches (2).

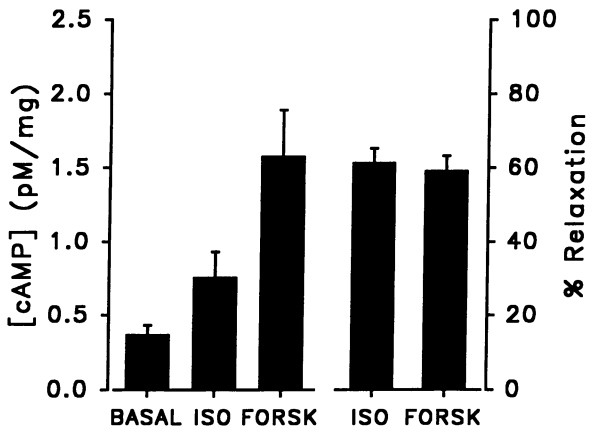


Figure 6. Relaxation of airway smooth muscle is not proportional to cAMP formation. Strips of equine trachealis were precontracted by methacholine (10 μM) and relaxed to an equivalent degree (~ 60%) with either isoproterenol (*ISO*, 1 μM) or forskolin (*FORSK*, 50 μM). Mean data from 6 control, 8 isoproterenol, and 6 forskolin strips are shown. cAMP was significantly higher in strips exposed to forskolin than in those exposed to isoproterenol ($P < 0.025$). The degree of relaxation was not different between forskolin and isoproterenol.

Since K_{Ca} channels are also stimulated by cAMP-mediated phosphorylation in smooth muscles and other tissues (1, 22, 32), we sought to examine the combined effects of each of these regulatory pathways in inside-out patches. PKA stimulated channel activity in a dose-dependent manner (Fig. 3 *B*). Stimulation was observed as low as 0.005 U/ml of the catalytic subunit of the kinase, a concentration well below that previously reported (1, 22, 33). 0.5 U/ml produced a nearly maximal response, and provided a stable level of stimulation for evaluation of additive G protein effects. Addition of α_S to inside-out patches in which channel activity had been near maximally stimulated by PKA led to a marked increase in K_{Ca} channel activity, well above the maximal level of stimulation produced by PKA (Fig. 4 *A* and *B*). As shown (Fig. 4 *B*), the fold increase by addition of α_S was significantly greater than that produced by the kinase alone ($P < 0.05$). Addition of α_S GTPγS to kinase-treated patches resulted in an augmentation of channel activity at all potentials, and a further apparent leftward shift in the relationship between voltage and nP_o (Fig. 4 *C*). These data provide further evidence for dual pathways of K_{Ca} channel regulation, i.e., a classical β-adrenergic receptor/cAMP-dependent kinase-mediated phosphorylation of the channel or a closely associated regulatory protein, and a membrane delimited stimulation of the channel or a closely associated regulatory protein by a protein–protein interaction with the GTP-activated α_S.

α_S produced a marked increase in channel activity in patches maximally stimulated by the kinase (Fig. 4 *A–C*), suggesting that each pathway may result in actions on unique channel regulatory sites, and that the stimulatory actions might thus be discriminated in terms of channel kinetics. Analysis of the modulation of gating kinetics by isoproterenol and α_S indicated a very similar effect on open-time kinetics, which consisted of an increase in the proportion of long duration openings (Tables I and II). Conversely, PKA stimulation of channel activity did not result from a marked shift in open-state proportions, but was accompanied by a modest change in mean open times (Table III). These results are consistent with the existence of two different regulatory sites on K_{Ca} channel proteins. We also attempted to analyze closed time distributions, but these could be fit by the sum of either two or three exponentials and were not consistent, probably due to the low absolute open-probabilities present in the physiologic recording conditions chosen (i.e., $pCa^{++} = 7$).

We attempted to examine cAMP-dependent and cAMP-independent coupling mechanisms at the level of force generation by determining the degree to which cellular cAMP content correlated with relaxation in airway smooth muscle strips. We

reasoned that if cAMP-independent mechanisms mediated some component of β-adrenergic relaxation, cellular cAMP content would be lower at equivalent relaxations in strips relaxed with a β-agonist, than with a direct activator of adenylyl cyclase such as forskolin (despite limitations associated with the measurement of cAMP content in multicellular tissue preparations). Our results indicated that, as predicted, cellular cAMP content was more than twofold higher when relaxed by forskolin than by isoproterenol, and the degree of stimulation over basal cAMP was significantly greater with forskolin. Discrepancies in cAMP content and relaxant effects using different agonists have previously been noted in smooth muscle and other tissues (34–37). While these findings are consistent with cAMP-independent mechanisms of β-adrenergic relaxation, they might also indicate that total cellular cAMP content does not accurately reflect the physiologically important pool of cAMP. Zhow et al. found that both isoproterenol and forskolin stimulated similar increases in PKA activity at the same degree of relaxation (34). Although the discrepancy between functional effects and cAMP concentration were reduced when PKA activity was analyzed, it was noted that forskolin produced a slightly greater increase in PKA activity at equivalent relaxation. Others have reported significant differences in PKA activity between the two stimuli (35). Thus, some evidence suggests that relaxation mechanisms independent of PKA stimulation might be important mediators of β-adrenergic bronchodilation.

In summary, we have demonstrated that K_{Ca} channels are stimulated by cAMP-dependent and cAMP-independent mechanisms. The finding that β-adrenergic receptor agonists stimulate channel activity in outside-out patches, independent of phosphorylation, is evidence for the physiologic significance of membrane-delimited pathways of channel regulation. The dual channel regulatory pathways are likely to occur independently, since the stimulatory effects at the level of the channel can be shown to occur simultaneously, with α_S strongly augmenting channel activity at approximately maximal PKA stimulation. In further support of the independent nature of channel stimulation by these mechanisms, we demonstrate that PKA and α_S have distinct effects on channel open-time kinetics. Finally, we show that at equivalent relaxations by isoproterenol and forskolin, cAMP content is less than one-half the level during β-adrenergic receptor relaxations, consistent with mechanisms of relaxation that occur independent of cAMP formation. These findings have important implications for the use of cAMP elevating agents such as phosphodiesterase inhibitors as bronchodilators. To the extent that membrane-delimited coupling mechanisms are important in mediating the relaxant effects of β-agonists, therapeutic strategies aimed at achieving elevations of cAMP may be less effective than those designed to more directly mimic the modulatory effects of β-adrenergic receptor agonists on target proteins. Moreover, strategies aimed at more directly stimulating these proteins in airway smooth muscle might avoid complications associated with the use of β-adrenergic agents for the treatment of asthma (38).

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