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Isoprenaline and aminophylline relax bronchial smooth muscle by cAMP-induced stimulation of large-conductance Ca^{2+}-activated K^+ channels[1]

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ABSTRACT

AIM: To investigate whether the relaxation of bronchial smooth muscle induced by isoprenaline and aminophylline is mediated by large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) via cAMP-dependent mechanism. **METHODS:** With isometric tension recording, the role of BK_{Ca} in relaxations of rat bronchial strips induced by isoprenaline and aminophylline was determined. With perforated patch-clamp technique, BK_{Ca} currents were observed in freshly isolated rat bronchial myocytes. **RESULTS:** Tetraethylammonium 5 mmol/L, a BK_{Ca} blocker, caused a significant rightward shift in the concentration-response curves of isoprenaline and aminophylline (about 4.26-fold and 3.78-fold, respectively) in methacholine-precontracted rat bronchial strips. Isoprenaline 1 μmol/L caused a significant increase in BK_{Ca} current from (94±15) pA/pF to (186±30) pA/pF (voltage steps from -60 mV to +50 mV, n=10, P <0.01), which was partly abolished by Rp-cAMP 100 μmol/L, a protein kinase A inhibitor. Furthermore, current-voltage relationship(I-V) curve exhibited an upward shift, and the peak current density was significantly raised (n=10, P<0.01) by ramp depolarization from -100 mV to +100 mV. Aminophylline 1 mmol/L caused a significant increase in BK_{Ca} current from (90±10) pA/pF to (166±25) pA/pF (voltage steps from -60 mV to +50 mV, n=11, P <0.01), which was partly abolished by Rp-cAMP 100 μmol/L. Furthermore, the I-V curve exhibited an upward shift, and the peak current density was significantly raised (n=11, P<0.01) by ramp depolarization from -100 mV to +100 mV. **CONCLUSION:** The relaxations induced by isoprenaline and aminophylline were, at least partly, mediated by cAMP-stimulation of BK_{Ca} in rat bronchial smooth muscle.

INTRODUCTION

Isoprenaline and aminophylline are well-known to relax airway smooth muscle and elevate the intracellular concentration adenosine 3',5'-cyclic monophosphate (cAMP) in that tissue[1]. Traditionally, cAMP has been thought to play a crucial role in regulating contraction of airway smooth muscle. Many evidences have shown that an increase in the level of cAMP is closely associated with relaxation of airway smooth muscle. This effect is presumably mediated by the action of cAMP-dependent protein kinase A (PKA)[2]. However, in air-

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way smooth muscle (ASM), the precise mechanism by which isoprenaline and aminophylline decrease Ca^{2+} concentration and produce relaxation remains unclear.

Large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}), ubiquitously distributed in smooth muscle, has been demonstrated to play an important role in regulation of smooth muscle contractility. In a variety of smooth muscle, including vessels and esophagus, BK_{Ca} channels play a role as a negative feedback mechanism to limit depolarization and contraction. Activation of BK_{Ca} leads to membrane hyperpolarization, which closes voltage-dependent Ca^{2+} channels and reduces Ca^{2+} influx, and results in a following reduction in intracellular Ca^{2+} concentration and relaxation[3-5]. As already stated in vascular smooth muscle, cAMP is confirmed to activate PKA, and then leads to BK_{Ca} channels activation[6]. BK_{Ca} activation has been demonstrated to play an important role in β_2-adrenoceptor stimulation-induced relaxation in artery smooth muscle[7]. In airway smooth muscle, similar finding has been made too[8], but, other finding with opposite results has been also obtained that PKA has no effect on BK_{Ca}[9]. Furthermore, the relevant investigations about BK_{Ca} were performed mainly in trachea instead of bronchus. So, in bronchial smooth muscle, whether the relaxation induced by isoprenaline and aminophylline was mediated by BK_{Ca} channels via PKA pathway remains unclear. Accordingly, in this study, we employed isometric tension recording and perforated-patch clamp techniques to determine whether the relaxation induced by isoprenaline and aminophylline was mediated by BK_{Ca} channels activation via PKA pathway.

MATERIALS AND METHODS

Drugs 8-Brom-cAMP, K_2ATP, nystatin, 4-aminopyridine (4-AP), tetraethylammonium (TEA), dithiothreitol, bovine serum albumin, Me_2SO, isoprenaline, methacholine (MCh), and aminophylline were purchased from Sigma. Papain, type I collagenase, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), egtazic acid were got from Gbico. Rp-cAMP (cAMP isomer, PKA inhibitor) was from Calbiochem.

Isolated bronchial strip segments Sprague-Dawley male rats weighing 180-250 g were bought from the Exeprimental Animal Center, Tongji Medical College of Huazhong University of Science and Technology (Grade II, Certificate No 19-053). The left and middle right lobus of lung were exicised and transferred to a petri dish of ice-cold Krebs-Henseleit solution containing (in mmol/L) KCl 4.7, NaCl 118, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_2$ 24, KH_2PO_4 1.2, glucose 11. Main bronchus was cleaned of connective tissue and cut into bronchial strips. The bronchial strips were mounted on stainless steel wires for isometric tension recording in organ baths containing Krebs-Henseleit solution maintained at 37 ° C and continuously gassed with 5 % CO_2 in oxygen. Force was recorded with force transducer (T-265, Nihonkhoden, Japan) connected to a polygraph recorder (Nihonkhoden, Japan). MCh was added to the buffer in order to induce a constant degree of tone. This concentration of MCh was present throughout the experiment and produced approximately 75 % of the maximum cholinergic contraction. The concentration represents final bath concentration. The strips were placed under a 400 mg tension and allowed to equilibrate for 90 min during which time they were washed every 15 min. Indomethacin (10 μmol/L) was added to the buffer to reduce the effect of spontaneous tone development due to released epithelium derived factors. Control cumulative concentration-response curves to isoprenaline and aminophylline were obtained in the absence (control) and presence of 5 mmol/L TEA, a BK_{Ca} blocker. All relaxations were expressed as a percentage of the maximum relaxation achieved with isoprenaline.

Cell preparation Single bronchial smooth muscle cell (BSMC) was obtained by the method of Snetkov *et al*[10] with some improvement. Briefly, the left and middle right lobi of lung were obtained as described above and transferred to a petri dish of ice-cold physiological salt solution (PSS) containing (in mmol/L): NaCl 135, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, glucose 10, HEPES 10, pH adjusted to 7.4 with NaOH and gently bubbled with 5 % CO_2 in oxygen. The bronchial trees were dissected free from lung parenchyma. The smooth muscle layer was obtained free of adherent advential, pulmonary artery and venous under a dissection microscope (Nanjing, China) using iris scissors. Airway epithelium was disrupted. Then BSM tissue was minced (1 mm×1 mm fragments) and incubated in 1 mL Ca^{2+}-free PSS containing collagenase 2 g/L, papain 1 g/L, bovine serum albumin 2 g/L, soybean trypsin inhibitor 1 g/L, and dithiothreitol 20 μmol/L at 36 ° C for 40-60 min. After digestion, single BSMC were dispersed by gentle trituration with a wide-bored Pasteur pipette, and the cell suspension was transferred to the cell chamber for study. This procedure provided a sufficient number of well-attached BSMC for experimental analysis.

To confirm the physiological responsiveness of

cells prepared in this way, we used methods of Yamakage *et al*[11] to measure visually the percentage of cells contracted by exposure to histamine 0.01 mmol/L in PSS solution. Histamine caused contractile response in 82.3 % of cells (n=58 cells from 8 rats).

Electrophysiological measurements Isolated myocytes were allowed to settle to the bottom of the chamber. A perforated-patch clamp whole cell recording technique was employed in the experiments. The tip of the patch pipette (3-5 MΩ) was filled with internal pipette solution containing (in mmol/L) KCl 130, $MgCl_2$ 2, $CaCl_2$ 1.8, K_2ATP 5, egtazic acid 2.5, HEPES 10 (pH 7.2 with KOH). K_2ATP was included to provide a substrate for energy-dependent process. The remainder of the pipette was backfilled with the same solution to which nystatin 200 mg/L was added. The perforated-patch clamp technique provides a measurement of stable whole cell currents without disrupting the cytoplasmic concentrations of divalent cations or metabolites[12]. Pipettes were prepared from capillary glass (Shanghai Brain Research Institute of Chinese Academy of Sciences) using a micropipette puller (PP-830, Narashige, Japan) and microforge (MF-830, Narashige, Japan). Tip resistance was obtained when filled with internal pipette solution. The seal resistance was usually between 1-4 GΩ. Whole-cell currents were recorded with an EPC-9 patch clamp amplifier (HEKA, Germany) in voltage-clamp mode. Membrane potential is recorded in current-clamp mode. Pipette and membrane capacitance and series resistance were electronically compensated. Voltage or current-clamp protocols were applied with Pulse 8.31 (HEKA, Germany). Data were filtered at 5 kHz, digitized with a analog-digital converter (PCI-16, Instrutech, America) and analyzed with Igor Pro 3.1 (Wavemetrics, America). Whole-cell current was normalized to cell capacitance and is expressed as picoamperes per picofarad (pA/pF). External solutions were changed with a rapid-exchange system and complete solution exchange was achieved in 1 s. All experiments were performed at room temperature (22 °C-25 °C)

Effects of isoprenaline and aminophylline on BK_{Ca} current were observed. BK_{Ca} currents were recorded by using pulse protocol and ramp protocol with perforated patch clamp whole-cell mode in presence of 4-AP 1 mmol/L which inhibits K_V currents. For the pulse protocol, the holding potential was -60 mV, membrane currents were activated by depolarizing pulse of 200 ms, from a holding potential of -60 mV to +50 mV in 10-mV step increments. For the ramp protocol, 300-ms voltage ramps from –100 mV to +100 mV were applied and the holding potential was maintained at –60 mV. Changes of BK_{Ca} current were observed in the absence and presence of isoprenaline or aminophylline.

Statistical analysis Individual pD_2 (the negative logarithm of the drug concentration causing 50 % of maximal effect) values for control or the treatment curves were calculated using linear regression analysis. Data were expressed as mean±SD. Statistical significance was determined with Student's t-test (paired and unpaired as applicable). P<0.05 was accepted as statistically significant. In electrophysiological experiment, n represents cells number.

RESULTS

Effects of TEA on MCh-induced contraction in bronchial strips TEA 0.1-10 mmol/L has no effect on the resting tension in isolated rat bronchial strips (n=8); In the presence of TEA 1 or 5 mmol/L, the MCh-induced baseline tension (75 % of the maximum) was significantly increased by (14±6) %, (21±5) %, respectively (n=7, P<0.01).

Effects of TEA on isoprenalilne and aminophylline-induced relaxation in MCh-precontracted bronchial strips Isoprenaline produced complete relaxation of MCh-induced tone. In the presence of TEA 5 mmol/L, the control concentration-response curves to isoprenaline were shifted to the right about 4.26-fold, with lower pD_2 (from 8.1±0.3 to 7.44±0.15, n=7, P<0.05, Fig 1A). Aminophylline resulted in complete concentration-dependent relaxation of MCh-induced tone. In the presence of TEA 5 mmol/L, the control concentration-response curves to isoprenaline were shifted to the right about 3.78-fold with lower pD_2 (from 3.92±0.13 to 3.34±0.07, n=8, P<0.05, Fig 1B).

Characterization of BK_{Ca} currents in rat BSMC Step depolarizations with pulse protocol elicited voltage-dependent outward currents, which could be significantly suppressed by TEA 1 mmol/L [from (94±16) pA/pF to (21±4) pA/pF, at +50 mV, n=12, P<0.01]. It was noninactivating and characterized by great noise (Fig 2A, B, and C). These characteristics are consistent with those of BK_{Ca} channels[13]. With ramp protocol, the peak current density was decreased from (118±14) pA/pF to (17±3) pA/pF (n=12, P<0.01, Fig 2D).

Effects of 8-Brom-cAMP on BK_{Ca} currents in rat BSMC In freshly isolated rat BSMC, application of

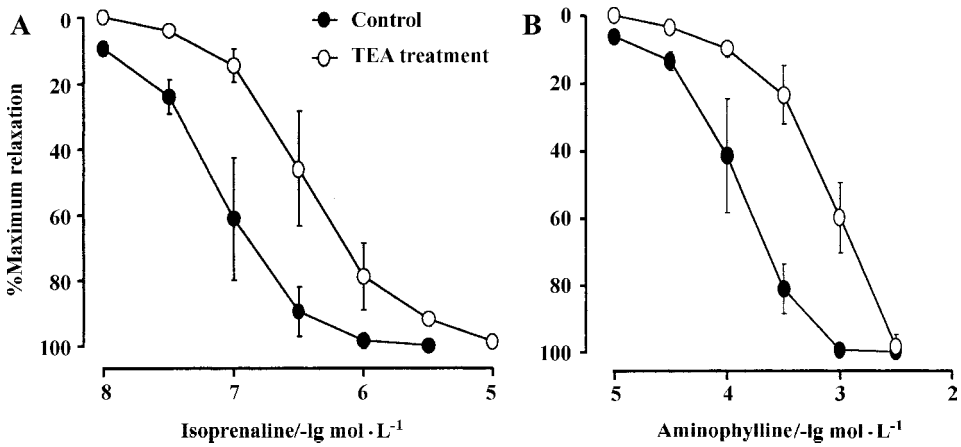

Fig 1. Effects of TEA 5 mmol/L on the suppression of the MCh-induced contraction by (A) isoprenaline (*n*=7) and (B) aminophylline (*n*=8). Mean±SD.

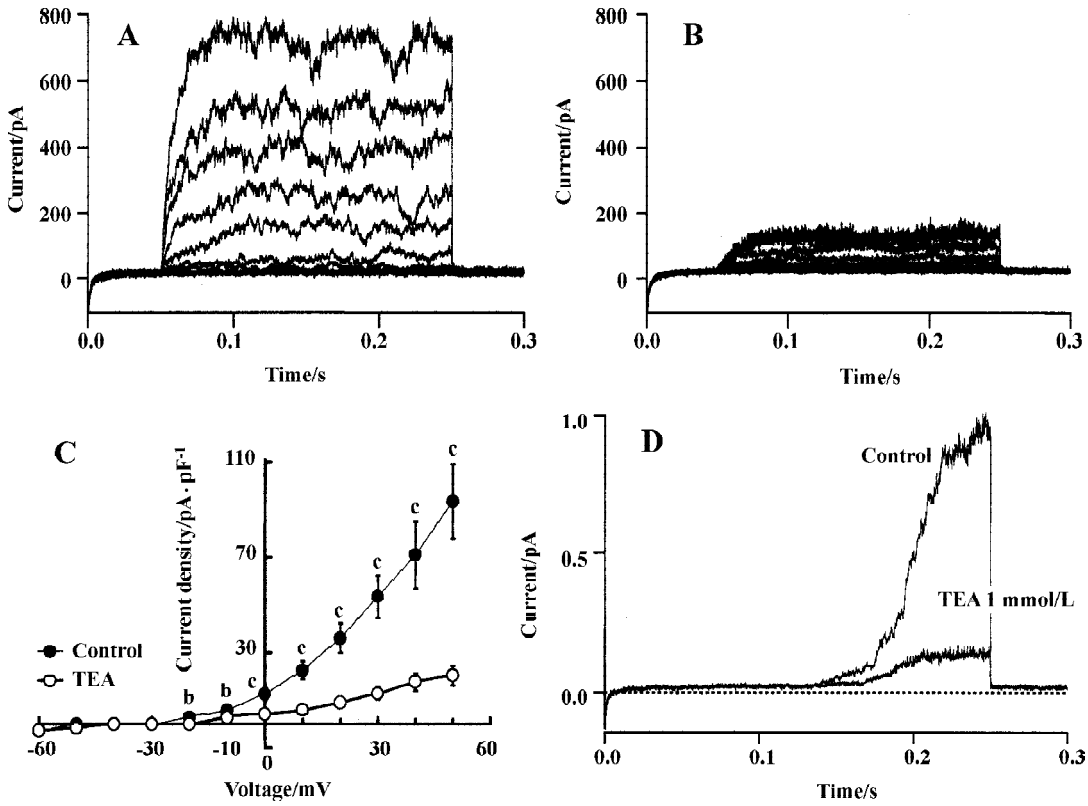

Fig 2. Identification of BK_{Ca} channel current. A and B: representative traces of whole-cell currents recorded from a single BSMC before (A) and after (B) TEA 1 mmol/L treatment. C: current-voltage relationships in absence and presence of TEA (*n*=12 from 9 rats). Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. D: representative BK_{Ca} current measured by ramp protocol in absence and presence of TEA 1 mmol/L.

8-Brom-cAMP 100 μmol/L caused a significant increase in the magnitude of the BK_{Ca} current (Fig 3A,B) from (89±12) pA/pF to (188±30) pA/pF (voltage steps from -60 mV to +50 mV, n=10, P<0.01), which was partly abolished by Rp-cAMP 100 μmol/L (a PKA inhibitor), (104±17) pA/pF, P<0.01, compared with those obtained in the presence of only 8-Brom-cAMP. Furthermore, the current-voltage relationship (I-V) curve exhibited an upward shift. With ramp protocol, similar results were obtained (Fig 3C). The peak current density was raised from (115±23) pA/pF to (213±32) pA/pF (n=10, P<0.01).

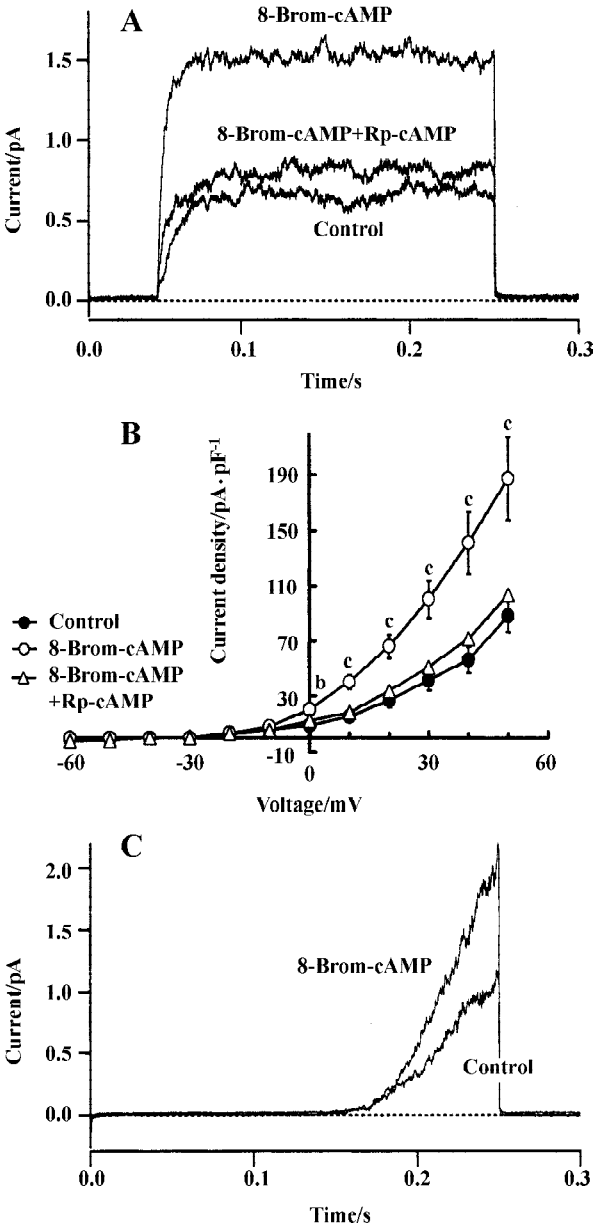

Fig 3. Effects of PKA activation on BK_{Ca} current in rat BSMC. A: representative current traces showing effects of 8-Brom-cAMP 100 μmol/L on BK_{Ca} current. B: current-voltage relationships in absence and presence of 8-Brom-cAMP and then 8-Brom-cAMP+ Rp-cAMP 100 μmol/L (*n*=7 from 5 rats). Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control. C: representative BK_{Ca} current measured by ramp protocol in absence and presence of 8-Brom-cAMP 100 μmol/L.

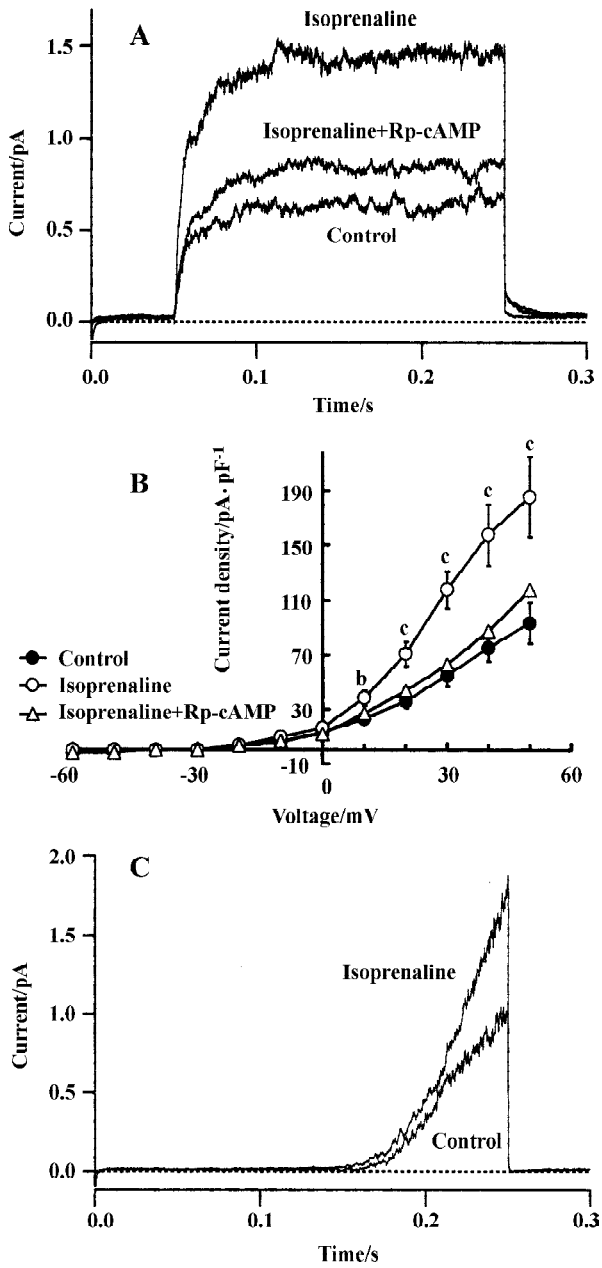

Fig 4. Effect of isoprenaline on BK_{Ca} current in rat BSMC. A: representative current traces showing effects of isoprenaline 1 μmol/L on BK_{Ca} current. B: current-voltage relationships in absence and presence of isoprenaline and then isoprenaline+Rp-cAMP 100 μmol/L (*n*=6 from 5 rats). Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control. C: representative BK_{Ca} current measured by ramp protocol in absence and presence of 8-Brom-cAMP 100 μmol/L.

Effects of isoprenaline on BK_{Ca} currents in rat BSMC Application of isoprenaline 1 μmol/L caused a significant increase in BK_{Ca} current (Fig 4A,B) from (94±15) pA/pF to (186±30) pA/pF (voltage steps from -60 mV to +50 mV, *n*=10, *P*<0.01), which was partly abolished by Rp-cAMP 100 μmol/L, (118±19) pA/pF, *P*<0.01, compared with those obtained in presence of isoprenaline only. Furthermore, the *I-V* curve exhibited an upward shift. With ramp protocol, similar results were obtained (Fig 4C). The peak current density was raised from (101±19) pA/pF to (197±23) pA/pF (*n*=10, *P*<0.01).

Effects of aminophylline on BK_{Ca} currents in rat BSMC Application of aminophylline 1 mmol/L

caused a significant increase (Fig5A,B) in BK_{Ca} current from (90±10) pA/pF to (166±25) pA/pF (voltage steps from –60 mV to +50 mV, n=11, P<0.01), which was partly abolished by Rp-cAMP 100 μmol/L, (99±14) pA/pF, P<0.01, compared with those obtained in the presence of only aminophylline. Furthermore, the *I-V* curve exhibited an upward shift. With ramp protocol, similar results were obtained (Fig 5C). The peak current density was raised from (114±21) pA/pF to (186±30) pA/pF (n=11, P<0.01).

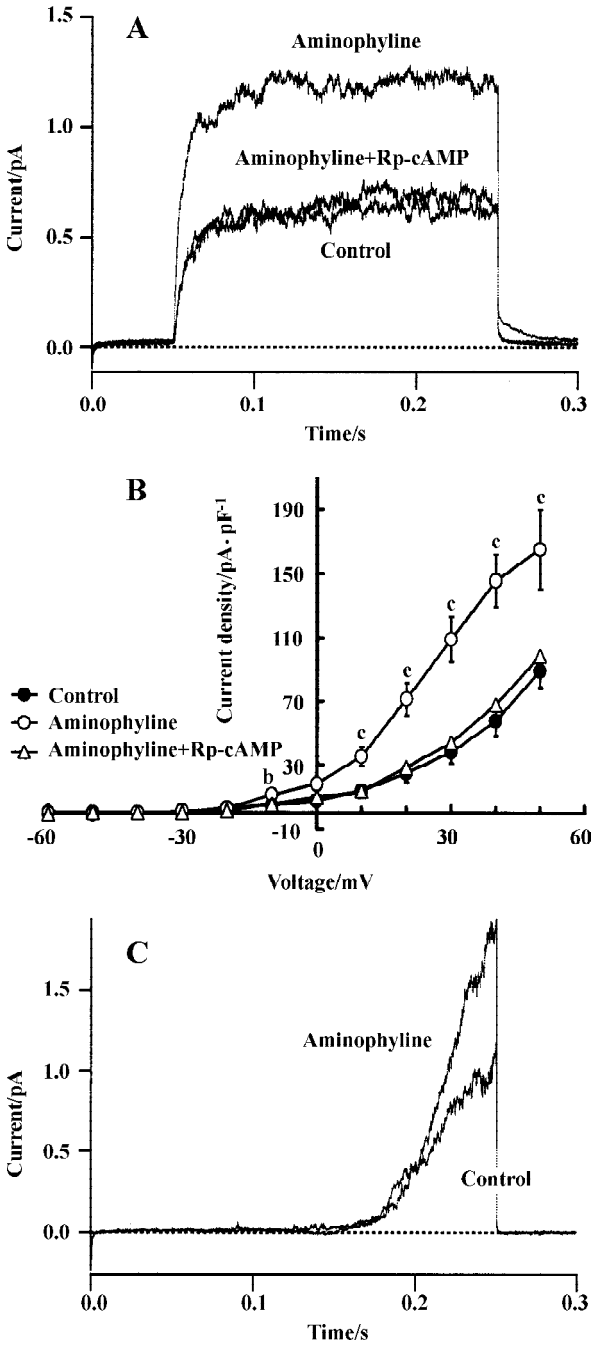

Fig 5. Effects of aminophylline on BK_{Ca} current in rat BSMC. A: representative current traces showing effects of aminophylline 1 mmol/L on BK_{Ca} current. B: current-voltage relationships in absence and presence of aminophylline and then aminophyllne+Rp-cAMP 100 μmol/L (n=7 from 6 rats). Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control. C: representative BK_{Ca} current measured by ramp protocol in absence and presence of aminophylline 1 mmol/L.

DISCUSSION

Functional roles for BK_{Ca} were investigated in smooth muscle strips with a channel blocker. We observed that TEA, a BK_{Ca} channel blocker, had no effect on resting tension, but caused marked augmentation of MCh-induced contraction. This findings suggested a role for BK_{Ca} in limiting excitation and contraction, although being unrelated to the regulation of resting tension, which is consistent with previous reports about BK_{Ca} in vascular smooth muscle[3]. Our findings had also shown that TEA caused a significant inhibition of the relaxations induced by isoprenaline and aminophylline. It was indicated that the relaxations were mediated, at least partly, by opening of BK_{Ca} channels, namely that BK_{Ca} channels were involved in this relaxation. But the mechanism underlying this relaxation mediated by BK_{Ca} channels is unknown.

The relaxations induced by both isoprenaline and aminophylline have been determined to be related well to intracellular cAMP concentration. The latter raises the concentration of cAMP through inhibiting cyclic nucleotide phosphodiesterase[2]. In vascular and prostatic smooth muscle, studies have demonstrated that PKA activation resulted in enhancement of BK_{Ca} channel activity via phosphorylation[6,14]. To determine the involvement of BK_{Ca} channels in the relaxation induced by isoprenaline and aminophylline via PKA pathway in bronchial smooth muscle, we employed perforated-patch clamp and freshly isolated bronchial smooth muscle cells to investigate the mechanisms. In the present experiment, BK_{Ca} channel currents in rat bronchial smooth muscle cells were successfully isolated and identified. PKA pathway was demonstrated to participate in the regulation of BK_{Ca} activity in rat BSMC because 8-Brom-cAMP, a PKA activator, resulted in a marked augmentation in BK_{Ca} current activity, which was partly reversed by a PKA inhibitor Rp-cAMP. These findings were consistent with the previous study in vascular smooth muscle[6]. Both isoprenaline and aminophylline significantly enhanced BK_{Ca} channel currents in freshly isolated rat BSMC, which was consistent with the above functional study results in the regulation of

tension of bronchial strips. The antagonism of Rp-cAMP, a PKA inhibitor, to the actions of isoprenaline and aminophylline in BSMC indicated that the enhancing effects of them on BK_{Ca} currents were at least partly mediated by PKA pathway. The exact mechanism by which PKA activation mediates activation of BK_{Ca} channels remains uncertain. It may involve direct phosphorylation of BK_{Ca} channel because multiple phosphorylation sites of PKA are present in BK_{Ca} channels[15], or, it may act indirectly via phosphatase because the activating effects of PKA on BK_{Ca} channels could be reversed by phosphatase inhibitor[16]. More experimens are needed to elucidate the exact mechanisms responsible for this phenomenon.

In conclusion, our results supported a functional role for BK_{Ca} in the isoprenaline- and aminophylline-induced relaxation by a mechanism involved in PKA in rat bronchial smooth muscle, although the exact mechanism by which PKA regulates BK_{Ca} currents remains uncertain.

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