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R- and S-terbutaline activate large conductance and Ca^{2+} dependent K^+ (BK_{Ca}) channel through interacting with β_2 and M receptor respectively

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ABSTRACT

This study investigated the effect of the β_2 receptor agonist terbutaline on the single channel activity of BK_{Ca} channel. The effects of racemate and two isomers of terbutaline were all assessed. β_2 adrenoceptors were stably overexpressed on HEK293 cells by lentiviral transduction method and chicken BK_{Ca} channels were transiently expressed on normal HEK293 cell line or HEK293 cells overexpressing β_2 receptors. Data showed that terbutaline significantly increased the single channel open probability of BK_{Ca} channel within 10 min. The channel activating effects of terbutaline are stereoselective and mainly stay with the R-enantiomers. The opening probability of BK_{Ca} channel at 10 min after drug application normalized to that just before drug application (Po10/Po0s) for R- and S-terbutaline were 7.85 ± 3.20 and 1.06 ± 0.45 respectively at 1 μM concentration, corresponding to 28.37 ± 9.96 and 2.68 ± 1.09 at the higher concentration of 10 μM. ICI 118551 blocked the effect of R- but not S-terbutaline (10 μM), whereas atropine blocked the channel activating effects of S-terbutaline of higher concentration. In addition, the muscarinic receptor agonist carbachol increased the BK_{Ca} channel activity in an atropine-sensitive manner as an positive control experiment, which indicate the involvement of M receptor in the channel activating effect of S-terbutaline.

1. Introduction

β-adrenoceptor agonists are an important kind of agents in the treatment of asthma and chronic obstructive airways disease. Of the established smooth muscle relaxant drugs, the β_2-selective sympathomimetics (e.g. terbutaline, salbutamol, fenoterol), are currently the most effective bronchodilators available and have remained the mainstay of asthma therapy [1]. β_2-adrenoceptor, as typical G-protein-coupled receptor (GPCR) with seven transmembrane domains, could transduce signals through interaction with both extracellular ligands and intracellular G protein [2,3]. The smooth muscle relaxant mechanism of β_2-adrenoceptor agonist is complicated. Traditionally, it is thought that the pathway initiated by β-adrenoceptor agonists include the Gs-cAMP-PKA-BK_{Ca} cascade [4,5]. The membrane hyperpolarization caused by BK_{Ca} channel activation results in a decrease in intracellular calcium, finally causing smooth muscle relaxation [6–8]. However, the above signaling transduction may not be the necessary and sole cascade. The β_2-AR agonists could activate both G_s and G_i intracellular pathways, some ligands even activate β_2-AR through a G protein independent manner [9]. Besides PKA, PKC and PKG also regulate channel functions by phosphorylation C-terminal of BK_{Ca} channel [10].

Many sympathomimetic drugs have one or two chiral centres in their structures and form two or more enantiomers. The chiral β-agonists used in the treatment of asthma include isoproterenol, salbutamol, formoterol, salmeterol and terbutaline. It was early found that the bronchodilating property of synthetic β-adrenoceptor agonists stayed mainly with the R-enantiomer (eutomer), which has been extensively studied for salbutamol and terbutaline in pharmacokinetics and pharmacodynamics [11–14]. In comparison with R-enantiomers, the S-enantiomers (distomer) of sympathomimetics are practically inert or even have deleterious effects. It has been generally assumed that the S-enantiomer of racemic β-adrenocepter agonists may cause airway hyper-reactivity and contribute to increase in asthma death [15–18]. Although most established chiral drugs used in treatment of asthma at present are still in racemic form for technical or economic reasons, the pure active enantiomers with improved antiasthmatic properties have been evaluated in preclinical laboratory study and introduced to market gradually.

This study directly investigated the effects of pure (R- and S-) enantiomers and racemic (rac-) terbutaline on BK_{Ca} single channel currents. Results showed that both R- and rac- terbutaline significantly increased the open probability of BK_{Ca} channel, with R-terbutaline exhibiting a prominently larger effect. The effects of R- terbutaline were blocked by β_2 receptor antagonist ICI 118551. S-terbutaline only increased the open probability of BK_{Ca} channel at relative higher concentration (10 μM), which was not affected by blocking β_2-adrenoceptor. However, the

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activating effect of S-ter (10 μM) on BK_{Ca} channels was completely blocked by 100 nM atropine - the antagonist of M receptor. The role the M receptor in the activation effect of S-terbutaline could be further supported by the result that the M receptor agonist-carbachol activated BK_{Ca} channels in an atropine-sensitive manner. These results indicated that terbutaline could activate BK_{Ca} channels stereoselectively with the R-enantiomer being more effective. The S- enantiomer at high concentration has a slight activity on BK_{Ca} channel, which is not mediated by β_2 adrenoceptor but by a pathway involving muscarinic receptor.

2. Materials and methods

2.1. Cell culture and transduction

HEK 293 cell line stably expressing β_2 adrenoceptors (HEK293-β_2) was obtained by standard lentiviral transduction method. Briefly, the human β_2 adrenoceptor coding region (NCBI accession number: BC073856) was cloned into the lentiviral expression vector pLVTHM. Recombinant lentivirus vector and packaging plasmids were cotransfected into HEK293 cells. Viral supernatant was collected and the titer was determined. HEK 293 cells were transfected with lentivirus expressing β_2 receptor. The stable cell line HEK-293-β_2 was selected with Hygromycin B (50 μg/ml). Both HEK293 and HEK293-β_2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) and maintained in a humidified chamber with 5% CO_2 at 37 °C. The chick BK_{Ca} coding sequence subcloned into vector *pcDNA* 3.1(−) was obtained as a gift from Prof. Qi (Medical College, Xiamen University, Xiangan Xiamen) and transiently cotransfected with EGFP (Clontech, Palo Alto, CA) into HEK293 or HEK293-β_2 cell lines 24 h before electrophysiological recording. The transient transfection was performed with LipofectAMINE Plus reagent (Invitrogen) following the manufacturer's instructions.

2.2. Quantitative real-time RT-PCR

Total RNA was extracted with the QIAamp RNeasy MiniKit according to the manufacturer's instructions (QIAGEN). Reverse transcription was performed with TAKARA Reverse Transcriptase XL. PCR was carried out using the following primers: SF: 5-CTGATGGTGTGGATTGTGTC-3′, SR: 5′-GCATAGGCTTGGTTCGTGA-3′. The real time PCR cycling program involved an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. All quantitative real-time PCR (qRT-PCR) was performed with the ABI 7500 (Applied Biosystems, USA). GAPDH mRNA was used as a reference standard for normalization of mRNA levels.

2.3. Western blot analysis

The expression levels of transiently transfected BK_{Ca} channel in HEK293-β_2 cell lines were assessed by western blot method. After treatment with lysis buffer, cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% stacking and 8% separating gels. Then, proteins were transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA) and blocked with 5% BSA for 60 min at 37 °C. Polyclonal rabbit anti-BK_{Ca} (1:1000, Abcam, Cambridge, MA) antibody and anti-β-actin antibody (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to label BK_{Ca} and β-actin protein respectively by incubating the membrane overnight at 4 °C. After washes in TBS containing 1‰ Tween-20 (TBST), goat anti-rabbit immunoglobulin (IgG)-horseradish peroxidase (HRP) secondary antibody at a dilution of 1:3000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied and incubated for 1 h at 37 °C. Immunoblots were visualized by chemiluminescence with Pierce™ ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL, USA) with exposure to autoradiograph film (X-OMAT AR; Eastman Kodak, Rochester, NY).

2.4. Electrophysiological recording

The single-channel recordings were performed at cell-attach patch-clamp configuration with an EPC-10 amplifier (HEKA, Lambrecht, Germany) at room temperature. Pipettes were pulled from borosilicate glass and had a resistance of between 4 and 7 MΩ. The pipette solution was (in mM): 140 KCl, 10 HEPES, 2 Mg-ATP, 3.3 $CaCl_2$, 5 EGTA, and the pH was adjusted to 7.2 with KOH. The extracellular solution was Hanks' balanced salts solution (HBSS, Sigma; in mM): 1.3 $CaCl_2$, 0.8 $MgSO_4$, 5.4 KCl, 0.4 KH_2PO_4, 136.9 NaCl, 0.3 Na_2HPO_4, 10 D-glucose and 4.2 $NaHCO_3$. Data were recorded with a sampling frequency of 10 kHz and low pass filtered at 1 kHz. Continuous recordings of at least 10 s were used to determine the single channel open probability (Po). The pipette voltage depolarized from −80 mV to −140 mV (transmembrane voltage: 0 mV to +60 mV, assuming a resting potential of −80 mV) to elicit the maximum channels opening in the clamping patch. A pipette voltage of −110 mV was used for final single-channel recording and comparison among different groups. TAC 4.1 (HEKA, Germany) were used for data analysis. The value of P_o based on the equation: $P_o = (1-P_C^{1/N})$, where P_C is the probability that all of the channels are in a closed state, N is the number of channels in the patch, which was estimated from the maximum number of channels opening observed at the pipette voltage of −140 mV. Iberiotoxin (IbTX, 100 nM) was used to identify BK_{Ca} single channel current. ICI 118551 (200 nM) and atropine (100 nM) were used to block β_2 and M receptors respectively. Carbachol is the agonist of M receptor. All chemicals and reagents, unless otherwise stated were obtained from Sigma.

2.5. Statistical analysis

The data were expressed as mean ± SD, n = number of independent experiments. Statistical analysis was performed by one-way ANOVA using a Bonferroni test. Significant differences between groups were defined at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. The expression and identification of β_2 adrenoceptor and BK_{Ca} channel

β_2 adrenoceptor was stably transfected into HEK293 cell line by lentiviral transduction method as described in materials and methods. To assess the overexpression of β_2 receptor, the qRT-PCR were carried out for ordinary HEK293 and HEK293-β_2 cell lines. For qRT-PCR analysis, Fig. 1A shows that the mRNA expression level of β_2-adrenoceptor was greatly increased for HEK293-β_2 cells compared with that of normal HEK293 cell line. The relative mRNA expression level of β2 receptor normalized to GAPDH was increased to 0.2645 ± 0.0133 ($n = 4$) for HEK293-β_2 from 0.0011 ± 0.0001 ($n = 4$) of normal HEK293 cells. The transiently expression of BK_{Ca} channels was identified by western blot analysis, Fig. 1B showed that the expression level of BK_{Ca} channels in HEK293-β_2-BK cells was apparently higher than that of HEK293 and HEK293-β_2 cell lines in which BK_{Ca} channels were not transfected. Furthermore, the expression of BK_{Ca} channel in HEK293-β_2 cell line was also examined by electrophysiological method. Fig. 2A showed the single channel currents of HEK293-β_2-BK cells at the pipette clamping voltage of −90 to −110 mV were completely blocked by 100 nM BK_{Ca} specific antagonist IbTX. Fig. 2B and C showed the currents of BK_{Ca} channel at different clamping voltages and single channel current-voltage curve respectively. The single channel conductance was calculated by linear fitting the IV curve of BK_{Ca} channel. The calculated single channel conductance of BK_{Ca} channel was about 179.3 pS, which is consistent with the common value of BK_{Ca} channels.