Identification of GIRK2-4 subunits in human esophageal smooth muscle cells

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Abstract. Acetylcholine (ACh) secreted from the vagus nerve contributes to the physiological and pathological regulation of the contraction and relaxation of human esophageal smooth muscle. Expression of acetylcholine-sensitive G protein-activated inwardly rectifying potassium channels (GIRKs) occurs widely in the heart, nervous system and gastrointestinal, but the role of GIRKs in the esophagus remains unclear. In the present study, expression of the GIRK1-4 subunits in mRNA and total protein was examined in human esophageal smooth muscle cells (SMCs) by reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. mRNA and protein expression of the GIRK2-4 subunits was detected in human esophageal longitudinal muscle (LM) and circular muscle (CM) cells. However, GIRK1 mRNA and protein were not observed in either the esophageal LM or CM. This study is the first to identify the expression of GIRK2-4 subunits in human esophageal SMCs.

Introduction

G protein-activated inwardly rectifying K+ (GIRK) channels are members of a family of inward-rectifier K+ (Kir) channels that includes 7 subfamilies (1,2). GIRK channels play a crucial role in the inhibitory regulation of neuronal excitability in most brain regions and of the heart rate through the activation of various G protein coupled receptors, such as opioid, cannabinoid and D2 dopamine receptors (3,4). In the heart, GIRK channels, previously named muscarinic K+ channels, are activated by M2 muscarinic and A1 adenosine receptors, which are coupled to pertussis toxin-sensitive G proteins, the Gi/o protein family (5). In the central nervous system (CNS), GIRK channels are activated by various Gi/o-protein-coupled receptors (Gi/oPCRs), such as a2 adrenergic and γ-aminobutyric acid type B (3,4). In mammals, 4 GIRK channel subunits have been identified. Neuronal GIRK channels are predominantly heteromultimers comprising of GIRK1 and 2 subunits in most brain regions or homomultimers comprising of GIRK2 subunits in the substantia nigra and ventral tegmental region, whereas atrial GIRK channels are heteromultimers comprising of GIRK1 and 4 subunits (3,6). Ablation of GIRK4 resulted in the functional elimination of I_{K,Ach} (7). In the stomach, small bowel and proximal colon, GIRKs were encoded by GIRK1 and 2 (8). The existence of GIRK channels in esophageal smooth muscle cells (SMCs) as well as the roles of GIRKs in the contraction of esophageal SMCs remain to be determined. Acetylcholine (ACh) secreted from the vagus nerve modulates the contractility of the esophageal SMCs.

Materials and methods

Preparation of tissue specimens and esophageal SMC cultures. Tissues were obtained from a disease-free region of the midportion of the distal third of the esophagus obtained from 18 esophageal cancer patients aged between 35 and 65 years (52.5±4.5, n=18). Esophageal manometry, 24-h pH monitoring and esophagoscopy were performed to exclude esophagitis in all of the patients. The patients received no treatment such as radiation or chemotherapy prior to surgery. Permission for use of all specimens in this study was obtained from each patient. Each patient signed informed consent forms for sample collection. The study was approved by the Ethics Committee of the Fourth Military Medical University and the investigation conformed to the principles outlined in the Declaration of Helsinki.
Histopathological analysis. Histopathological analysis was carried out on the Hematoxylin eosin (H&E)- and immunohistochemistry-stained tissue slides in a blinded manner.

Immunostaining was carried out using a streptavidin-labeled peroxidase (S-P) kit (KIT9730) according to the manufacturer's instructions. The primary antibodies used in this study included those against epithelial membrane antigen (EMA), cytokeratin (CK), high-MW-CK, desmin, nerve specificity enolase (NSE), smooth muscle actin (SM-actin), vimentin, CD34, S-100 protein and CD117. The reagents used for immunostaining were supplied by Maxim Biotechnology Corporation Limited (Fuzhou, China).

RNA isolation and RT-PCR. Total RNA was extracted from the LM and CM groups of SMCs using a TRizol kit (Invitrogen, Carlsbad, CA, USA) and acid guanidinium thiocyanate-phenol-chloroform extraction.

Total RNAs (4 µg) were used to generate the first strand cDNA by reverse transcription (Invitrogen) according to the manufacturer's instructions. cDNA reaction mixture (3 µl) was used in each polymerase chain reaction (PCR). PCR was performed in a 50 µl reaction containing PCR buffer, 2 mM MgCl₂, 200 µM dNTPs, 0.1 nM of each primer, and 2 units of Taq DNA polymerase (Qiagen, Valencia, CA, USA). denaturation (33 cycles; 94˚C, 30 sec) annealing (55-65˚C, 30 sec), and extension (72˚C, 60 sec) were conducted in a PCR thermal cycler (MJ PTC100, USA). Table I shows the primers for human GIRK1-4. PCR primers for GAPDH were used to confirm fidelity of the PCR reaction and to detect genomic DNA contamination.

The PCR products were then visualized by ultraviolet illumination after electrophoresis on 1% agarose-TAE [10 mM tris (pH 7.5), 5.7% glacial acetic acid and 1 mM EDTA] gels containing 0.5 µg/ml ethidium bromide. Gel images were then taken by a multianalyzer (Bio-Rad, Hercules, CA, USA). GAPDH was used as the internal control.

Western blotting. To detect GIRK subunit proteins, SMCs were rinsed with cold PBS. SMCs were washed in PBS and homogenized (Brinkmann Polytron) in 10 mM HEPES buffer (pH 7.0) containing 1 mM dithiothreitol and mini complete protease inhibitor cocktail tablets (1 tablet/25 ml buffer, Boehringer Mannheim, IN, USA).

Crude protein homogenates were denatured by boiling for 15 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. The extract was centrifuged at 12,000 x g for 20 min. Bradford protein assay reagent (BioColor Bioscience, Shanghai, Inc., China) was used to determine the protein concentration of the samples. Samples were resolved by SDS-PAGE on a 12% gel and transferred onto a polyvinylidene difluoride membrane. The membranes were incubated in PBS buffer containing 5% non-fat dry milk for 1 h at room temperature in order to block non-specific binding. After washing 3 times in PBS, the blots were incubated with primary antibodies in PBS solution containing 0.1% BSA at 4˚C overnight. The primary antibodies used were: anti-GIRK1 (1:600), anti-GIRK2 (1:600), anti-GIRK3 (1:600), anti-GIRK4 (1:600) (Santa Cruz, CA, USA) and anti-GAPDH (1:300) (Bioss, Shanghai, China). The membranes were washed with PBS containing 0.05% Tween-20. The appropriate secondary antibody was used for each primary antibody and a signal was developed using Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) for 1 h. The signal was collected in a Bio-Rad Fluor-S Max detection system and quantified by densitometry analysis using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data were expressed as the means ± SD. Statistical differences between groups were determined by one-way ANOVA followed by the LSD post-hoc test. P<0.05 was considered to be statistically significant.

Results

Identification of GIRK1-4 subunit mRNAs in human esophageal SMCs. PCR primers were designed to amplify sequences specific for GIRK1-4 subunits. mRNA expression of GIRK subunits was found in human esophageal SMCs in LM and CM layers. Transcripts for GIRK2-4 subunits were identified.
in cells from both human esophageal LM and CM layers (Fig. 1A). GIRK1 was not expressed in human esophageal SMCs or LM or CM layers (Fig. 1A). The identity of the amplified products was confirmed by direct DNA sequencing.

Compared with the GAPDH group, the mRNA expression of GIRK2-4 subunits in the human LM layer was 0.116±0.017, 0.273±0.025 and 0.678±0.031 for GIRK2, GIRK3 and GIRK4, respectively. The mRNA expression of GIRK2-4 subunits in the human esophageal CM layer was 0.120±0.0135, 0.308±0.031 and 0.703±0.037, respectively (Fig. 1B).

Compared with the expression of GIRK4 subunit mRNA, the expression of GIRK2-3 subunits was significantly lower (P<0.05, Fig. 1B). However, no difference was found between mRNA expression of GIRK2-3 subunits (P>0.05, Fig. 1B). In addition, mRNA expression of GIRK2-4 subunits was not significantly different between human esophageal LM and CM layers (P>0.05, Fig. 1B).

Identification of GIRK1-4 subunit protein in human esophageal SMCs. To further investigate the expression of the GIRK subunit protein in human esophageal SMCs, the protein expression of GIRK1-4 subunits was determined by immunoblot analysis in human esophageal SMCs.

Specific polyclonal antibodies directed at each GIRK1-4 subunit were used to examine their expression in human esophageal LM and CM SMCs (Fig. 2A). Immunoreactive protein bands corresponding to each were identified and their molecular weights were estimated to be: GIRK1, 57; GIRK2, 49; GIRK3, 44; GIRK4, 48 and GAPDH, 37. Following isolation of the enriched membrane protein using a Bio-Rad kit, the protein expression of GIRK2-4 was determined (Fig. 2A). However, the GIRK1 protein expression was not observed in human esophageal SMCs (Fig. 2A). Signal was collected in a Bio-Rad Fluor-S Max detection system and quantified by densitometry analysis using Quantity One software (Bio-Rad).

Similarly, total protein expression of GIRK2-4 subunits in human esophageal LM layer was 0.056±0.007, 0.153±0.019 and 0.495±0.029, respectively. Protein expression of GIRK2-4 subunits in human esophageal CM layer was 0.070±0.0085, 0.188±0.028 and 0.495±0.029, respectively (Fig. 2B).

Compared with the expression of GIRK4 subunit protein, the expression of GIRK2-3 subunits was significantly lower...
force of contraction and an increased rate of smooth muscle motility via M2 and M3 receptors on the SMC surface (23,24). ACh is able to depolarize the membrane of SMCs mediated by the M2 receptor (25,26).

First, the M2 receptor couples to the Gi/Go-type G proteins and stimulates phosphoinositide metabolism, which is linked to Ca^{2+}-signaling events. Intracellular events lead to the depolarization and activation of L-type Ca^{2+} channels (27), resulting in smooth-muscle contraction (28). Second, M2 receptor stimulation indirectly induces a contraction by inhibiting the adenyl cyclase and protein kinase A (PKA), which would otherwise have an excitatory effect due to the stimulatory effect of PKA on a number of K^+ channels in SMCs (29). Third, M2 receptor also inhibits potassium channels and opens non-specific cation channels to induce contraction (30). Under physiological ionic conditions, the muscarinic receptor-mediated current is carried with a reversal potential between -10 and 0 mV, whereby depolarization is produced; thus, the discharge of action and slow-wave potentials initiates or accelerates unless the depolarization is extremely strong (31,32). Previous studies have shown that ACh activates and modulates the activity of GIRK channels in esophageal SMCs (33,34).

In addition, the possibility of a basally active \( I_{K_{\text{ca}}^+} \)-like conductance contributes to resting membrane potential along with the activity of GIRK channels (35). In brief, GIRK channels play a significant role in the control and modulation of esophageal SMC function. Our data showed that M2 receptor was down-regulated in the esophagus in acid-perfused rabbits (unpublished data), indicating that GIRK channels may undergo plastic changes under pathological conditions. Further studies are required to explore the molecular basis of the GIRKs in esophageal SMCs and to elucidate their regulatory mechanisms under physiological and pathological conditions.

In conclusion, our study provides evidence that mRNA and the protein expression of GIRK2-4 subunits were present in human esophageal LM and CM cells, whereas GIRK1 was not. GIRK channels in the human esophagus may be potential therapeutic targets in GERD treatment.

References