Int J Clin Exp Pathol 2017;10(3):3851-3856
www.ijcep.com /ISSN:1936-2625/IJCEP0045304

Original Article

Expression changes of small conductance Ca^{2+} activated K^+ channel in atrial myocytes caused by calumenin silence

Xiaoxue Cui[1*], Yu Wang[1,2,4*], Xiaotong Shan[1], Liying Xuan[1], Yilin Wang[1], Chengxi Wei[1,2,4], Ming Zhao[1,3,4]

[1]Inner Mongolia University for The Nationalities, Tongliao, Inner Mongolia, P. R. China; [2]Medicinal Chemistry and Pharmacology Institute, Inner Mongolia University for The Nationalities, Tongliao, Inner Mongolia, P. R. China; [3]First Clinical Medical of Inner Mongolia University for Nationalities, Tongliao, Inner Mongolia, P. R. China; [4]Inner Mongolia Provincial Key Laboratory of Mongolian Medicine Pharmacology for Cardio-cerebral Vascular System, Tongliao, Inner Mongolia, P. R. China. [*]Equal contributors.

Received November 28, 2016; Accepted November 30, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Small conductance Ca^{2+} activated K^+ channels have been shown to contribute to the pathogenesis of atrial fibrillation (AF). ER resident calcium binding calumenin has been shown to have a cardioprotective role. In this study, the suckling mouse atrial myocytes was identified by Immunohistochemical method. Calumenin protein was down-regulated in mouse neonatal cardiomyocytes (atrial myocytes). Ca^{2+} concentration was tested with fluorescence spectrophotometer and the expression of SK2 was detected with Real Time PCR and Western blotting. Compared with the control group, the concentration of Ca^{2+} was increased and the expression of SK2 mRNA and protein was increased in Calumenin silence model group. This study highlights calumenin affected Ca^{2+} and SK2 expression in mouse atrial myocytes, which may play an important role in the occurrence and development of atrial fibrillation.

Keywords: Atrial fibrillation, calumenin, small conductance calcium activated potassium channel, suckling mouse

Introduction

Atrial fibrillation (AF), one of the most common clinical arrhythmia, is a serious threat to human survival and health. AF can trigger thromboembolic disease, increase patient mortality, make the risk of stroke increase 4 to 5 times and that of dementia increase two times [1] When Atrial fibrillation occurs, the rapid stimulation can make the systolic phase relatively prolonged, the frequency of action potential increased and the intracellular Ca^{2+} increased through ICa-L, thus induce the release of sarcoplasmic reticulum Ca^{2+}, Finally lead to the intracellular Ca^{2+} overload [2], Small conductance calcium activated potassium channels (SK) is one of the calcium activated potassium channels (KCa), which is insensitive to voltage and sensitive to calcium. In recent years, the research found that SK channels existed in the cardiomyocytes, molecular evidence indicated that SK2 differently expressed in human and mouse atrium and ventricular, mainly in the atrium. SK2 channels is a kind of KCa channels, which is highly sensitive to intracellular free Ca^{2+} and rapidly responses to Ca^{2+} change and quickly converts the change of intracellular Ca^{2+} concentration into cell membrane potential changes. Therefore, we speculated that intracellular calcium overload may affect SK2 channel function in AF atrial myocytes.

Calumenin is a Ca^{2+} binding protein, which is located in the endoplasmic reticulum/sarcoplasmic reticulum of mammalian cardiomyocytes and belongs to the CREC family with many EF-hand structures. Calumenin interacts with Ryanodine and SERCA2a regulate cardiomyocytes calcium release, calcium uptake and calcium storage and maintain the steady state of calcium cycle [3]. At present, it has not been seen the related reports on the correlation about calumenin, SK2 and mechanism of atrial fibrillation, this study intends to observe the SK2 expression changes in suckling mouse atrial myocytes with or without calumenin

silence, to study a new pathophysiological mechanism of atrial fibrillation.

Materials and methods

Animal drug and instrument

1-3d, Balb/csuckling mice were purchased from Basic Medical School of Jilin University animal center, license number: scxk (Ji) 2011-0004. This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

KCNN2 antibody was purchased from Abcam Company, calumenin antibody was purchased from Bioss Company, β-actin was purchased from Wanleibio Company. Lentiviral transfection plasmid was synthesized by Shenyang WANLEI life Science Company.

Isolation and culture of suckling mouse atrial myocytes

Placed suckling mouse in culture dishes and washed them with PBS repeatedly. Cut atrial tissue into 1-3 mm^3 small pieces. Added 0.1% type II collagenase and 0.1% trypsin mixture, After digestion with 37°C for 25 min, moved it into the 15 ml centrifuge tube to centrifuge with 1500 r/min for 7 min and discarded supernatant, 1000 r/min after suspending precipitation with PBS and pipetting repeatedly through a straw centrifuged 5 min, culture medium suspended cell, a 200 mesh screen was used to remove chunks of tissue. PBS cleaned cell 2 times, 1000 r/min centrifuged 10 min, removed the supernatant and kept precipitation. Culture medium suspended cell again, then placed it into culture plate after cell counting. Fiber cells adherent faster, a large number of fiber cells can be removed for 2 h, pure myocardial cells was in supernatant suspension fluid. Cells were placed in culture plate after 24 hours without moving, then every two days for changing liquid. Cultured cells to a density of about 90%, PBS cleaned 2 times. According to the experiment contents, the cells were inoculated in 6-well plates, concentration was 5×10^4/ml, and placed in the incubator with temperature of 37 and CO_2 concentration of 5% overnight. Transfection was allowed after 24 h, the cell density in general in 70% is appropriate.

Identification of suckling mouse atrial myocytes by immunohistochemistry

To detect the expression of α-SCA protein in cultured suckling mouse atrial myocytes by immunohistochemistry. The steps as follow: (1) 0.1% TritonX-100 incubation; (2) 3% hydrogen peroxide incubation education; (3) serum blocking; (4) first antibody incubation; (5) secondary antibody incubation; (6) Horseradish peroxidase; (7) DAB color rendering; (8) hematoxylin staining; (9) microscopic examination.

Calumenin interference plasmid construction

Sample source status ShRNA oligidic nucleotide fragment; shRNA lentiviral vector plasmid. The following experimental procedures: (1) Experiment product preparation; (2) The oligonucleotide annealing; (3) The plasmid enzyme digestion; (4) The plasmid recombinant; (5) Positive clones screening; (6) Sequencing and identification.

The interference fragment information is as follows:

Name	Target
Calumenin -sh1	ggatggagacctaattgcc
NC	ttctccgaacgtgtcacgt

Calumenin interference lentiviral plasmid transfected the target cells

Prepare virus: Taken out the lentiviral preserved in 4°C and shaken before used gently. Infected target cells: After the virus was ready, the cells were removed from the incubator to observe the cell growth state, if the cell state was well then started the experiment. Used the pipettes absorb a certain amount (the number of virus and cell ratio of 100: 1) of the virus liquid and add into the medium and added ploybrene in the medium (6 g/ml) to improve the efficiency of infection. Sucked original culture medium and added the new culture medium containing virus fluid to lentivirus transfection cells and control cells respectively. Incubated cells were mixed and incubated in 37°C, 5% CO_2. After 24 h, normal culture medium (containing 10% serum DMEM culture medium) replaced containing lentiviral medium, cultured in 37°C, 5% CO_2 incubator.

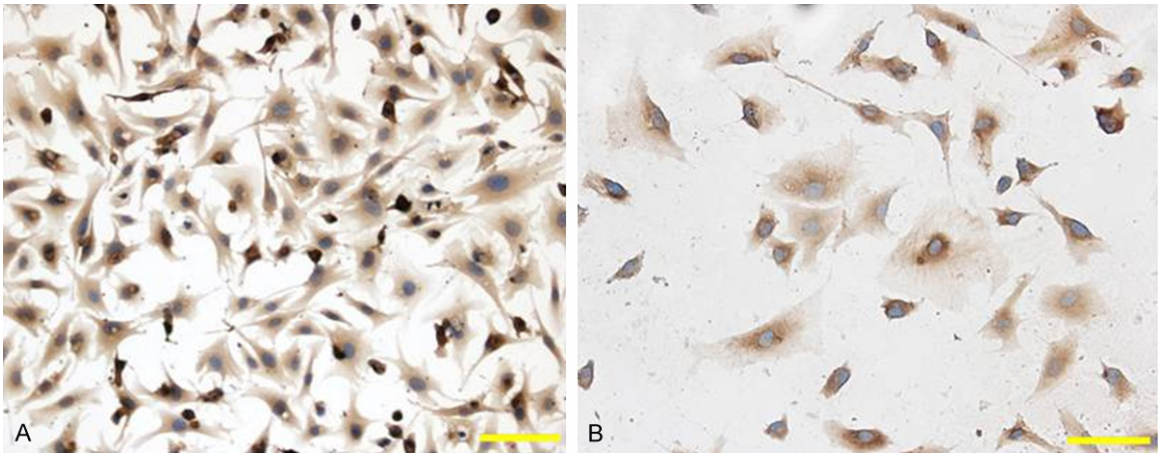

Figure 1. Atrial muscle cells were analyzed by α-SCA protein. A: Culturing atrial muscle cell. B: The immunocytochemistry of culturing atrial muscle cell α-SCA.

Real time PCR detected the expression of calumenin and SK2 mRNA

Suckling mouse atrial myocytes were homogenized in TRIzol reagent and the total RNA was isolated. Total RNA were used to synthesize the first-strand cDNA, according to the Invitrogen manufacurers' instructions. The cDNAs synthesized were then used for quantitative real-time PCR analysis.

The ABI 7300 Detection System (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR using the SYBR Green PCR Master Mix (TianGen), GAPDH served as a reference control and the $2^{-\Delta\Delta ct}$ method was used.

Measurement of calcium concentration

Intracellular Ca^{2+} levels were detected by the Ca^{2+}-sensititive fluorochrome Fluo-3/acetoxymethyl ester (Fluo-3/AM) by fluorescence spectrophotometer. Suckling mouse atrial myocytes were loaded with Fluo-3/AM for 10 min. cells were passed through 340 nm filter. Fluorescence emissions were detected and recorded (Deficiency in TLR4 signal transduction ameliorates cardiac injury and cardiomyocyte contractile dysfunction during ischemia).

Western blot detected the expression of calumenin and SK2 in each group

Each group suckling mouse atrial myocytes experiment following the steps of the experiment. Protein extraction, protein quantification with the BCA method, take 15 μL samples for SDS-PAGE, transferred to a PVDF membrane, closed, Incubated first antibody, Incubated secondary antibody, ECL substrate luminescence, saved the image. Common antibody blocking buffer, resisting incubation liquid and secondary antibody incubation liquid were 5% skim milk powder.

Statistical analyses

Experimental results were shown Means ± SE, using SSPS11.5 statistical analysis software for data analysis, different groups using paired t test.

Results

Immunohistochemical identification of suckling mouse myocardial cells

Suckling mouse cardiac muscle cells were detected by immunohistochemistry experiment, checking specific α-SCA protein, identified as atrial muscle cells (**Figure 1**)

Quantitative real-time PCR detected the expression of calumenin and SK2 mRNA in cardiac myocytes in each group

Quantitative real-time PCR was used to evaluate the expression of calumenin and SK2 mRNA. The mRNA levels of calumenin were decreased in cardiac myocytes with calumenin silence (**Figure 2A**). The mRNA levels of SK2 in cardiac myocytes with calumenin silence were 2.6 times more than SK2 in cardiac myocytes

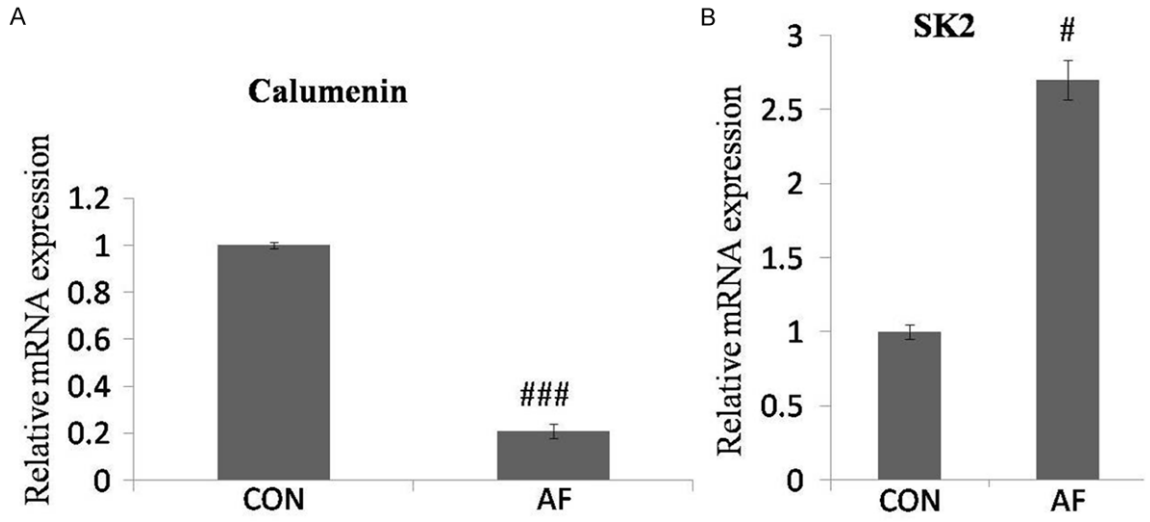

Figure 2. mRNA expression of calumenin and SK2 were analyzed by real-time PCR in atrial myocytes. All data shown as mean ± SE (n = 3 per group). #P<0.5, ###P<0.001.

Table 1. Effect of calumenin on $[Ca^{2+}]i$ in atrial myocytes (n = 6)

	$[Ca^{2+}]i$ (nmol/L)
CON	96.38 ± 2.34
CON + calimenin silence	109.76 ± 1.29**

All data are shown as mean ± SE (n = 6 per group). **P<0.01.

(**Figure 2B**). According to the paired t-test, the mRNA expression levels of SK2 were higher in cardiac myocytes with calumeinin silence than in cardiac myocytes.

Fluorescence spectrophotometer detected calcium concentration

To explore the potential mechanism involved calumenin protein, intracellular Ca^{2+} was evaluated using the fluorescence dye fura-3/AM. Compared with control group, the intracellular Ca^{2+} levels increased in atrial myocytes with calumenin silence (**Table 1**). The results revealed that down-regulated calumenin elevated the intracellular Ca^{2+} levels in atrial myocytes.

Western blotting detected the expression of calumenin and SK2 in cardiac myocytes in each group

Western blotting was applied to measure the atrial myocytes calumenin expression in two groups, in order to verify the lentiviral transfection calumenin silence plasmid was successful or not. The experimental results showed that: Compared with the control group, the calumenin silence model group atrial myocytes calumenin expression was significantly decreased (**Figure 3A**). At the same time, compared with the control group, the calumenin silence model group atrial myocytes SK2 was increased significantly (**Figure 3B**).

Discussion

At present, the main treatment of AF is drug therapy, interventional therapy, surgical treatment, but the effect is not ideal. This is due to the unknown AF mechanism. The current study on the mechanism of atrial fibrillation included "Multiple-wavelet hypothesis" theory, "drive with fibrillation like conduction" theory, "pulmonary venous wave" theory and "atrial fibrillation begets atrial fibrillation". The so-called "atrial fibrillation begets atrial fibrillation" refers to that after the atrial electrical remodeling (AER); AF tends to a state of self sustaining. AER refers to that the atrial muscle ERP shortened, the dispersion increased, the frequency adaptation decline, disappeared or reverse changed caused by repeated AF attacks or continuous electrical stimulation [4, 5]. The occurrence of AF is closely related to the shortening of ERP in AP, and AP is determined by different ion channel activity in cardiac muscle cells. Studies have shown that rapid pacing in primary culture of rat atrial myocytes may lead to the mRNA

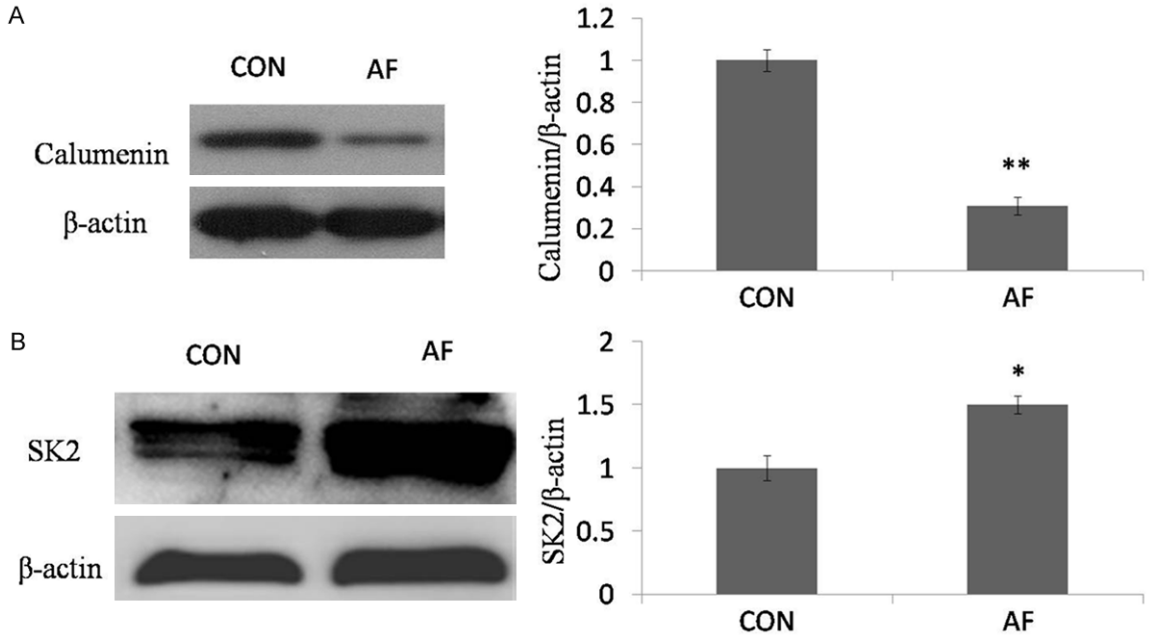

Figure 3. Calumenin regulates proteins expression of SK2 in atrial myocytes. Protein levels of calumenin and SK2 were analyzed from lysates derived. All data are shown as mean ± SE (n = 3 per group). *P<0.5, **P<0.01.

and protein expression of L-type calcium channel and potassium channel decline in different degrees. When AF occurs, because of the expression of L-type calcium channel decreased its density down regulated [6-9]. All of these results suggested that the changes of ion channel function might be related to the occurrence and maintenance of AF.

SK2 channel, consist of α subunit, calmodulin (CAM) and protein kinase CK2 (CK2) and protein phosphatase 2A (PP2A) (10), has the characteristics of K^+ selectivity, Ca^{2+} sensitivity and voltage independence [11, 12]. The expression of SK2 in the atrium is more abundant than ventricle [13]. The role of SK2 channel in the development of AF has become a hot topic in current research. The calumenin is a Ca^{2+} binding protein, which is located in the endoplasmic reticulum/reticulum of mammalian cardiac muscle cells and belongs to the CREC family with multiple EF-hand structures. Ca^{2+}, as the second messenger, transports the extracellular signal to the intracellular and participates in the regulation of a variety of ways. EF-hand protein plays an important role in this process. EF-hand protein, as the intracellular calcium sensor, in different ranges, precisely induces the intracellular calcium concentration change and then transformates these simple regulatory signals into a variety of functional response. We in previous work found that Calumenin maintain calcium homeostasis in cardiac myocytes by the way of combining with the SERCA2a of endoplasmic reticulum in cardiac muscle cells. In VMC mouse, cardiac cavity expanded, contraction function decreased, myocardial calumenin expression decreased, the binding capacity of cardiomyocytes Calumenin and endoplasmic reticulum SERCA2a reduced. Reported in the literature: in myocardial cell, closed calumenin gene could increase the SERCA2 activity, change the affinity of the SERCA2 to Ca^{2+}, increase the amplitude of mouse myocardial cell calcium transient, shorten Ca^{2+} reach peak time and reduce diastolic Ca^{2+} 50% -reduction process.

In this experiment, we observed that SK2 expression increased significantly in silence calumenin atrial myocytes, suggesting that silence calumenin atrial myocytes may cause the intracellular Ca^{2+} concentration significantly increased, the SK2 expression increased. It activated SK2 channels and increased SK2 currents, affected the ERP of AP duration in atrial muscle cells, resulted in atrial fibrillation. As for the silent calumenin atrial myocytes calcium concentration is how to change, as well as its effect on the SK2 current and ERP

of AP, further elucidation is needed in future experiments.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 8136-0587), Natural Science Foundation of Inner Mongolia (No. 2016BS0806) and the Mongolian medicine systems biology science and technology innovation team plan of Inner Mongolia.

Disclosure of conflict of interest

None.

Address correspondence to: Chengxi Wei, Medicinal Chemistry and Pharmacology Institute, Inner Mongolia University for the Nationalities, 22 Holin He Street, Tongliao 028002, Inner Mongolia, P. R. China. Tel: 86-475-8134245; Fax: 86-475-8134245; E-mail: weichengxi1224@163.com; Ming Zhao, First Clinical Medical of Inner Mongolia University for Nationalities, No. 1472 Holin He Street, Tongliao 028002, Inner Mongolia, P. R. China. Tel: 86-475-8267852; Fax: 86-475-8267852; E-mail: langzhe73@163.com

References

[1] American College of Cardiology Foundation; American Heart Association; European Society of Cardiology; Heart Rhythm Society. Management of patients with atrial fibrillation (compilation of 2006 ACCF/AHA/ESC and 2011 ACCF/AHA/HRS recommendations): A report of the American College of Cardiology/American Heart Association Task Force on practice guidelines. Circulation 2013; 127: 1916-1926.

[2] Caballero R, de la Fuente MG, Gómez R, Barana A, Amorós I, Dolz-Gaitón P, Osuna L, Almendral J, Atienza F, Fernández-Avilés F, Pita A, Rodríguez-Roda J, Pinto A, Tamargo J, Delpón E. In humans, chronic atrial fibrillation decreases the transient outward current and ultrarapid component of the delayed rectifier current differentially on each atria and increases the slow component of the delayed rectifier current in both. J Am Coll Cardiol 2010; 55: 2346-2354.

[3] Sahoo SK, Kim DH. Characterization of calumenin in mouse heart. BMB Rep 2010; 43: 158-1563.

[4] Scridon A, Tabib A, Barrès C, Julien C, Chevalier P. Left atrial endocardial fibrosis and intra-atrial thrombosis-landmarks of left atrial remodeling in rats with spontaneous atrial tachyarrhythmias. Morphol Embryol 2013; 54: 405-411.

[5] Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. Circulation 1995; 92: 1954-1968.

[6] Workman AJ, Kane KA, Rankin AC. Cellular bases for human atrial fibrillation. Heart Rhythm 2008; 5 Suppl: S1-6.

[7] Nattel S, Frelin Y, Gaborit N, Louault C, Demolombe S. Ion-channel mRNA-expression profiling: Insights into cardiac remodeling and arrhythmic substrates. Mol Cell Cardiol 2010; 48: 96-105.

[8] Heinzel FR, Bito V, Biesmans L, Wu M, Detre E, von Wegner F, Claus P, Dymarkowski S, Maes F, Bogaert J, Rademakers F, D'hooge J, Sipido K. Remodeling of T-tubules and reduced synchrony of Ca2+ release in myocytes from chronically ischemic myocardium. Circ Res 2008; 102: 338-346.

[9] Louch WE, Bito V, Heinzel FR, Macianskiene R, Vanhaecke J, Flameng W, Mubagwa K, Sipido KR. Reduced synchrony of Ca2+ release with loss of T-tubules-a comparison to Ca2+ release in human failing cardiomyocytes. Cardiovasc Res 2004; 62: 63-73.

[10] Allen D, Fakler B, Maylie J, Adelman JP. Organization and regulation of small conductance Ca2+-activated K+ channel multiprotein complexes. Neurosci 2007; 27: 2369-2376.

[11] Bildl W, Strassmaier T, Thrum H. Protein kinase CK2 is coassembled with small conductance Ca^{2+}-activated K+ channelsl and regulates channel gating. Neuron 2004; 43: 847-858.

[12] Allen D, Fakler B, Maylie J, Adelman JP. Organization and regulation of small conductance Ca2+-activated K+ channels multiprotein complexes. Neuroscience 2007; 27: 2369-2376.

[13] Tuteja D, Xu D, Timofeyev V, Lu L, Sharma D, Zhang Z, Xu Y, Nie L, Vázquez AE, Young JN, Glatter KA, Chiamvimonvat N. Differential expression of small-conductance Ca2+-activated K+ channels SK1, SK2, and SK3 in mouse atrial and ventricular myocytes. Physiol Heart Circ Physiol 2005; 289: H2714-H2723.