Calcium-activated potassium channel family in coronary artery bypass grafts

Wen-Tao Sun, PhD,[a] Hai-Tao Hou, MPhil,[a] Huan-Xin Chen, MPhil,[a] Hong-Mei Xue, PhD,[a] Jun Wang, MPhil,[a] Guo-Wei He, MD, PhD, DSc,[a,b,c] and Qin Yang, MD, PhD[a]

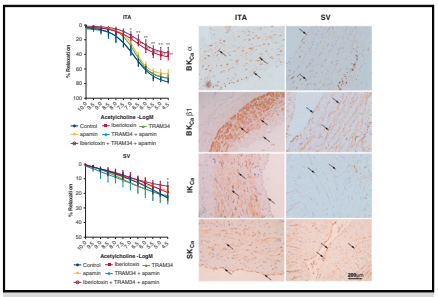
ABSTRACT

Objectives: We examined the expression, distribution, and contribution to vasodilatation of the calcium-activated potassium (K_{Ca}) channel family in the commonly used coronary artery bypass graft internal thoracic artery (ITA) and saphenous vein (SV) to understand the role of large conductance K_{Ca} (BK_{Ca}), intermediate-conductance K_{Ca} (IK_{Ca}), and small-conductance K_{Ca} (SK_{Ca}) channel subtypes in graft dilating properties determined by endothelium-smooth muscle interaction that is essential to the postoperative performance of the graft.

Methods: Real-time polymerase chain reaction and western blot were employed to detect the messenger RNA and protein level of K_{Ca} channel subtypes. Distribution of K_{Ca} channel subtypes was examined by immunohistochemistry. K_{Ca} subtype-mediated vasorelaxation was studied using wire myography.

Results: Both ITA and SV express all K_{Ca} channel subtypes with each subtype distributed in both endothelium and smooth muscle. ITA and SV do not differ in the overall expression level of each K_{Ca} channel subtype, corresponding to comparable relaxant responses to respective subtype activators. In ITA, BK_{Ca} is more abundantly expressed in smooth muscle than in endothelium, whereas SK_{Ca} exhibits more abundance in the endothelium. In comparison, SV shows even distribution of K_{Ca} channel subtypes in the 2 layers. The BK_{Ca} subtype in the K_{Ca} family plays a significant role in vasodilatation of ITA, whereas its contribution in SV is quite limited.

Conclusions: K_{Ca} family is abundantly expressed in ITA and SV. There are differences between these 2 grafts in the abundance of K_{Ca} channel subtypes in the endothelium and the smooth muscle. The significance of the BK_{Ca} subtype in vasodilatation of ITA may suggest the potential of development of BK_{Ca} modulators for the prevention and treatment of ITA spasm during/after coronary artery bypass graft surgery. (J Thorac Cardiovasc Surg 2021;161:e399-409)



Differences in the distribution and functionality of K_{Ca} channels between ITA and SV.

CENTRAL MESSAGE

All calcium-activated potassium channel subtypes are distributed in the endothelial and smooth muscle layers of internal thoracic artery and saphenous vein. Internal thoracic artery and saphenous vein differ in calcium-activated potassium channel subtype abundance in the 2 layers. Large-conductance calcium-activated potassium channels play a critical role in internal thoracic artery dilatation.

PERSPECTIVE

By revealing the distribution profile and physiological role of the calcium-activated potassium channel family in internal thoracic artery and saphenous vein, this study provided new mechanistic insight into the intrinsic biological differences between the arterial and venous coronary artery bypass graft that may influence the postoperative graft function and suggested the potential of development of large-conductance calcium-activated potassium channel modulators for the prevention and treatment of internal thoracic artery spasm during/after coronary artery bypass graft surgery.

See Commentaries on pages e411 and e412.

From the [a]Center for Basic Medical Research, & Department of Cardiovascular Surgery, TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China; [b]School of Pharmacy, Wannan Medical College, Wuhu, Anhui, China; and [c]Department of Surgery, Oregon Health and Science University, Portland, Ore.

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Address for reprints: Qin Yang, MD, PhD, TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 61, 3rd Avenue, TEDA, Tianjin, China (E-mail: qyanghk@163.com).

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Abbreviations and Acronyms

BK_{Ca} = Large-conductance calcium-activated potassium
CABG = coronary artery bypass graft
EC_{50} = half maximal effective concentration
EDHF = endothelium-derived hyperpolarizing factor
EDRF = endothelium-derived relaxing factor
H-score = histoscore
IK_{Ca} = intermediate-conductance calcium-activated potassium
ITA = internal thoracic artery
K_{Ca} = calcium-activated potassium
mRNA = messenger RNA
NO = nitric oxide
Q-PCR = quantitative polymerase chain reaction
R_{max} = Maximal response
SK_{Ca} = small-conductance calcium-activated potassium
SV = saphenous vein

▶ Video clip is available online.

Internal thoracic artery (ITA) and saphenous vein (SV) are the most commonly used coronary artery bypass grafts (CABG) for the treatment of ischemic heart disease. ITA is superior to SV in long-term patency, although it has the tendency to develop spasm during surgical dissection and the perioperative period. In addition to the intrinsic structure difference, ITA and SV are known to be different in native matrix metalloproteinase characteristics[1] and endothelial function. Previous studies from our group have demonstrated that compared with SV, the ITA produces more nitric oxide (NO) and shows greater biofunctionality of endothelium-derived hyperpolarizing factor (EDHF).[2] These divergent properties are believed to underlie the differences between ITA and SV in postoperative graft function and long-term patency rates.

Calcium-activated potassium (K_{Ca}) channels, including large-conductance K_{Ca} (BK_{Ca}), intermediate-conductance K_{Ca} (IK_{ca}), and small-conductance K_{Ca} (SK_{Ca}), are involved in the regulation of vascular tone. Being composed of a symmetrical tetramer containing four pore-forming α subunits and 4 regulatory β subunits,[3] BK_{Ca} channels in the vasculature were reported to be mainly distributed in the plasma membrane of smooth muscle cells,[4] whereas the existence and function of BK_{Ca} in the vascular endothelium remains controversial.[5,6] In comparison, SK_{Ca} and IK_{Ca} channels were found to be predominantly expressed in endothelial cells in various vasculatures,[7-9] with sporadic reports of function and expression in the smooth muscle.[10,11] Opening of BK_{Ca} channels in smooth muscle cells causes hyperpolarization that limits calcium ion entry through voltage-activated calcium ion channels, providing a negative-feedback mechanism opposing vasoconstriction.[12] BK_{Ca} channels act as a smooth muscle effector of endothelium-derived relaxing factors (EDRF),[13,14] in particular NO. Numerous studies have demonstrated that activation of endothelial IK_{Ca} ($K_{Ca}3.1$) and SK_{Ca} ($K_{Ca}2.3$) underlies the classical EDHF pathway,[15] and these channels are also involved in the regulation of NO bioavailability.[16]

Despite extensive research in animal vasculatures, studies regarding the role of K_{Ca} channels in human vessels are limited. It was reported that BK_{Ca} channels are expressed in smooth muscle cells of human coronary arteries and mediate coronary artery dilation.[17] Modulation of endothelial SK_{Ca} and IK_{Ca} channels were found to take part in coronary arteriolar dysfunction in pathological/diseased conditions such as laminar shear stress, ischemia-reperfusion, and diabetes.[18-20] Expressions or function of K_{Ca} channels were also demonstrated in human mesenteric,[7] gastroepiploic,[21] and pulmonary arteries.[22] Nevertheless, these studies either focused on specific K_{Ca} subtype or investigated the overall function of K_{Ca} channels, none of them looked into the distribution profile and the respective contribution to vasorelaxation made by K_{Ca} subtypes.

There has been a certain amount of studies concerning the effects of chemically synthesized compounds and natural substances on CABG. By using pharmacological tools, some studies, including those from our group, have identified the functional role of K_{Ca} channels in human ITA. For example, blockade of K_{Ca} channels with tetraethylammonium enhanced the contractile effect of endogenous vasopressin.[23] Relaxation induced by propofol and flavanol compound (–)–epicatechin was significantly inhibited by the selective BK_{Ca} blocker iberiotoxin in ITA.[24,25] Procyanidin B2-induced relaxation of ITA was slightly reduced by the IK_{Ca} inhibitor TRAM-34, whereas almost abolished by iberiotoxin.[26] Few studies investigated the role of K_{Ca} channels in the venous graft SV. Zhang and colleagues[27] reported that the total patch current was significantly suppressed by tetraethylammonium and iberiotoxin in smooth muscle cells isolated from human SV.[27] So far, no efforts have been made to compare the localization and function of different subtypes of K_{Ca} channels between ITA and SV.

The present study aimed to examine the expression, localization, and the respective contribution to relaxation

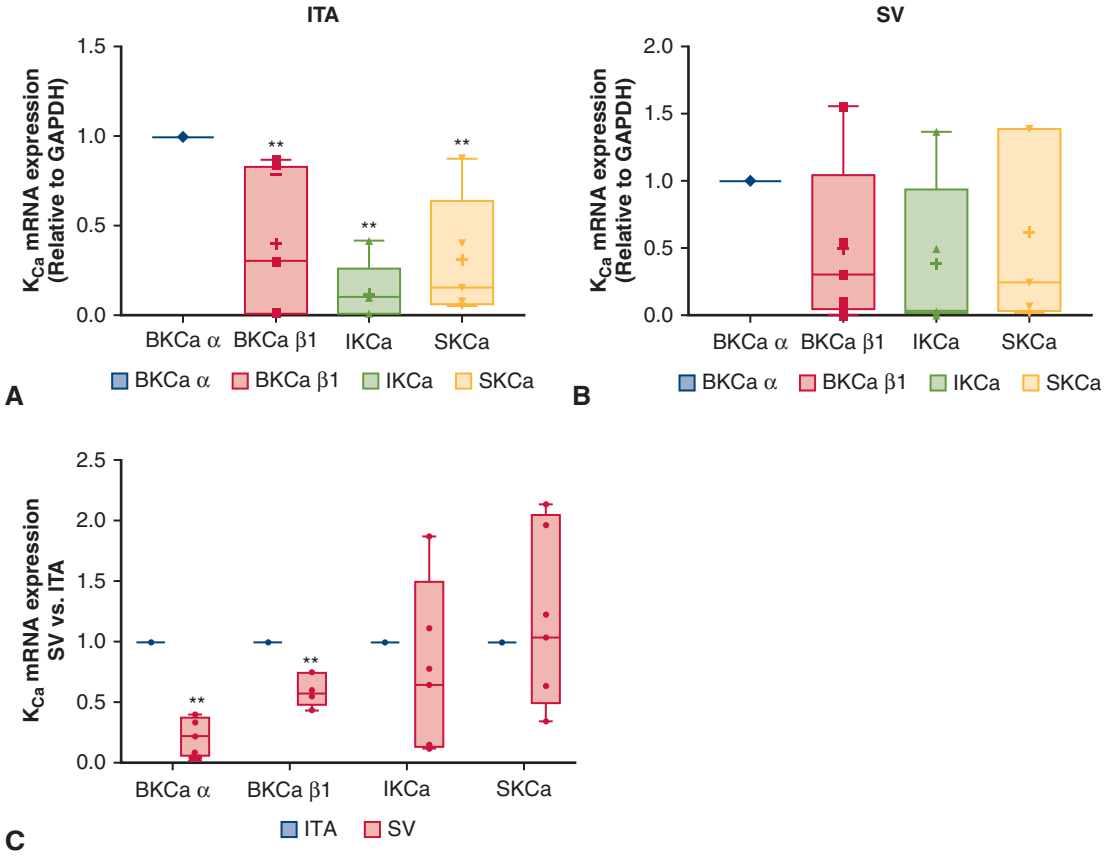

FIGURE 1. Comparison of the messenger RNA (*mRNA*) expression level of calcium-activated potassium (K_{Ca}) channels, including large-conductance K_{Ca} (BK_{Ca}) (composed of α and β1 subunits), intermediate-conductance K_{Ca} (IK_{Ca}), and small-conductance K_{Ca} (SK_{Ca}) channels. A, In the internal thoracic artery (*ITA*). B, In the saphenous vein (*SV*). C, Comparison between ITA and SV with respect to the mRNA expression level of each K_{Ca} channel subtype. Both ITA and SV express all 3 subtypes of K_{Ca} channels. The ITA shows more mRNA abundance of the BK_{Ca} subtype than the IK_{Ca} and SK_{Ca} subtypes, whereas in the SV the mRNA expression level of the 3 subtypes are of no statistical difference. At the mRNA level, ITA expresses more BK_{Ca} subtype than SV. Data are representative of 5 independent experiments. In each experiment, the ITA and the SV samples were taken from the same patient. A and B, Values are expressed as fold-difference relative to $BK_{Ca}\alpha$ for comparison within the same graft. C, Values are expressed relative to corresponding K_{Ca} subtype in the ITA for comparison between ITA and SV. *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase. $^{**}P < .01$.

of K_{Ca} channel subtypes in both ITA and SV. By using multiple methods, including western blot, real-time polymerase chain reaction (Q-PCR), immunohistochemistry, and myograph recording, we were able to clarify the differences between the arterial and venous grafts regarding the distribution profile and physiological role of the K_{Ca} family.

MATERIALS AND METHODS

ITA (n = 50) and SV (n = 50) grafts were harvested from patients undergoing CABG surgery. The residual segments of ITA and SV that would otherwise be discarded were collected with the consent of the patients. The study protocol was approved by the Institutional Ethics Review Board of TEDA International Cardiovascular Hospital (No. [2018]-0626-2).

Q-PCR

Total RNA was extracted from ITA and SV segments using TRIzol reagent (ThermoFisher Scientific, Irvine, Calif) and reverse transcription and Q-PCR amplification were performed in LightCycler 96 (Roche, Basel, Switzerland) employing TransScript Green Two-Step qRT-PCR SuperMix system (Transgen, Beijing, China). The details of PCR cycle conditions, primers used for amplification of $BK_{Ca}\alpha$, $BK_{Ca}\beta1$, $K_{Ca}2.3$, and $K_{Ca}3.1$, and quantification methods are described in Appendix E1.

Western Blot

Whole-cell protein of ITA and SV segments were extracted and the proteins of interest, including $BK_{Ca}\alpha$, $BK_{Ca}\beta1$, $K_{Ca}2.3$, and $K_{Ca}3.1$ were detected using specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies, as described elsewhere.[28,29] The details of the procedure are described in Appendix E1.

Histology and Immunohistochemistry Staining

ITA and SV segments were fixed in 4% paraformaldehyde, decalcified, dehydrated, and embedded in paraffin. The embedded tissue samples were sliced into 4- to 5-μm sections and stained with hematoxylin and eosin after deparaffination. Immunohistochemistry staining was performed with the primary antibody against $BK_{Ca}\alpha$, $BK_{Ca}\beta1$, $K_{Ca}2.3$, or $K_{Ca}3.1$, as described in Appendix E1.

Immunostaining was evaluated by 2 independent pathologists using a blind protocol design. For each specimen, the histoscore (H-score) of

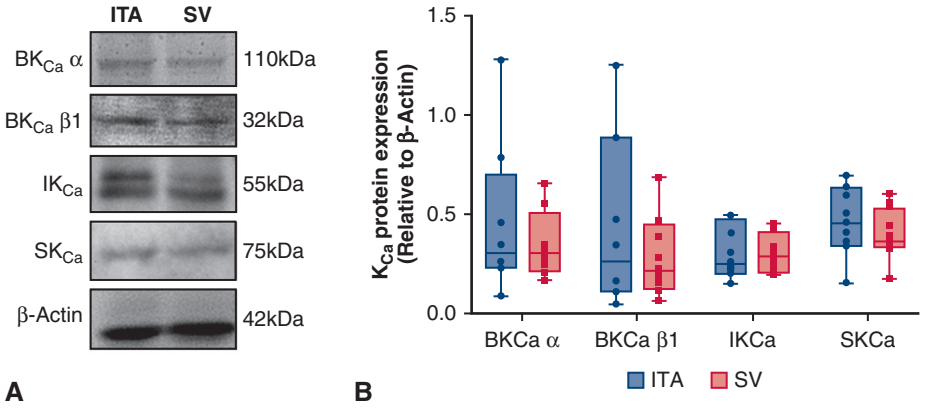

FIGURE 2. The protein expression of calcium-activated potassium (K_{Ca}) channel subtypes in internal thoracic artery (*ITA*) and saphenous vein (*SV*). A, Representative blots of K_{Ca} channel subtypes in ITA and SV, including large-conductance K_{Ca} (BK_{Ca}) (composed of α and $\beta 1$ subunits), intermediate-conductance K_{Ca} (IK_{Ca}), and small-conductance K_{Ca} (SK_{Ca}). B, Protein expression levels of K_{Ca} channel subtypes obtained from 8 independent experiments, each employing ITA and SV samples from the same patient. No significant differences were observed in the overall protein expression level of each K_{Ca} subtype between ITA and SV.

each K_{Ca} channel subtype was calculated as the sum of staining intensity (negative staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3) multiplied by the percentage of stained cells (0%-100%).

Isometric Force Study

ITA and SV segments were cut into 3-mm rings and mounted in a 4-channel Mulvany myograph (Model 620M; J. P. Trading, Aarhus, Denmark). The details of myograph experiment have been extensively published in our previous studies.[28-30] Cumulative dose–response curves to BK_{Ca} activator NS1619 (–9 to –4.5 LogM), IK_{Ca}/SK_{Ca} activator NS309 (–9 to –4.5 LogM), and acetylcholine (–10 to –4.5 LogM) were established in ITA and SV rings precontracted by U46619. In the study of acetylcholine-induced relaxation, vessels were incubated with the specific blocker for BK_{Ca} (100 nmol/L iberiotoxin), IK_{Ca} (1 μmol/L TRAM34), or SK_{Ca} (100 nmol/L apamin) before U46619 precontraction. Relaxant responses of ITA and SV to acetylcholine were also studied after combined application of TRAM34 and apamin, or TRAM34 and apamin plus iberiotoxin.

Statistical Analysis

Expression of targets of interest was normalized to the expression of β-actin for protein analysis, and to the level of glyceraldehyde 3-phosphate dehydrogenase for messenger RNA (mRNA) analysis. Relaxation was expressed as the percentage decrease in isometric force induced by U46619. Data were expressed as mean ± standard error of the mean. One-way analysis of variance was used to assess differences among groups followed by Scheffe post hoc test (SPSS version 20; IBM-SPSS Inc, Armonk, NY). When 2 groups were compared, differences were assessed by Student *t* test.

RESULTS

K_{Ca} Channel Expression in ITA and SV

mRNA expression of K_{Ca} channel subtypes: ITA versus SV. Q-PCR experiments enrolling 5 pairs of ITA and SV (each pair from the same individual) showed that all 3 subtypes of K_{Ca} channels are expressed in both the arterial and venous grafts. Further analysis indicated that the mRNA level of the BK_{Ca} subtype is significantly higher than that of IK_{Ca} and SK_{Ca} in ITA (Figure 1, *A*), whereas in SV, the mRNA expressions of the 3 K_{Ca} channel subtypes are of no statistical difference (Figure 1, *B*). In comparison with ITA, SV expresses less BK_{Ca} subtype, shown by lower mRNA levels of α and $\beta 1$ subunits of BK_{Ca} (Figure 1, *C*).

Protein expression of K_{Ca} channel subtypes: ITA versus SV. Protein detection using 8 pairs of ITA and SV further demonstrated the endogenous expression of the K_{Ca} family in these grafts (Figure 2, *A* and *B*). The protein levels of BK_{Ca}, IK_{Ca}, and SK_{Ca} were not significantly different from each other in ITA, which was inconsistent with the results from Q-PCR showing significant higher mRNA expression of BK_{Ca}, suggesting a significant translational control of K_{Ca} expression in this arterial graft. In comparison, the protein expression levels of the 3 K_{Ca} subtypes in SV was in line with the mRNA expression pattern, showing no differences in the protein content of BK_{Ca}, IK_{Ca}, and SK_{Ca}. Further comparison between ITA and SV showed a relatively lower protein level of BK_{Ca} in SV, whereas the difference was statistically insignificant.

Distribution Profile of K_{Ca} Channel Subtypes in ITA and SV

Immunohistochemistry staining revealed the localization of K_{Ca} family in ITA and SV, showing the distribution of all 3 subtypes of K_{Ca} in both endothelium and smooth muscle layers (Figure 3, *A*). H-score calculated from 5 independent pairs of ITA and SV samples indicated no significant differences in the overall staining of each K_{Ca} subtype (Figure 3, *B*), which was in agreement with the results of western blot experiment showing comparable protein level of each K_{Ca} subtype in the 2 grafts (Figure 2, *B*). Further analysis of the distribution of each K_{Ca} subtype in the endothelial and the smooth muscle layer showed that in ITA, BK_{Ca} is more abundantly expressed in smooth muscle

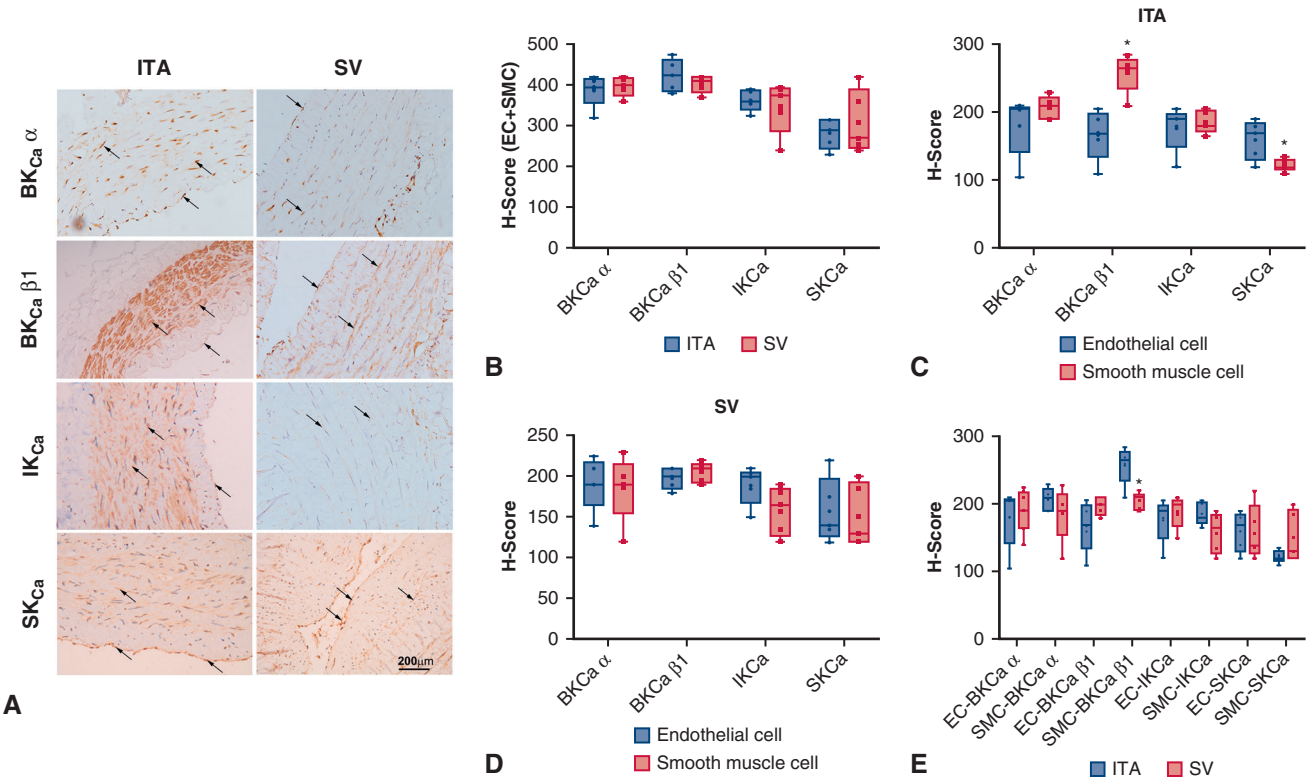

FIGURE 3. Distribution of calcium-activated potassium (K_{Ca}) channel subtypes in internal thoracic artery (*ITA*) and saphenous vein (*SV*). A, Immunohistochemistry images showing the distribution of K_{Ca} channel subtypes, including large-conductance K_{Ca} (BK_{Ca}) (composed of α and β1 subunits), intermediate-conductance K_{Ca} (IK_{Ca}), and small-conductance K_{Ca} (SK_{Ca}) in both the endothelium and smooth muscle layers in ITA and SV. Positive staining of specific K_{Ca} channel subtype is indicated by *black arrows*. B, Comparison between ITA and SV with respect to the overall histoscore (H-score) of each K_{Ca} channel subtype in both endothelial (*EC*) and smooth muscle cells (*SMC*), which shows no significant differences. C and D, Comparison of the H-score of each K_{Ca} subtype between the EC layer and the SMC layer in ITA and SV. BK_{Ca} is more abundantly expressed in SMC than in EC in the ITA, whereas SK_{Ca} exhibits more abundance in the EC layer. D, SV exhibited no significant differences in the K_{Ca} distribution in the EC and the SMC layers. E, Comparison between ITA and SV of the H-score of each K_{Ca} channel subtype in ECs and SMCs, respectively, shows a significant lower expression of BK_{Ca} β1 in the SMC layer of the SV. Data were obtained from 5 independent experiments, each employing ITA and SV samples from the same patient. *$P < .05$.

cells than in endothelial cells ($P = .018$), whereas SK_{Ca} exhibits more abundance in the endothelial layer ($P = .020$) (Figure 3, *C*). Different from ITA, SV exhibited no significant differences in the K_{Ca} distribution in the endothelial and the smooth muscle layer, though the abundance of IK_{Ca} tends to be slightly more in the endothelium than in the smooth muscle ($P = .058$) (Figure 3, *D*). Comparison between ITA and SV showed a significantly lower H-score of BK_{Ca} β1 in the smooth muscle layer of the SV ($P = .021$) (Figure 3, *E*).

Role of K_{Ca} Channel Subtypes in the Vasoactivity of ITA and SV

Relaxant response of ITA and SV to K_{Ca} channel openers. The BK_{Ca} channel agonist NS1619 and the IK_{Ca}/SK_{Ca} channel opener NS309 elicited dose–dependent relaxations in both ITA and SV. Relaxant responses of ITA and SV to NS1619 were similar in terms of the maximal response (51.0% ± 3.1% and 56.4% ± 8.6%, respectively) (Figure 4, *A*). The half maximal effective concentration (EC_{50}) value for NS1619 in ITA was –5.45 ± 0.11 LogM and –5.84 ± 0.08 LogM in SV ($P = .033$). The maximal response (R_{max}) and sensitivity to NS309 did not differ between ITA and SV (R_{max}, 79.0% ± 6.7% vs 75.0% ± 7.3%; EC_{50}, –5.38 ± 0.04 vs –5.35 ± 0.08 LogM) (Figure 4, *B*).

K_{Ca}-mediated relaxation in response to acetylcholine in ITA and SV. The contribution of K_{Ca} subtypes in the relaxant response of ITA and SV to acetylcholine was further studied with the use of specific K_{Ca} blockers. Acetylcholine induced dose–dependent relaxation in both grafts, with R_{max} of 76.2% ± 3.0% in ITA and 23.2% ± 1.5% in SV (Figure 4, *C* and *D*). Application of the BK_{Ca} blocker iberiotoxin significantly decreased acetylcholine-induced relaxation in ITA from 76.2% ± 3.0% to 41.9% ± 5.4% ($P < .01$). In comparison, inhibition of IK_{Ca} with TRAM-34 or inhibition of SK_{Ca} with apamin did not significantly suppress acetylcholine-

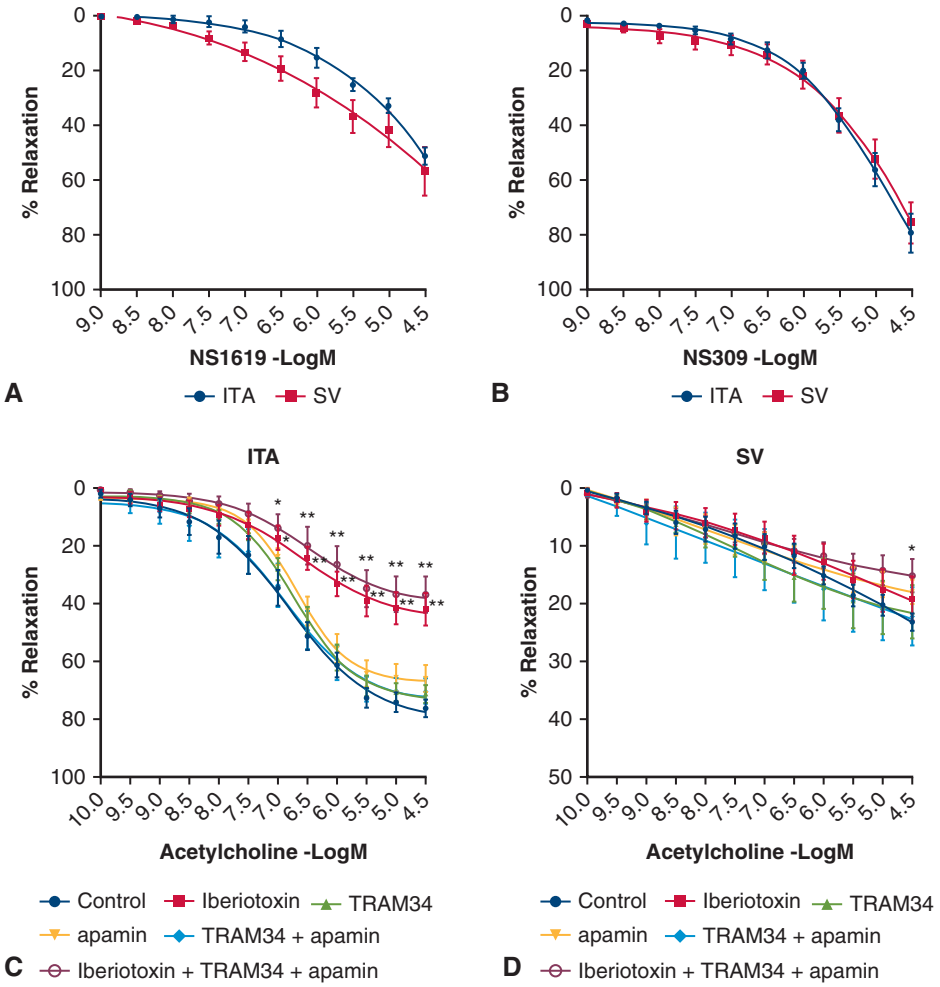

FIGURE 4. Vasorelaxant response mediated by calcium-activated potassium (K_{Ca}) channels in internal thoracic artery (*ITA*) and saphenous vein (*SV*). A and B, Cumulative concentration-response curves of ITA and SV to the large-conductance K_{Ca} (BK_{Ca}) channel opener NS1619 and the intermediate/small-conductance K_{Ca} (IK_{Ca}/SK_{Ca}) channel opener NS309. C and D, Role of K_{Ca} subtypes in ITA and SV in acetylcholine-induced relaxation. The relaxant response to acetylcholine was studied in ITA and SV with or without pretreatment with selective channel blockers either individually or in combined use. BK_{Ca} subtype plays a predominant role in mediating the relaxant response of ITA to acetylcholine, whereas its role in SV is minor. IK_{Ca} and SK_{Ca} channel subtypes are unessential to the relaxation induced by acetylcholine in both ITA and SV. For each group, data represents 8 independent experiments. *$P < .05$ versus control. **$P < .01$ versus control. *Iberiotoxin*, Large-conductance calcium-activated potassium (BK_{Ca}) channel blocker; *TRAM-34*, intermediate-conductance calcium-activated potassium (IK_{Ca}) channel blocker; *apamin*, small-conductance calcium-activated potassium (SK_{Ca}) channel blocker.

induced relaxation in ITA, although minor decreases were observed. Except a slight decrease in the maximal response, 71.2% ± 3.0% in the TRAM-treated group and 66.0% ± 4.6% in the apamin-treated group, inhibition of IK_{Ca} and SK_{Ca} tended to blunt the response of ITA to acetylcholine at the low concentration range (Figure 4, *C*). Application of TRAM-34 in conjunction with apamin showed no further inhibition on relaxation compared with the use of TRAM-34 or apamin alone. Further combination of TRAM-34, apamin, and iberiotoxin resulted in a dramatic attenuation in acetylcholine-induced relaxation (36.9% ± 6.1%), which was only slightly less than the relaxation in the ITA treated with iberiotoxin alone (Figure 4, *C*), suggesting the predominant role of BK_{Ca} subtype in the K_{Ca} family in mediating the relaxant response of ITA to endogenous vasodilators.

Compared with ITA, SV showed significantly less relaxant response to acetylcholine (23.2% ± 1.5% vs 76.2% ± 3.0%; $P < .001$). Blockade of different K_{Ca} subtype, respectively, caused no obvious inhibition on acetylcholine-induced relaxation in SV. Even triple application of BK_{Ca}, IK_{Ca}, and SK_{Ca} blockers showed quite limited inhibitory effect on the relaxant response, with significant suppression only observed at the highest concentration of acetylcholine (15.2% ± 2.8% vs 23.2% ± 1.5% in the control group; $P = .02$).

Treatment with respective K_{Ca} blockers individually did not significantly affect the resting force and

TABLE 1. Resting force and U46619-induced contraction of internal thoracic artery (ITA) and saphenous vein (SV)

Group	Resting force (mN)		U Contraction (mN)	
	ITA	SV	ITA	SV
Control	16.3 ± 1.3	5.0 ± 0.6	53.3 ± 4.7	29.4 ± 4.4
Iberiotoxin	11.7 ± 2.2	6.2 ± 1.0	66.9 ± 10.7	21.4 ± 6.3
TRAM34	14.5 ± 3.7	4.9 ± 0.5	38.4 ± 7.0	21.3 ± 4.8
Apamin	17.5 ± 3.1	4.0 ± 0.9	49.3 ± 6.7	20.4 ± 2.9
TRAM34 + apamin	12.0 ± 1.8	4.8 ± 0.9	47.5 ± 9.5	18.8 ± 2.9
Iberiotoxin + TRAM34 + apamin	18.1 ± 2.5	6.0 ± 2.1	76.3 ± 10.2*	27.5 ± 6.5

Iberiotoxin, Large-conductance calcium-activated potassium (BK_{Ca}) channel blocker; *TRAM-34*, intermediate-conductance calcium-activated potassium (IK_{Ca}) channel blocker; *apamin*, small-conductance calcium-activated potassium (SK_{Ca}) channel blocker. *$P < .05$ versus control.

U46619-precontraction of ITA and SV except that in the ITA, combined use of BK_{Ca}, IK_{Ca}, and SK_{Ca} blockers enhanced U46619-induced precontraction (Table 1). The sensitivity of ITA and SV to acetylcholine was barely influenced by the K_{Ca} blockers, as indicated by the unaltered EC_{50} values (Table 2).

DISCUSSION

In this study, by using Q-PCR, western blot, and immunohistochemistry for localization and quantification of K_{Ca} subtypes, and isometric force measurement for functional investigation, we for the first time compared the distribution profile and contribution to vasodilatation of the members of K_{Ca} family between ITA and SV (Video 1). Results derived from this study demonstrated that both ITA and SV express all 3 subtypes of K_{Ca} channels, which are distributed in both endothelial and smooth muscle layers. ITA does not differ from SV in the overall expression level of each K_{Ca} channel subtype, ITA and SV show differences in the abundance of K_{Ca} channel subtypes in endothelial and smooth muscle layers, and the BK_{Ca} subtype in the K_{Ca} family plays a significant role in endothelium-dependent vasodilatation of ITA, whereas its contribution in SV is quite limited. Figure 5 provides a schematic summary of the present study.

TABLE 2. Half maximal effective concentration (EC_{50}) values for acetylcholine in internal thoracic artery (ITA) and saphenous vein (SV) with or without pretreatment with different calcium-activated potassium (K_{Ca}) channel blockers

Group	EC_{50} (–LogM)	
	ITA	SV
Control	6.97 ± 0.19	6.73 ± 0.29
Iberiotoxin	7.02 ± 0.25	7.21 ± 0.38
TRAM34	6.73 ± 0.08	7.13 ± 0.25
Apamin	6.67 ± 0.08	7.35 ± 0.31
TRAM34 + apamin	6.99 ± 0.21	7.13 ± 0.34
Iberiotoxin + TRAM34 + apamin	6.45 ± 0.21	7.21 ± 0.31

Iberiotoxin, Large-conductance calcium-activated potassium (BK_{Ca}) channel blocker; *TRAM-34*, intermediate-conductance calcium-activated potassium (IK_{Ca}) channel blocker; *apamin*, small-conductance calcium-activated potassium (SK_{Ca}) channel blocker.

As the commonly used CABG, the ITA and SV are obviously different in anatomic structure and hemodynamic characteristics. In addition, they are different in the response to constrictor and dilator agents. For example, the inotropic/vasodilator compound levosimendan relaxed ITA but failed to relax SV completely.[31] Prostaglandin E_2 induced vasoconstriction in ITA whereas vasodilatation in SV, which was attributed to the mediation of different prostaglandin E_2 receptors.[32] Compared with SV, ITA exhibited greater endothelium-dependent relaxation,[33] which was again demonstrated in the present study. The EDRF stimulus acetylcholine evoked approximately 80% relaxation in ITA but <25% relaxant response in SV. Our previous efforts to understand the differences between ITA and SV in the degree of endothelium-dependent relaxation revealed that ITA releases more NO and exhibits more hyperpolarization than SV to EDRF stimuli.[2] In this study, with a further attempt to dissect the molecular basis of such intrinsic differences in endothelial function between the 2 grafts, we studied and compared the expression and function of the K_{Ca} channel family, which has been suggested to largely take part in the generation/action of EDRF, including EDHF and NO.[8,13,16,34]

To gain more precise knowledge about whether K_{Ca} channels differ between ITA and SV in the expression and distribution, Q-PCR, western blot, and immunohistochemistry experiments were performed using paired grafts, namely, each pair of ITA and SV samples was taken from the same patient. This ruled out the influence of clinical characteristics of different patients on the K_{Ca} channels and thus enabled the revealing of the intrinsic differences, if any, in this channel family between ITA and SV. Our results suggest that ITA and SV do not differ from each other with respect to the overall expression levels of the members of the K_{Ca} family, evidenced by the comparable protein level and H-score of each K_{Ca} channel subtype in these 2 grafts. At the mRNA level, ITA was found to express more BK_{Ca} subtype than SV. The inconsistent mRNA and protein data may suggest that ITA differs from SV in the

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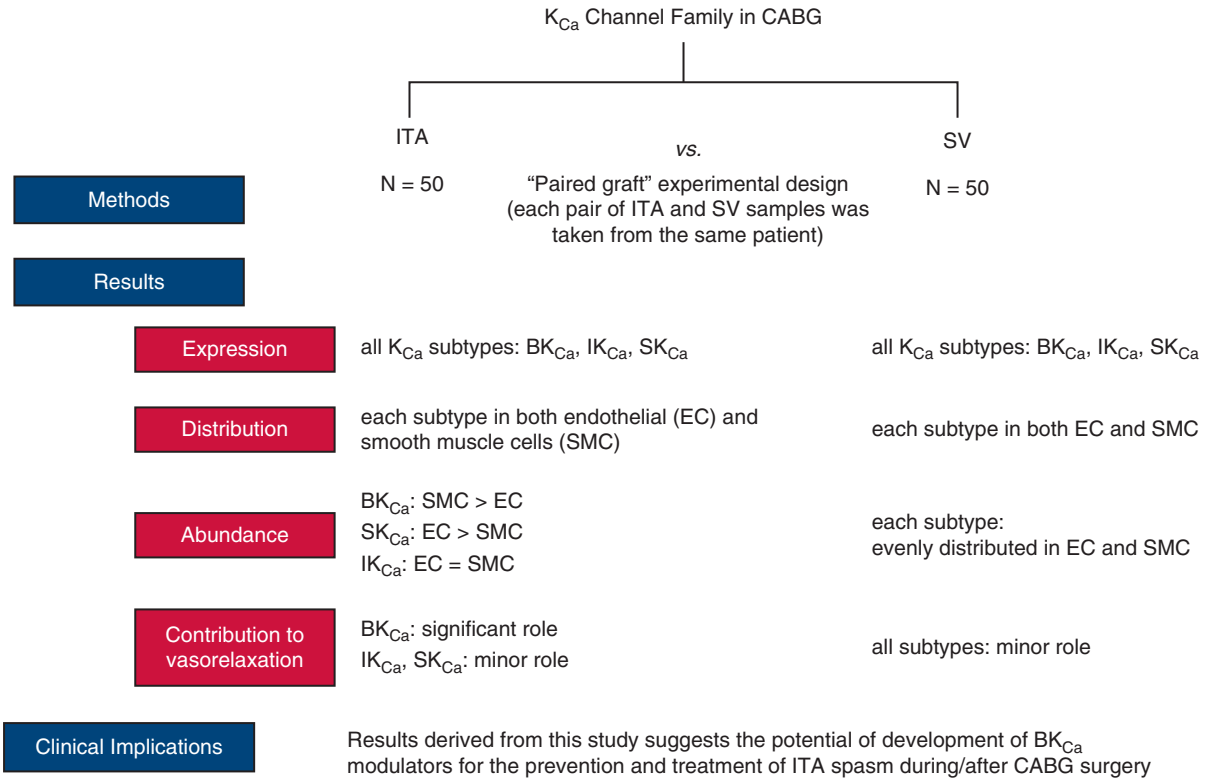
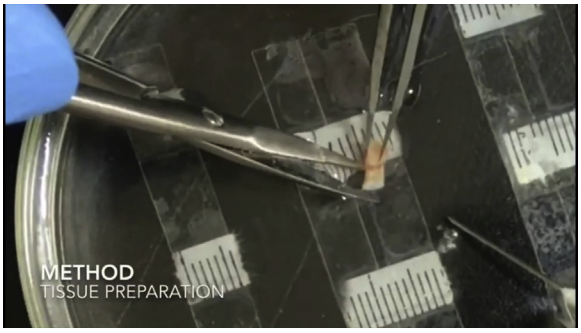


FIGURE 5. Schematic summary of the expression and distribution profile of calcium-activated potassium (*K_{Ca}*) channel family in coronary artery bypass grafts (*CABG*) and their role in the dilatory function of the grafts. *BK_{Ca}*, Large-conductance K_{Ca}; *IK_{Ca}*, intermediate-conductance K_{Ca}; *SK_{Ca}*, small-conductance K_{Ca}; *EC*, endothelial cell, *SMC*, smooth muscle cell.

translational control of BK_{Ca} expression. Whether this is due to the differences in oxygen tension and shear stress in the arterial and venous system is a topic for future investigation. The higher BK_{Ca} mRNA content in ITA may serve as a reservoir for maintaining or repairing BK_{Ca} channel function in the arterial graft in pathological conditions. The comparable expression of K_{Ca} subtypes in ITA and SV was also supported by the similar degree of responses of the 2 grafts to the BK_{Ca} opener NS1619 and the IK_{Ca}/SK_{Ca} opener NS309.

Immunohistochemistry experiments showed that in ITA and SV, IK_{Ca}, SK_{Ca}, and BK_{Ca} channel subtypes are localized in both endothelial and smooth muscle cells and they present in similar abundance in these 2 grafts, as suggested by western blot and H-score analysis. Although ITA and SV share similar expression and localization profile for K_{Ca} channels, they are different in the distribution abundance of K_{Ca} channel subtypes. In ITA, BK_{Ca} is more abundantly expressed in smooth muscle cells than in endothelial cells, whereas SK_{Ca} exhibits more abundance in the endothelial layer, which is in accordance with the finding of BK_{Ca} and SK_{Ca} distribution in various vasculatures. In comparison, SV exhibits even distribution of K_{Ca} channel subtypes in the endothelial and the smooth muscle layer, although IK_{Ca} tends to be slightly more in abundance in the endothelium. Another important finding of the present study is that compared with ITA, SV expresses less BK_{Ca} $\beta 1$ in the smooth muscle, shown by the lower H-score of BK_{Ca} $\beta 1$ staining. Considering the essential role of the $\beta 1$ subunit in the function of BK_{Ca} channels, this may indicate a difference in the physiological performance of BK_{Ca} channels between ITA and SV.

Functional studies of the K_{Ca} channels using acetylcholine suggested that in ITA, BK_{Ca} subtype plays a significant role in endothelium-dependent vasodilatation, whereas the participation of IK_{Ca} and SK_{Ca} is limited. Single blockade of IK_{Ca} or SK_{Ca} and combined blockade of these 2 subtypes only inhibited the relaxation to a small extent. The insignificant contribution of IK_{Ca} and SK_{Ca} was not in line with our expectation. Taking into account the interaction between NO and EDHF,[35] we assumed that under physiological condition, the predominant NO synthase 3–NO signaling masks the role of IK_{Ca} and SK_{Ca} channels, the molecular basis for EDHF, in the vasodilatation of ITA. In fact, previous studies, including our study in ITA showing the role of EDHF[2] were conducted in the presence of NO synthase 3 and prostaglandin I_2 inhibitors. The abundant expression of BK_{Ca} channels in the smooth muscle of ITA may also imply the critical role of NO in acetylcholine-induced relaxation



VIDEO 1. A video clip depicting the aim, methods, results, and clinical implication of the study on calcium-activated potassium (K_{Ca}) family in coronary artery bypass grafts. Video available at: https://www.jtcvs.org/article/S0022-5223(19)33456-7/fulltext.

in this arterial graft because BK_{Ca} is a well-recognized smooth muscle effector of NO.

We previously reported that SV produces less NO and EDHF than ITA,[2] which explains the dramatically weaker relaxation of SV compared with ITA in response to acetylcholine. Similar to ITA, blockade of IK_{Ca} and SK_{Ca} channels in SV barely influenced the endothelium-dependent relaxation induced by acetylcholine. The BK_{Ca} channel blocker iberiotoxin only caused minor inhibition on acetylcholine-induced relaxation in SV, which was in contrast to the significant inhibition by iberiotoxin in ITA. Less expression of BK_{Ca} $\beta 1$ in the smooth muscle of SV may provide an explanation for the weaker inhibitory effect of iberiotoxin in this graft.

FUTURE PERSPECTIVES AND IMPLICATIONS

Inconsistent with the common notion that IK_{Ca} and SK_{Ca} channels are generally specifically expressed in vascular endothelium, whereas BK_{Ca} is expressed in smooth muscle, the present study provided solid evidence that all 3 subtypes of K_{Ca} channels are abundantly expressed in both endothelial and smooth muscle cells of ITA and SV. This raises 2 questions for future research: What is the respective role and contribution of endothelial K_{Ca} and smooth muscle K_{Ca} channels to the vasoactivity of ITA and SV? And, Does a certain K_{Ca} subtype in the endothelium behave the same as the subtype in the smooth muscle? Further studies using endothelium-denuded preparation and electrophysiological approaches are warranted to provide more comprehensive understanding of the role of K_{Ca} family in the function of CABG. In addition, although in this study IK_{Ca} and SK_{Ca} channels were shown to be unessential to relaxation of ITA and SV, considering their abundance in the grafts, are these channels involved in the regulation of inflammatory process to influence the postoperative performance and long-term patency of the graft? Moreover, the finding of the differences in BK_{Ca} subtype (significant vs minor contribution to vasorelaxation in ITA and SV, correlated to higher smooth muscle BK_{Ca} $\beta 1$ level in ITA than SV) suggests the potential of BK_{Ca} modulator in the prevention and treatment of ITA spasm, which cannot be achieved by IK_{Ca} and SK_{Ca} channel modulators.

CONCLUSIONS

By revealing for the first time the distribution profile and the respective contribution of K_{Ca} channel subtypes in vasorelaxation in ITA and SV, this study demonstrated that K_{Ca} channel family is abundantly expressed in ITA and SV. There are differences between these 2 grafts in the abundance of K_{Ca} channel subtypes in the endothelium and the smooth muscle. The significance of the BK_{Ca} channel subtype in vasodilatation of ITA may suggest the potential of development of BK_{Ca} modulators for the prevention and treatment of ITA spasm during and after CABG surgery.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: calcium-activated potassium channels, coronary artery bypass graft, internal mammary artery, saphenous vein

APPENDIX E1. MATERIALS AND METHODS

Quantitative Polymerase Chain Reaction

Extraction of total RNA from internal thoracic artery (ITA) and saphenous vein (SV) segments using TRIzol reagent (ThermoFisher Scientific, Irvine, Calif) was performed according to the manufacturer's instructions. Reverse transcription and quantitative polymerase chain reaction (PCR) amplification were performed in LightCycler 96 (Roche, Basel, Switzerland) employing TransScript Green Two-Step qRT-PCR SuperMix system (Transgen, Beijing, China) under optimal PCR cycle conditions: 94°C for 30 seconds, 45 cycles of 5 seconds at 94°C, 50°C for 15 seconds, 72°C for 10 seconds, and melting at 95°C for 10 seconds, 65°C for 10 seconds and 97°C for 1 second. The following primers were used for amplification: large-conductance calcium-activated potassium channel (BK_{Ca}) α, 5′-ACAGCATCGGAGTCTTG-3′ (forward) and 5′-GTCATCA
TCATCGTCTTGG-3′ (reverse); $BK_{Ca}\beta 1$, 5′-GACCAGAACCAGCAG
TG-3′ (forward), and 5′-GGAGAAGCAGTAGAAGACC-3′ (reverse); small-conductance calcium-activated potassium channel (SK_{Ca}) (SK2.3) 5′-TGGAGAAGCAGATTGG-3′ (forward) and 5′-GATGATGGCAGACA
GG-3′ (reverse); intermediate-conductance calcium-activated potassium channel (IK_{Ca}), 5′-AGCCTGGATGTTCTAC-3′ (forward) and 5′-ATG-GAGTTC
ACTTGTTC-3′ (reverse). Glyceraldehyde 3-phosphate dehydrogenase was amplified in parallel as an internal loading control with the primers 5′-CATCCCTGCCTCTACTG-3′ (forward) and 5′-GCTTCACCACC
TTCTTG-3′ (reverse). Qualitative PCR was performed in triplicate for each gene. The threshold cycle (Ct) values were calculated and statistically evaluated by SPSS version 20 (IBM-SPSS Inc, Armonk, NY). Expression of the target messenger RNAs was normalized to glyceraldehyde 3-phosphate dehydrogenase levels and relative differences were determined using the comparative Ct ($\Delta\Delta$Ct) method, and fold expression was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct represents ΔCt values normalized with the mean ΔCt of control samples.

Western Blot

Whole cell protein of ITA and SV segments were extracted with radioimmunoprecipitation assay buffer containing protease inhibitor (Solarbio, Beijing, China). Protein extracts were determined for concentration by bicinchoninic acid assay, aliquoted, and fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (40 μg/lane), followed by electrotransferred to a polyvinylidene difluoride membrane (ThermoFisher Scientific) for detection of the protein of interest. Details of the procedures are published elsewhere.[28,29] The polyvinylidene difluoride membrane was probed overnight at 4°C with primary antibodies (Abcam, Cambridge, Mass) against the protein of interest, including $BK_{Ca}\alpha$ (1:1000), $BK_{Ca}\beta 1$ (1:200), $K_{Ca}2.3$ (1:500), and $K_{Ca}3.1$ (1:500), followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G or horse antimouse immunoglobulin G secondary antibodies (1:3000) (Cell Signaling Technology, Danvers, Mass) for 1 hour at room temperature. β-actin (1:2000) (Absin, Shanghai, China) was used as internal loading control. Color development was performed with electrochemiluminescence kit (Beyotime, Nanjing, China), followed by imaging using G:BOX gel doc system (Syngene, Cambridge, United Kingdom), and analyzing by Quantity One imaging system version 4.6.6 (Bio-Rad, Irvine, Calif).

Histology and Immunohistochemistry Staining

ITA and SV segments were fixed in 4% paraformaldehyde, decalcified, dehydrated, and embedded in paraffin. The embedded tissue samples were sliced into 4- to 5-μm sections and stained with hematoxylin and eosin after deparaffination.

For immunohistochemistry staining, slides were heated at 100°C in 10 mmol/L tannic acid/1 mmol/L ethylenediaminetetraacetic acid solution (ZSGB-BIO, China) to retrieve antigens. Endogenous peroxidase activity was quenched by a 10 min-incubation with hydrogen peroxide (ZSGB-Biotechnology, Beijing, China) (3% in phosphate-buffered saline). Sections were incubated for 60 minutes at 37°C with primary antibodies against $BK_{Ca}\alpha$ (1:4000), $BK_{Ca}\beta 1$ (1:500), $K_{Ca}2.3$ (1:800), and $K_{Ca}3.1$ (1:800) and followed by 30-minute incubation with goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody at room temperature for signal detection of K_{Ca} subtypes. Afterward, the specimens were washed in phosphate buffered saline and stained with a liquid 3,3'-diaminobenzidine peroxidase substrate kit (ZSGB-Biotechnology). Counterstaining was performed with Mayer's hematoxylin (ZSGB-Biotechnology). Negative controls were immunostained without the primary antibody. The immunostained images were captured using a microscope (Olympus BX43, Tokyo, Japan).

Immunostaining was evaluated by two independent pathologists using a blind protocol design. For each specimen, the histoscore of each K_{Ca} subtype was calculated as the sum of staining intensity (negative staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3) multiplied by the percentage of stained cells (0%-100%).

Isometric Force Study

ITA and SV segments were cut into 3-mm rings and mounted in a 4-channel Mulvany myograph (Model 620M; J. P. Trading, Aarhus, Denmark). The details of myograph experiment have been extensively published in our previous studies.[28-30] Cumulative dose-response curves to BK_{Ca} activator NS1619 (–9 to –4.5 Log M), IK_{Ca}/SK_{Ca} activator NS309 (–9 to –4.5 Log M), and acetylcholine (–10 to –4.5 Log M) were established in ITA and SV rings precontracted by U46619. In the study of acetylcholine-induced relaxation, vessels were incubated with the specific blocker for BK_{Ca} (iberiotoxin, 100 nmol/L), IK_{Ca} (TRAM34, 1 μmol/L), or SK_{Ca} (apamin, 100 nmol/L) before U46619-precontraction. Relaxant responses of ITA and SV to acetylcholine were also studied after combined application of TRAM34 and apamin, or TRAM34 and apamin plus iberiotoxin.