

# Calcium-activated potassium channel family in coronary artery bypass grafts

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

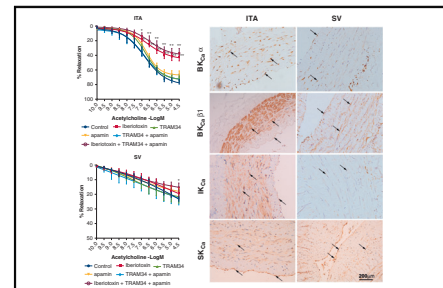
## ABSTRACT

**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* 2019; ■:1-10)



**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 Commentary on page XXX.

From the <sup>a</sup>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; <sup>b</sup>School of Pharmacy, Wannan Medical College, Wuhu, Anhui, China; and <sup>c</sup>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](mailto:qyanghk@163.com)). 0022-5223/\$36.00

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**Abbreviations and Acronyms**

BK <sub>Ca</sub>	= Large-conductance calcium-activated potassium
CABG	= coronary artery bypass graft
EC <sub>50</sub>	= half maximal effective concentration
EDHF	= endothelium-derived hyperpolarizing factor
EDRF	= endothelium-derived relaxing factor
H-score	= histoscore
IK <sub>Ca</sub>	= intermediate-conductance calcium-activated potassium
ITA	= internal thoracic artery
K <sub>Ca</sub>	= calcium-activated potassium
mRNA	= messenger RNA
NO	= nitric oxide
Q-PCR	= quantitative polymerase chain reaction
R <sub>max</sub>	= Maximal response
SK <sub>Ca</sub>	= small-conductance calcium-activated potassium
SV	= saphenous vein



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page to access supplementary information.

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<sup>1</sup> 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).<sup>2</sup> These divergent properties are believed to underlie the differences between ITA and SV in postoperative graft function and long-term patency rates.

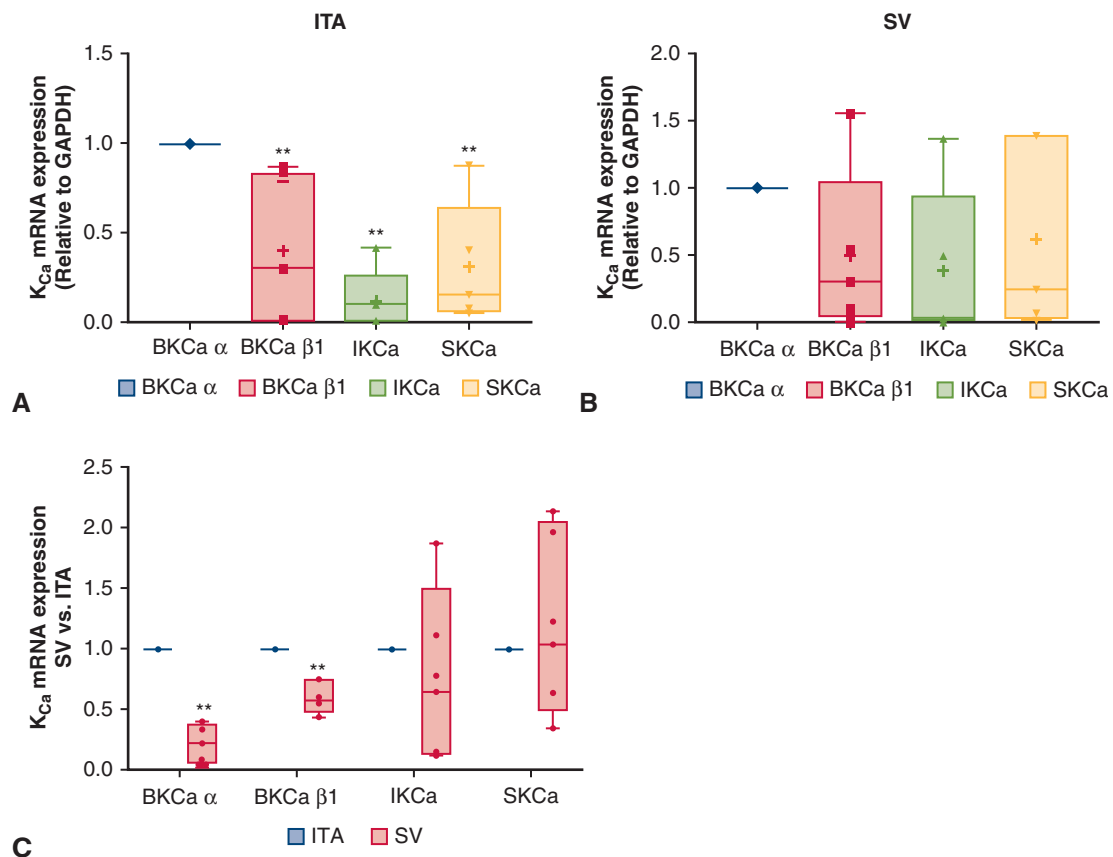
Calcium-activated potassium (K<sub>Ca</sub>) channels, including large-conductance K<sub>Ca</sub> (BK<sub>Ca</sub>), intermediate-conductance K<sub>Ca</sub> (IK<sub>Ca</sub>), and small-conductance K<sub>Ca</sub> (SK<sub>Ca</sub>), are involved in the regulation of vascular tone. Being composed of a symmetrical tetramer containing four pore-forming  $\alpha$  subunits and 4 regulatory  $\beta$  subunits,<sup>3</sup> BK<sub>Ca</sub> channels in the vasculature were reported to be mainly distributed in the plasma membrane of smooth muscle cells,<sup>4</sup> whereas

the existence and function of BK<sub>Ca</sub> in the vascular endothelium remains controversial.<sup>5,6</sup> In comparison, SK<sub>Ca</sub> and IK<sub>Ca</sub> channels were found to be predominantly expressed in endothelial cells in various vasculatures,<sup>7-9</sup> with sporadic reports of function and expression in the smooth muscle.<sup>10,11</sup> Opening of BK<sub>Ca</sub> 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.<sup>12</sup> BK<sub>Ca</sub> channels act as a smooth muscle effector of endothelium-derived relaxing factors (EDRF),<sup>13,14</sup> in particular NO. Numerous studies have demonstrated that activation of endothelial IK<sub>Ca</sub> (K<sub>Ca</sub>3.1) and SK<sub>Ca</sub> (K<sub>Ca</sub>2.3) underlies the classical EDHF pathway,<sup>15</sup> and these channels are also involved in the regulation of NO bioavailability.<sup>16</sup>

Despite extensive research in animal vasculatures, studies regarding the role of K<sub>Ca</sub> channels in human vessels are limited. It was reported that BK<sub>Ca</sub> channels are expressed in smooth muscle cells of human coronary arteries and mediate coronary artery dilation.<sup>17</sup> Modulation of endothelial SK<sub>Ca</sub> and IK<sub>Ca</sub> channels were found to take part in coronary arteriolar dysfunction in pathological/diseased conditions such as laminar shear stress, ischemia-reperfusion, and diabetes.<sup>18-20</sup> Expressions or function of K<sub>Ca</sub> channels were also demonstrated in human mesenteric,<sup>7</sup> gastroepiploic,<sup>21</sup> and pulmonary arteries.<sup>22</sup> Nevertheless, these studies either focused on specific K<sub>Ca</sub> subtype or investigated the overall function of K<sub>Ca</sub> channels, none of them looked into the distribution profile and the respective contribution to vasorelaxation made by K<sub>Ca</sub> 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<sub>Ca</sub> channels in human ITA. For example, blockade of K<sub>Ca</sub> channels with tetraethylammonium enhanced the contractile effect of endogenous vasopressin.<sup>23</sup> Relaxation induced by propofol and flavanol compound (–)-epicatechin was significantly inhibited by the selective BK<sub>Ca</sub> blocker iberiotoxin in ITA.<sup>24,25</sup> Procyanidin B2-induced relaxation of ITA was slightly reduced by the IK<sub>Ca</sub> inhibitor TRAM-34, whereas almost abolished by iberiotoxin.<sup>26</sup> Few studies investigated the role of K<sub>Ca</sub> channels in the venous graft SV. Zhang and colleagues<sup>27</sup> reported that the total patch current was significantly suppressed by tetraethylammonium and iberiotoxin in smooth muscle cells isolated from human SV.<sup>27</sup> So far, no efforts have been made to compare the localization and function of different subtypes of K<sub>Ca</sub> 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  $\alpha$  and  $\beta 1$  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  $BK_{Ca}\alpha$ ,  $BK_{Ca}\beta 1$ ,  $K_{Ca}2.3$ , and  $K_{Ca}3.1$ , and quantification methods are described in [Appendix E1](#).

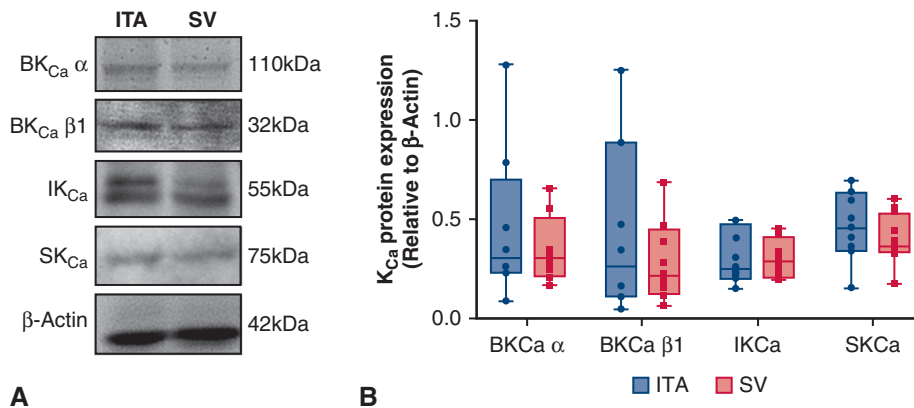
## Western Blot

Whole-cell protein of ITA and SV segments were extracted and the proteins of interest, including  $BK_{Ca}\alpha$ ,  $BK_{Ca}\beta 1$ ,  $K_{Ca}2.3$ , and  $K_{Ca}3.1$  were detected using specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies, as described elsewhere.<sup>28,29</sup> 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- $\mu$ m sections and stained with hematoxylin and eosin after deparaffination. Immunohistochemistry staining was performed with the primary antibody against  $BK_{Ca}\alpha$ ,  $BK_{Ca}\beta 1$ ,  $K_{Ca}2.3$ , 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  $\alpha$  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.<sup>28–30</sup> 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  $\mu$ mol/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  $\beta$ -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  $\pm$  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

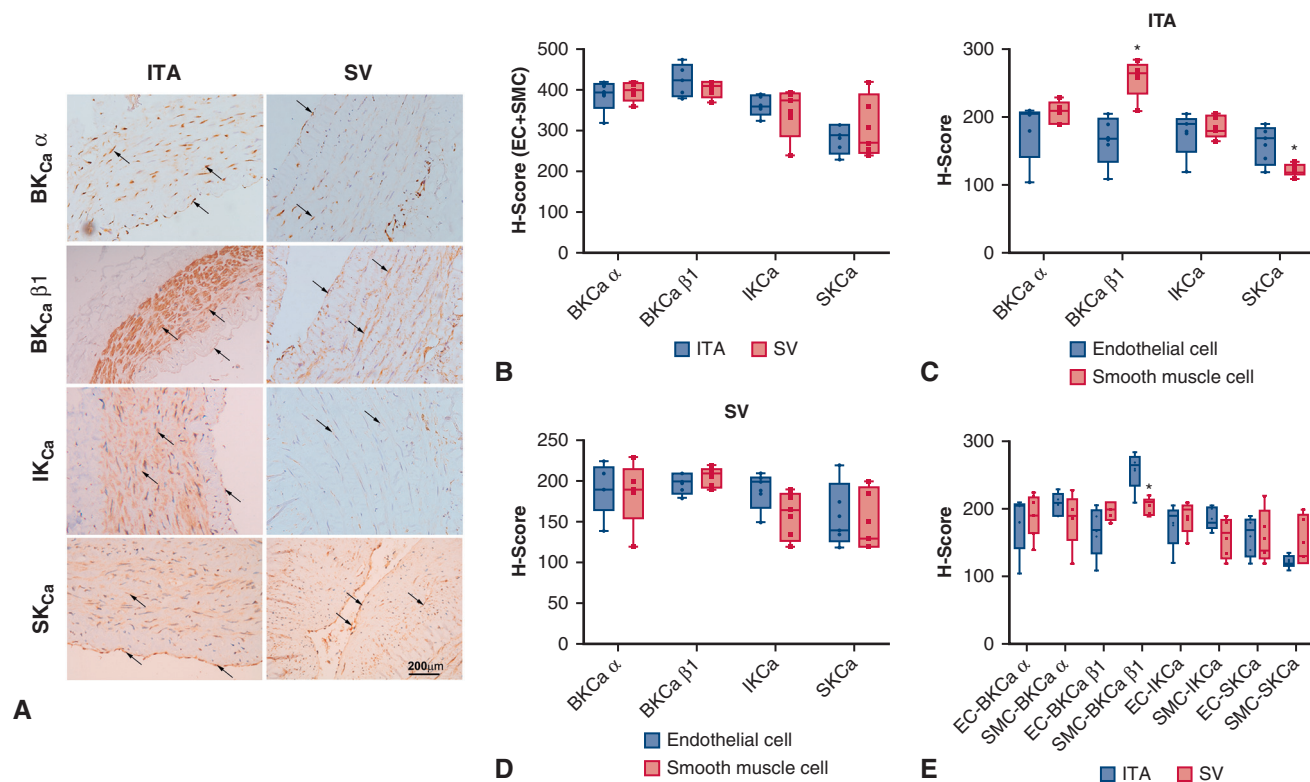
**mRNA expression of  $K_{Ca}$  channel subtypes: ITA versus SV.** Q-PCR experiments enrolling 5 pairs of ITA and SV (each pair from the same individual) showed that all 3 subtypes of  $K_{Ca}$  channels are expressed in both the arterial and venous grafts. Further analysis indicated that the mRNA level 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  $\alpha$  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  $\alpha$  and  $\beta 1$  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  $BK_{Ca}$   $\beta 1$  in the SMC layer of the SV. Data were obtained from 5 independent experiments, each employing ITA and SV samples from the same patient. \* $P < .05$ .

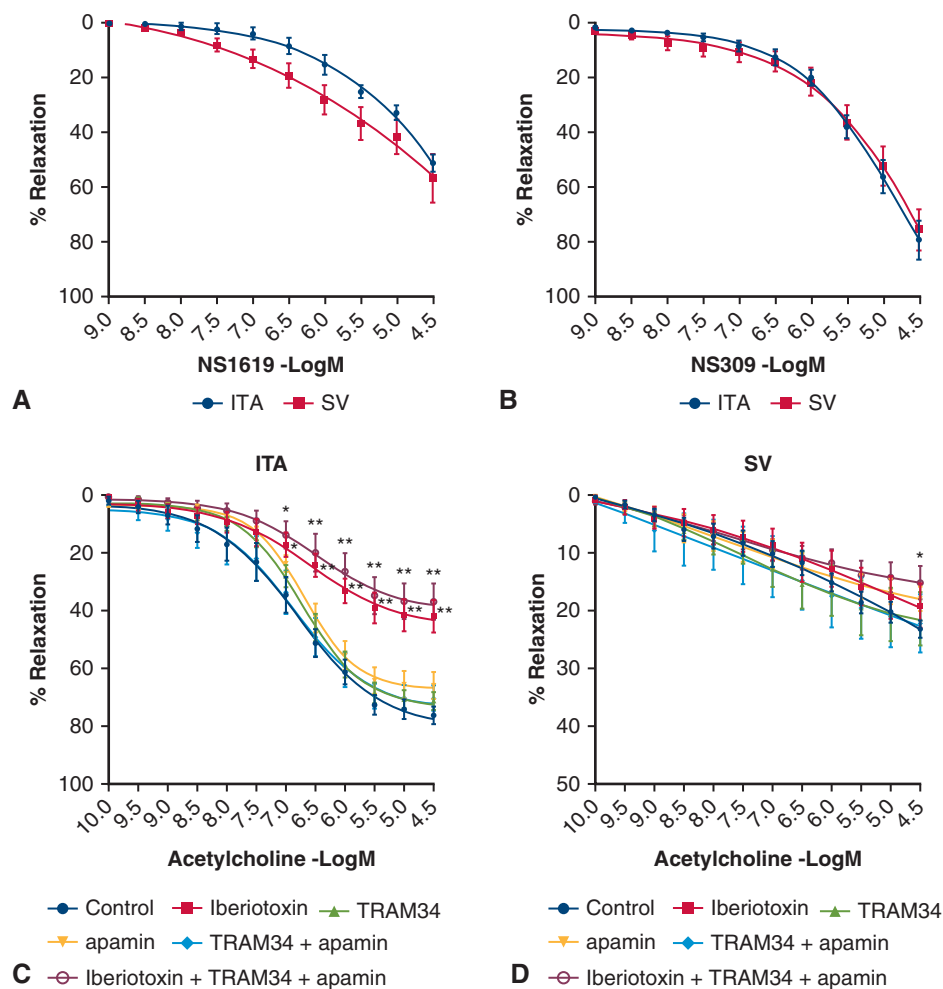
cells than in endothelial cells ( $P = .018$ ), whereas  $SK_{Ca}$  exhibits more abundance in the endothelial layer ( $P = .020$ ) (Figure 3, C). Different from ITA, SV exhibited no significant differences in the  $K_{Ca}$  distribution in the endothelial and the smooth muscle layer, though the abundance 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  $BK_{Ca}$   $\beta 1$  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 ( $51.0\% \pm 3.1\%$  and

$56.4\% \pm 8.6\%$ , respectively) (Figure 4, A). The half maximal effective concentration ( $EC_{50}$ ) value for NS1619 in ITA was  $-5.45 \pm 0.11$  LogM and  $-5.84 \pm 0.08$  LogM in SV ( $P = .033$ ). The maximal response ( $R_{max}$ ) and sensitivity to NS309 did not differ between ITA and SV ( $R_{max}$ ,  $79.0\% \pm 6.7\%$  vs  $75.0\% \pm 7.3\%$ ;  $EC_{50}$ ,  $-5.38 \pm 0.04$  vs  $-5.35 \pm 0.08$  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  $76.2\% \pm 3.0\%$  in ITA and  $23.2\% \pm 1.5\%$  in SV (Figure 4, C and D). Application of the  $BK_{Ca}$  blocker iberiotoxin significantly decreased acetylcholine-induced relaxation in ITA from  $76.2\% \pm 3.0\%$  to  $41.9\% \pm 5.4\%$  ( $P < .01$ ). 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,  $71.2\% \pm 3.0\%$  in the TRAM-treated group and  $66.0\% \pm 4.6\%$  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 ( $36.9\% \pm 6.1\%$ ), 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 ( $23.2\% \pm 1.5\%$  vs  $76.2\% \pm 3.0\%$ ;  $P < .001$ ). 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 ( $15.2\% \pm 2.8\%$  vs  $23.2\% \pm 1.5\%$  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<sub>Ca</sub>) channel blocker; *TRAM-34*, intermediate-conductance calcium-activated potassium (IK<sub>Ca</sub>) channel blocker; *apamin*, small-conductance calcium-activated potassium (SK<sub>Ca</sub>) channel blocker. \**P* < .05 versus control.

U46619-precontraction of ITA and SV except that in the ITA, combined use of BK<sub>Ca</sub>, IK<sub>Ca</sub>, and SK<sub>Ca</sub> blockers enhanced U46619-induced precontraction (Table 1). The sensitivity of ITA and SV to acetylcholine was barely influenced by the K<sub>Ca</sub> blockers, as indicated by the unaltered EC<sub>50</sub> values (Table 2).

## DISCUSSION

In this study, by using Q-PCR, western blot, and immunohistochemistry for localization and quantification of K<sub>Ca</sub> 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<sub>Ca</sub> family between ITA and SV (Video 1). Results derived from this study demonstrated that both ITA and SV express all 3 subtypes of K<sub>Ca</sub> 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<sub>Ca</sub> channel subtype, ITA and SV show differences in the abundance of K<sub>Ca</sub> channel subtypes in endothelial and smooth muscle layers, and the BK<sub>Ca</sub> subtype in the K<sub>Ca</sub> 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.

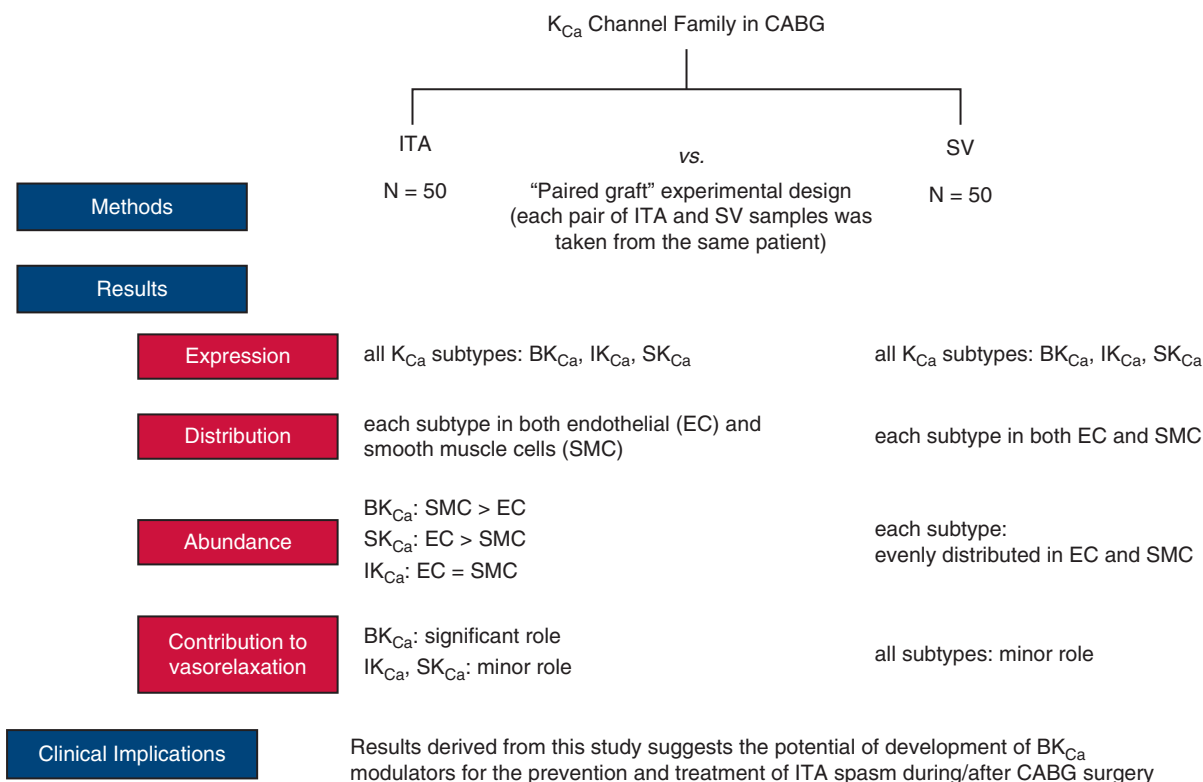
As the commonly used CABG, the ITA and SV are obviously different in anatomic structure and hemodynamic characteristics. In addition, they are different in the response to constrictor and dilator agents. For example, the inotropic/vasodilator compound levosimendan relaxed ITA but failed to relax SV completely.<sup>31</sup> Prostaglandin E<sub>2</sub> induced vasoconstriction in ITA whereas vasodilatation in SV, which was attributed to the mediation of different prostaglandin E<sub>2</sub> receptors.<sup>32</sup> Compared with SV, ITA exhibited greater endothelium-dependent relaxation,<sup>33</sup> 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.<sup>2</sup> 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<sub>Ca</sub> channel family, which has been suggested to largely take part in the generation/action of EDRF, including EDHF and NO.<sup>8,13,16,34</sup>

To gain more precise knowledge about whether K<sub>Ca</sub> 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<sub>Ca</sub> 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<sub>Ca</sub> family, evidenced by the comparable protein level and H-score of each K<sub>Ca</sub> channel subtype in these 2 grafts. At the mRNA level, ITA was found to express more BK<sub>Ca</sub> subtype than SV. The inconsistent mRNA and protein data may suggest that ITA differs from SV in the

**TABLE 2. Half maximal effective concentration (EC<sub>50</sub>) values for acetylcholine in internal thoracic artery (ITA) and saphenous vein (SV) with or without pretreatment with different calcium-activated potassium (K<sub>Ca</sub>) channel blockers**

Group	EC <sub>50</sub> (-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<sub>Ca</sub>) channel blocker; *TRAM-34*, intermediate-conductance calcium-activated potassium (IK<sub>Ca</sub>) channel blocker; *apamin*, small-conductance calcium-activated potassium (SK<sub>Ca</sub>) channel blocker.



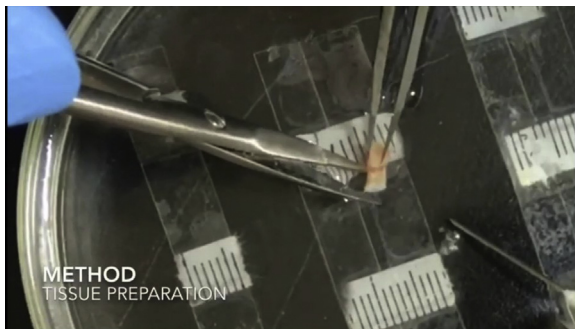
**FIGURE 5.** Schematic summary of the expression and distribution profile of calcium-activated potassium ( $K_{Ca}$ ) channel family in coronary artery bypass grafts (CABG) and their role in the dilatory function of the grafts. BK<sub>Ca</sub>, Large-conductance K<sub>Ca</sub>; IK<sub>Ca</sub>, intermediate-conductance K<sub>Ca</sub>; SK<sub>Ca</sub>, small-conductance K<sub>Ca</sub>; EC, endothelial cell, SMC, smooth muscle cell.

translational control of BK<sub>Ca</sub> 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<sub>Ca</sub> mRNA content in ITA may serve as a reservoir for maintaining or repairing BK<sub>Ca</sub> channel function in the arterial graft in pathological conditions. The comparable expression of K<sub>Ca</sub> subtypes in ITA and SV was also supported by the similar degree of responses of the 2 grafts to the BK<sub>Ca</sub> opener NS1619 and the IK<sub>Ca</sub>/SK<sub>Ca</sub> opener NS309.

Immunohistochemistry experiments showed that in ITA and SV, IK<sub>Ca</sub>, SK<sub>Ca</sub>, and BK<sub>Ca</sub> 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<sub>Ca</sub> channels, they are different in the distribution abundance of K<sub>Ca</sub> channel subtypes. In ITA, BK<sub>Ca</sub> is more abundantly expressed in smooth muscle cells than in endothelial cells, whereas SK<sub>Ca</sub> exhibits more abundance in the endothelial layer, which is in accordance with the finding of BK<sub>Ca</sub> and SK<sub>Ca</sub> distribution in various vasculatures. In comparison, SV exhibits even distribution of K<sub>Ca</sub> channel subtypes in the endothelial and the smooth muscle layer, although IK<sub>Ca</sub> 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<sub>Ca</sub>  $\beta$ 1 in the smooth muscle, shown by the lower H-score of BK<sub>Ca</sub>  $\beta$ 1 staining. Considering the essential role of the  $\beta$ 1 subunit in the function of BK<sub>Ca</sub> channels, this may indicate a difference in the physiological performance of BK<sub>Ca</sub> channels between ITA and SV.

Functional studies of the K<sub>Ca</sub> channels using acetylcholine suggested that in ITA, BK<sub>Ca</sub> subtype plays a significant role in endothelium-dependent vasodilatation, whereas the participation of IK<sub>Ca</sub> and SK<sub>Ca</sub> is limited. Single blockade of IK<sub>Ca</sub> or SK<sub>Ca</sub> and combined blockade of these 2 subtypes only inhibited the relaxation to a small extent. The insignificant contribution of IK<sub>Ca</sub> and SK<sub>Ca</sub> was not in line with our expectation. Taking into account the interaction between NO and EDHF,<sup>35</sup> we assumed that under physiological condition, the predominant NO synthase 3–NO signaling masks the role of IK<sub>Ca</sub> and SK<sub>Ca</sub> 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<sup>2</sup> were conducted in the presence of NO synthase 3 and prostaglandin I<sub>2</sub> inhibitors. The abundant expression of BK<sub>Ca</sub> 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](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,<sup>2</sup> 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<sub>Ca</sub>)  $\alpha$ , 5'-ACAGCATCGGAGTCTTG-3' (forward) and 5'-GTCATCA TCATCGTCTTGG-3' (reverse); BK<sub>Ca</sub> $\beta$ 1, 5'-GACCAGAACCAGCAG TG-3' (forward), and 5'-GGAGAAGCAGTAGAAGACC-3' (reverse); small-conductance calcium-activated potassium channel (SK<sub>Ca</sub>) (SK2.3) 5'-TGGAGAAGCAGATTGG-3' (forward) and 5'-GATGATGGCAGACA GG-3' (reverse); intermediate-conductance calcium-activated potassium channel (IK<sub>Ca</sub>), 5'-AGCCTGGATGTTCTAC-3' (forward) and 5'-ATG-GAGTTC ACTTGTTTC-3' (reverse). Glyceraldehyde 3-phosphate dehydrogenase was amplified in parallel as an internal loading control with the primers 5'-CATCCCTGCCTCTACTG-3' (forward) and 5'-GCTTACCACC 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  $\Delta$ Ct values normalized with the mean  $\Delta$ Ct 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  $\mu$ g/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.<sup>28,29</sup> The polyvinylidene difluoride membrane was probed overnight at 4°C with primary antibodies (Abcam, Cambridge, Mass) against the protein of interest, including BK<sub>Ca</sub> $\alpha$  (1:1000), BK<sub>Ca</sub> $\beta$ 1 (1:200), K<sub>Ca</sub>2.3 (1:500), and K<sub>Ca</sub>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.  $\beta$ -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- $\mu$ m 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<sub>Ca</sub> $\alpha$  (1:4000), BK<sub>Ca</sub> $\beta$ 1 (1:500), K<sub>Ca</sub>2.3 (1:800), and K<sub>Ca</sub>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<sub>Ca</sub> 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<sub>Ca</sub> 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.<sup>28-30</sup> Cumulative dose-response curves to BK<sub>Ca</sub> activator NS1619 (-9 to -4.5 Log M), IK<sub>Ca</sub>/SK<sub>Ca</sub> 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<sub>Ca</sub> (iberiotoxin, 100 nmol/L), IK<sub>Ca</sub> (TRAM34, 1  $\mu$ mol/L), or SK<sub>Ca</sub> (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.

**000 Calcium-activated potassium channel family in coronary artery bypass grafts**

*Wen-Tao Sun, PhD, Hai-Tao Hou, MPhil, Huan-Xin Chen, MPhil, Hong-Mei Xue, PhD, Jun Wang, MPhil, Guo-Wei He, MD, PhD, DSc, and Qin Yang, MD, PhD, Tianjin and Wuhu, Anhui, China, and Portland, Ore*

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 the abundance of calcium-activated potassium channel subtypes in the 2 layers.

Large-conductance calcium-activated potassium channels play a critical role in the dilatation of internal thoracic artery.