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Differential modulation of SK channel subtypes by phosphorylation

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Abstract

Small-conductance Ca^{2+}-activated K^+ (SK) channels are voltage-independent and are activated by Ca^{2+} binding to the calmodulin constitutively associated with the channels. Both the pore-forming subunits and the associated calmodulin are subject to phosphorylation. Here, we investigated the modulation of different SK channel subtypes by phosphorylation, using the cultured endothelial cells as a tool. We report that casein kinase 2 (CK2) negatively modulates the apparent Ca^{2+} sensitivity of SK1 and IK channel subtypes by more than 5-fold, whereas the apparent Ca^{2+} sensitivity of the SK3 and SK2 subtypes is only reduced by ~2-fold, when heterologously expressed on the plasma membrane of cultured endothelial cells. The SK2 channel subtype exhibits limited cell surface expression in these cells, partly as a result of the phosphorylation of its C-terminus by cyclic AMP-dependent protein kinase (PKA). SK2 channels expressed on the ER and mitochondria membranes may protect against cell death. This work reveals the subtype-specific modulation of the apparent Ca^{2+} sensitivity and subcellular localization of SK channels by phosphorylation in cultured endothelial cells.

Introduction

Small-conductance Ca^{2+}-activated K^+ (SK) channels are a unique group of K^+ ion channels[1], activated exclusively by intracellular Ca^{2+} [1–4]. Calmodulin (CaM) is constitutively associated with SK channels and serves as the Ca^{2+} sensor of the SK-CaM complex[1]. There are four subtypes in the SK channel family encoded by the *KCNN* mammalian genes, including *KCNN1* for SK1 ($K_{Ca}2.1$), *KCNN2* for SK2 ($K_{Ca}2.2$), *KCNN3* for SK3 ($K_{Ca}2.3$) and *KCNN4* for IK ($K_{Ca}3.1$ or SK4) channels. SK channels play an important role in the vasculature[5–9] and the central nervous system[2]. In neurons, SK channels expressed on the neuronal cell surface contribute to the medium after

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hyperpolarization (mAHP) and regulate the firing frequency of neurons[2,10]. SK channels were also reported to express on the intracellular endoplasmic reticulum (ER) membrane and play a protective role against cell death in human dopaminergic neurons[11] and immortalized mouse hippocampal-derived HT-22 cells[12]. SK channels were also identified in mitochondria of neurons[13] and cardiomyocytes[14] where they exert protective roles.

In blood vessels, SK channels are expressed in endothelial cells[15]. It is well documented that the activation of SK channels expressed on the endothelial cell surface can reduce vascular tone. The cell surface expression of these channels is especially important in vasodilation mediated by endothelium-dependent hyperpolarization (EDH)[16–18]. Using transgenic SK3 mice, it was found that expression of SK3 channels modulates arterial tone and blood pressure[5]. Genetic deficit of both SK3 and IK channels in mice abolishes the EDH response and causes hypertension[19]. Compromised SK channel activity may be a contributing factor to hypertension[7]. In contrast to the enormous body of research on the role of cell surface SK channels in vasodilation, it is not clear whether any SK channel subtypes are expressed on the ER and mitochondria membranes in endothelial cells and whether they can protect endothelial cells from apoptosis.

In neurons, casein kinase 2 (CK2) phosphorylates the CaM complexed with the SK2 channel and leads to decreased apparent Ca^{2+} sensitivity[20–23], whereas cyclic AMP-dependent protein kinase (PKA) mediates phosphorylation at the C-terminus of SK2 channels and reduces their cell surface expression[24–26]. A CK2 inhibitor 4,5,6,7-tetrabromo-2-azabenzotriazole (TBB) increased apparent Ca^{2+} sensitivity of SK2 channels[22], while a PKA inhibitor (H89) improved surface expression of SK2 channels[24]. But it is not clear if these kinases regulate the other SK channel subtypes differently. In vascular endothelial cells, even less is known. We therefore investigated the modulation of different SK channel subtypes utilizing the cultured endothelial cells as a tool. In previous studies, SK3 and IK channel subtype expression has been readily detected through reverse transcriptase PCR and then confirmed through electrophysiology and pharmacological experiments in vascular endothelial cells from humans, pigs and cattle[27–33]. In contrast, expression of SK1 channels in isolated and cultured endothelial cells was rarely detected[33]. Most interestingly, the mRNA of SK2 channels was readily detectable[31–33], but functional SK2 channel current on the plasma membrane was rarely identifiable in isolated and cultured endothelial cells[33].

Here, we took advantage of an immortalized mouse aorta endothelial cell line that we established and characterized previously[34–36], to study the modulation of different SK channel subtypes by phosphorylation[34–36]. The objective is to compare the modulation of different subtypes by phosphorylation, utilizing the cultured endothelial cells as a tool. We found that the apparent Ca^{2+} sensitivity of SK1 and IK channel subtypes is more strongly negatively modulated than that of the SK3 and SK2, presumably through phosphorylation of CaM by CK2. On the other hand, the cell surface expression of SK2 channel subtype is very limited, partly as a result of phosphorylation at its C-terminus by PKA. SK2 channels express on the ER and mitochondria membranes, which may be associated with its protective role against palmitate-mediated cell death.

Results

Modulation of SK channels' apparent Ca^{2+} sensitivity by CK2 phosphorylation.

We first compared apparent Ca^{2+} sensitivity of SK channels in HEK293 and cultured endothelial cells. SK channels were heterologously expressed in HEK293 cells for inside-out patch-clamp experiments to determine apparent Ca^{2+} sensitivity of these channels as we previously reported[20,37–40]. To investigate the differential modulation of SK channel subtypes, we utilized an immortalized mouse aorta endothelial cell line that we previously established and characterized[34–36]. Exposing the inside-out patches of the non-transfected cultured endothelial cells to 10 μM Ca^{2+} could not induce any changes in current compared to nominal 0 Ca^{2+}, suggesting that the endogenous Ca^{2+}-activated channel current in this cultured endothelial cell line is too small to be detected in inside-out patches (Fig. S1). We then transfected cultured endothelial cells with SK channel subtype cDNAs and stable clones were obtained through selection by puromycin and enrichment using repeated GFP fluorescence-activated cell sorting. The same electrophysiology method used for HEK293 cells was utilized to measure apparent Ca^{2+} sensitivity of the SK channel subtypes in cultured endothelial cells (Fig. S2). The leak recorded at nominal 0 Ca^{2+} was subtracted from SK channel currents recorded at various Ca^{2+} concentrations, before Ca^{2+} concentration-response curves were constructed.

Surprisingly, all SK channel subtypes exhibited significantly lower apparent Ca^{2+} sensitivity in cultured endothelial cells than in HEK293 cells. The apparent Ca^{2+} sensitivity of SK channel subtypes expressed in HEK293 cells seems comparable to each other (Fig. 1A), whereas SK channel subtypes expressed in cultured endothelial cells exhibit larger differences in their apparent Ca^{2+} sensitivity (Fig. 1B). Among the four SK channel subtypes heterologously expressed in HEK293 cells, there is no statistically significant difference between their EC_{50} values to Ca^{2+}. However, the SK1 channel exhibits significantly greater EC_{50} value for Ca^{2+} than SK2 ($P < 0.0001$) and SK3 ($P = 0.0006$) channel subtypes, when expressed in cultured endothelial cells. The IK channel also exhibits a lower apparent Ca^{2+} sensitivity than SK2 ($P < 0.0001$) and SK3 ($P = 0.0058$) channel subtypes in cultured endothelial cells (Fig. 1C).

The SK1 channel subtype expressed in cultured endothelial cells showed a 4.6-fold lower apparent Ca^{2+} sensitivity than the same subtype expressed in HEK293 cells (Fig. S3A). The EC_{50} value for Ca^{2+} to activate SK1 channels in cultured endothelial cells (1.19 ± 0.068 μM, n = 8) was ~4.6-fold greater than in HEK293 cells (0.26 ± 0.017 μM, n = 6, P = 0.00000007). For the SK2 channel subtype, only a 1.5-fold difference in apparent Ca^{2+} sensitivity was found between channels expressed in cultured endothelial cells (EC_{50} = 0.48 ± 0.044 μM; n = 9) and those in HEK293 cells (EC_{50} = 0.31 ± 0.020 μM; n = 6, P = 0.039) (Fig. S3B). For the SK3 channel, a 2.1-fold difference was found between SK3 channels expressed in cultured endothelial cells (EC_{50} = 0.63 ± 0.08 μM; n = 8), and those in HEK293 cells (EC_{50} = 0.30 ± 0.024 μM; n = 6, P = 0.001) (Fig. S3C). For IK channels, an ~5-fold difference was found between IK channels expressed in cultured endothelial cells (EC_{50} = 1.07 ± 0.25 μM; n = 11) and those in HEK293 cells (EC_{50} = 0.25 ± 0.023 μM; n = 10, P = 0.00001) (Fig. S3D). Thus, every SK channel subtype exhibits a reduced apparent

Ca^{2+} sensitivity when expressed in cultured endothelial cells compared to HEK293 cells. In addition, the apparent Ca^{2+} sensitivity of SK1 and IK channel subtypes is more negatively modulated than that of the SK3 and SK2 subtypes in cultured endothelial cells.

We postulate that the reduced apparent Ca^{2+} sensitivity of SK channels in cultured endothelial cells is caused by post-translational modification. In neurons, CK2 phosphorylates CaM, which is constitutively associated with the SK2 channel and therefore decreases its apparent Ca^{2+} sensitivity[20–22]. To test whether CK2 is also responsible for decreased apparent Ca^{2+} sensitivity of SK channels in cultured endothelial cells, we utilized the CK2 inhibitor TBB (Millipore-Sigma), a cell-permeable CK2 inhibitor previously used in the studies of CK2 regulation of SK2 channels[22]. In our excised inside-out patches, the intracellular kinases can no longer access the SK channels on the membrane, whereas CK2 is co-assembled with the channels[21]. The IC_{50} of TBB on rodent CK2 is about 0.9 μM[41]. For complete inhibition of CK2 co-assembled with the channels, cells were incubated with TBB (10 μM) in the bath solution before and during the electrophysiological recordings (Fig. S4). The treatment with TBB drastically enhanced apparent Ca^{2+} sensitivity of the SK1 channel in cultured endothelial cells by ~5.4 fold from 1.19 ± 0.068 μM (n = 8) to 0.22 ± 0.023 μM (n = 6; P = 0.00000005; Fig. S5A). The effect of TBB on the apparent Ca^{2+} sensitivity of the SK2 channel is only slightly more than 2-fold, from 0.48 ± 0.044 μM (n = 9) to 0.22 ± 0.020 μM (n = 6, P = 0.004; Fig. S5B). TBB increased apparent Ca^{2+} sensitivity of the SK3 channel by ~2.6-fold from 0.63 ± 0.08 μM (n = 8) to 0.24 ± 0.0099 μM (n = 8, P = 0.0003; Fig. S5C). The effect of TBB on the apparent Ca^{2+} sensitivity of the IK channel is ~8-fold, changing from 1.07 ± 0.25 μM (n = 11) to 0.13 ± 0.029 μM (n = 8, P = 0.00006; Fig. S5D). Hence, every channel subtype exhibits an increased apparent Ca^{2+} sensitivity in the presence of TBB. CK2 is the kinase that causes post-translational modification of the SK channel complexed with CaM and negatively modulates their apparent Ca^{2+} sensitivity in cultured endothelial cells.

Even though the SK channel subtypes expressed in cultured endothelial cells exhibit diverse apparent Ca^{2+} sensitivity (Fig. 1B), their Ca^{2+}-dependent activation curves are very similar to each other in the presence of TBB (Fig. 2A). When compared among the channel subtypes, the apparent Ca^{2+} sensitivity of the four channel subtypes are comparable to each other in the presence of TBB (Fig. 2B). TBB abolished the statistically significant difference between apparent Ca^{2+} sensitivity of SK channel subtypes in cultured endothelial cells. With a structurally unrelated and more potent CK2 inhibitor, CX4945 (silmitasertib, 5-[(3-chlorophenyl)amino]-benzo[c]-2,6-naphthyridine-8-carboxylic acid)[42], we were able to obtain similar results on the SK channel subtypes (Fig. S6). In the presence of CX4945 (Cayman Chemicals), the apparent Ca^{2+} sensitivity of the four channel subtypes are similar to each other (Fig. S6). These results indicate that CK2 negatively modulates apparent Ca^{2+} sensitivity of SK1 and IK channel subtypes more than the SK3 and SK2 subtypes.

Expression of SK2 channels on intracellular membranes.

In immortalized mouse hippocampal-derived HT-22 cells, SK channels were found to express on the intracellular ER[12] and mitochondria[13] membranes. In cultured endothelial cells, SK channels on the intracellular membranes remain unexplored. Our stably-transfected

cell lines express SK channel subtypes as fusion proteins with green fluorescent protein (GFP). We further transfected the cells with cDNAs of the ER membrane targeted red fluorescence protein (DsRed2-ER)[43]. We examined the colocalization of the GFP-tagged SK1 (Fig. S7A), SK2 (Fig. S7B), SK3 (Fig. S7C) and IK (Fig. S7D) with DsRed2-ER. In order to compare the levels of co-localization between DsRed-ER and different SK channel subtypes, histograms were made using the grey values measured for the area indicated by the white arrows (Fig. S7A–D). We further calculated the Pearson correlation coefficients from multiple histograms of each channel subtype (Fig. S7E). The SK2 channel subtype has a significantly greater correlation coefficient (0.79 ± 0.055, $n = 7$) compared with other channel subtypes (Fig. 3E), suggesting a higher level of co-localization with DsRed2-ER. Certainly, a Pearson correlation coefficient of ~0.79 does not mean exclusive expression of SK2 channels on the ER membrane. We then performed cell fractionation of the stable cell lines of SK channel subtypes and prepared mitochondria (Mit), plasma membrane (PM) and ER fractions using the same technique that we previously described[44,45]. Immunoblots for markers of the mitochondria (Cytochrome C), plasma membrane (Na^+-K^+ ATPase) and the ER (GRP-78) were performed (Fig. 3A). Densitometry with ImageJ program of the immunoblots indicates distribution of Cytochrome C in the mitochondria (Fig. 3B), Na^+-K^+ ATPase on the plasma membrane (Fig. 3C) and GRP-78 in the microsome (Fig. 3D). Anti-GFP antibodies were used to detect the SK channel subtypes in the fractions (Fig. 3E). All four channel subtypes were found on the plasma membrane and the intracellular membranes (Fig. 3F–I). Noticeably, the SK2 channel subtype is expressed abundantly on the ER membrane, even though SK2 channels were also detected on plasma membrane and mitochondria (Fig. 3G).

To detect the endogenous SK channel subtypes in the cultured endothelial cells, non-transfected cells were fractionated and then subjected to immunoblots with specific antibody for each SK channel subtypes. Endogenous SK3 channels were the only channel subtype detected in this particular endothelial cell line, with more prominent expression of SK3 channels on the plasma membrane than the mitochondria and ER (Fig. S8A,B), which is similar to the results of heterologously expressed SK3 channels (Fig. 3). Other channel subtypes were not detected in this cell line (Fig. S8C).

Role of SK2 channels on the intracellular membranes.

Our results show that the SK2 channel subtype is abundantly expressed in the ER fraction and to a lesser extent in the mitochondrial fraction of cultured endothelial cells (Fig. 3). Because the SK channel expression on the ER and mitochondria membranes may be linked with protection against apoptosis mediated by ER stress[12] and reactive oxygen species[13,14], we challenged cultured endothelial cell lines stably expressing the different SK channel subtypes with palmitate-induced apoptosis (Fig. 4). Palmitate induces cell death through a pathway related to ER stress and oxidative stress[46]. Compared to the control parent cell line, no protective effect against palmitate-induced cell death was seen for the SK1, SK3 and IK channels. However, a significant protective effect was found for the SK2 channel (Fig. 4A). The heterologous expression of SK2 channels significantly reduced 60 μM palmitate-mediated endothelial cell death from 38.5 ± 2.8 % ($n = 5$) to 20.2 ± 1.8 % ($n = 5$, $P = 0.008$). Cell death mediated by 80 μM palmitate decreased even more drastically from 50.8 ± 2.6

% (n = 5) to 26.6 ± 1.3 % (n = 5, P = 0.0002) as a result of SK2 channel heterologous expression (Fig. 4B).

Phosphorylation of SK2 channels.

In cultured endothelial cells, the SK2 channel subtype seems to show less plasma membrane expression (Fig. 3). PKA phosphorylates the C-terminus of the SK2 channel[24] and reduces its cell surface expression in neurons[25,26]. We isolated the SK2 channel protein from the plasma membrane of the cultured endothelial cells stably expressing SK2 channels through immunoprecipitation using GFP-Trap_A beads (Chromotek). SK2 channel proteins were cleaved off the GFP tag from the immunoprecipitants and then separated on SDS-PAGE gel (Fig. 5A). Protein bands at >170 kDa and ~50 kDa were both identified as SK2 channels by tandem mass spectrometry (LC-MS/MS) detection. The protein bands at >170 kDa might be the high molecular weight complex of SK2 channels[47]. A protein band at ~17 kDa was identified as CaM (Fig. S9 and Table S1). No prominent phosphorylation of CaM at T79 was found in the peptide corresponding to CaM residues ^{76}MKDTDSEEEIR^{86} detected by LC-MS/MS. Instead, LC-MS/MS revealed that phosphate groups were associated with a peptide corresponding to SK2 channel residues ^{567}RSSSTAPPTSSESS^{580} (Fig. 5B,C). Phosphorylation of residues S569 and S570 was identified. In contrast to the significantly higher expression of WT SK2 channels on the ER membrane than on the plasma membrane (Fig. 3G), the alanine mutations of the three adjacent serine residues S568, S569 and S570 (SK2-AAA) increased the expression of the mutant SK2 channels on the plasma membrane to a level comparable to their expression on the ER membrane (Fig. S10).

Discussion

SK channels are a unique group of potassium channels that are activated exclusively by CaM when it is bound with Ca^{2+} [48]. These channels play an important role in the cardiovascular system and the central nervous system. The pore-forming subunits and the associated CaM are subject to phosphorylation by PKA[24–26] and CK2[20–23], respectively (Fig. 6). Here, we utilize an immortalized mouse aorta endothelial cell line to study the differential phosphorylation of SK channel subtypes. We report that SK channel subtypes are differentially modulated by phosphorylation when heterologously expressed in cultured endothelial cells. The apparent Ca^{2+} sensitivity of SK1 and IK channel subtypes is more strongly decreased than that of the SK3 and SK2 subtypes by CK2 phosphorylation, when expressed on the plasma membrane of cultured endothelial cells (Fig. 2). In addition, the SK2 channel subtype expresses on the intracellular membranes (Fig. 3) and protects cultured endothelial cells against cell death (Fig. 4).

In endothelial cells, the basal intracellular free Ca^{2+} concentration is estimated to be lower than 0.1 μM[49]. Ca^{2+} release from the ER[50] and Ca^{2+} influx through calcium-permeable channels[9] triggers local Ca^{2+} signals to activate SK channels in cultured endothelial cells. The apparent Ca^{2+} sensitivity of the SK channels will require fine-tuning for precise intracellular signaling towards EDH-mediated vasodilation. Phosphorylation at threonine79 (T79) of CaM complexed with SK channels inhibits the apparent Ca^{2+} sensitivity of SK channels[21]. When expressed in HEK293, all four channel subtypes exhibited EC_{50} values

for Ca^{2+} in a narrow range from 0.25 to 0.31 μM (Fig. 1C), presumably because of low phosphorylation levels by CK2 in HEK293 cells. Thus, HEK293 cells may not be an ideal cell line to study the phosphorylation of SK-CaM complex. When expressed in cultured endothelial cells, the channel subtypes exhibited very diverse EC_{50} values for Ca^{2+} from 0.48 to 1.19 μM (Fig. 1C). This diversity in apparent Ca^{2+} sensitivity of SK channel subtypes is caused by different levels of CK2 phosphorylation, as the CK2 inhibitors TBB and CX4945 completely abolished the diversity in apparent Ca^{2+} sensitivity among the subtypes (Fig. 2 and Fig. S6). In neurons, cell signaling pathways of neurotransmitters can influence the phosphorylation of the SK-CaM complex by CK2[51,52]. The cell signaling pathways regulating the phosphorylation by CK2 and dephosphorylation by PP2A in endothelial cells are still unclear. Nonetheless, our results definitively indicate that differences exist in the modulation of apparent Ca^{2+} sensitivity among the SK channel subtypes in cultured endothelial cells.

Among the SK channel subtypes, the SK2 channel is the most sensitive to apamin, a bee venom toxin, with an IC_{50} of ~70 pM[53–55]. The SK3 channel is less sensitive to apamin with an IC_{50} of ~10 nM[56,57]. The SK channel current on the cell surface of endothelial cells is typically comprised of a charybdotoxin-sensitive IK component and an apamin-sensitive component[27–33]. Usually it requires apamin at nM levels to inhibit this apamin-sensitive component, suggesting its molecular identity as predominantly the SK3 channel subtype. It is puzzling that the mRNA of SK2 channels was readily detectable in isolated and cultured endothelial cells[31–33], whereas functional SK2 channel current on the plasma membrane was rarely identified. What is limiting the expression of SK2 channels on the endothelial cell surface? PKA phosphorylates the C-terminus of the SK2 channel[24]. In lateral amygdala pyramidal neurons, β-adrenoceptor activation regulates synaptic SK2 channel expression on the dendrites, through activation of PKA[25]. In mouse hippocampus, long-term potentiation (LTP) induction activates PKA and causes SK2 channel internalization[26]. Tonic PKA activity is also involved in the enrichment of SK channels on the dendrites compared to the soma of cultured hippocampal neurons[58,59]. PKA phosphorylation of a serine residue (S465) in the CaM binding domain may also underlie the functional recruitment of dormant SK2 channels in ventricular myocytes[60]. Our mass spectrometry studies show that the SK2 channel protein isolated from the plasma membrane of cultured endothelial cells undergoes prominent phosphorylation in its C-terminus, more specifically at amino acid residues S569 and S570 (Fig. 5). In a previous study, mass spectrometry detected phosphorylation of the same serine residues from purified channel C-terminus fragment treated with PKA catalytic subunit[24]. Mutations of these serine residues prevented the decrease in cell surface expression of SK2 channels caused by PKA stimulation in COS7 cells[24]. The phosphorylation might also be one of the causes of the limited plasma membrane expression of SK2 channels in cultured endothelial cells, as mutations of these residues to alanine increased the expression of SK2 channels on the plasma membrane to a level comparable to their ER expression (Fig. S10). Different from the previous mass spectrometry studies[24], our mass spectrometry experiments were performed from isolated full-length SK2 channel protein without any artificial kinase treatment, suggesting naturally occurring phosphorylation.

The SK2 channel expression on the cell surface is very limited (Fig. 3). Where are SK2 channels expressed in cultured endothelial cells? Our results suggest the expression of SK2 channels on the ER and mitochondria membranes (Fig. 3). The SK2 channels expressed on these intracellular membranes are associated with a protective role against cell death in immortalized mouse hippocampal-derived HT-22 cells[12,13] and cardiomyocytes[14]. Our results revealed a protective role of SK2 channels against the palmitate-mediated endothelial cell death (Fig. 4), echoing their expression on the intracellular membranes (Fig. 3).

The objective of this report is to compare the modulation of different SK channel subtypes by phosphorylation. Our studies were performed with immortalized mouse aorta endothelial cells, which limits the application of the findings reported here only to the cells examined. Our findings here revealed that the modulation of the apparent Ca^{2+} sensitivity and subcellular localization by phosphorylation is channel subtype specific. In addition to studies on the SK channels, research focusing on the subtype-specific modulations of these channels by kinases and phosphatases will be needed in future studies of endothelial cells. Given the roles of SK3 and IK channels in the EDH-mediated vasodilatory response[16,61,62] and the protection by SK2 channels against endothelial cell death which we just reported (Fig. 4), there is no doubt about the importance of the SK channels in the vasculature. Pharmacological interventions targeted at SK channels might lead to promising strategies not only to relax vessels and reduce blood pressure[63] but also to protect the vascular endothelium.

Methods

Cultured endothelial cells

We utilized an immortalized mouse aorta endothelial cell line that we previously established and characterized, as a tool to study the differential modulation of SK channel subtypes. The generation and characterization of this immortalized cell line are descibed as follows.

Cell line generation: When we first planned to generate a mouse endothelial cell line, our strategy was to allow these cells to behave like primary cells isolated from an intact artery as much as possible[35]. The approach was to use transgenic C57BL/6J-Tg(SV)7Bri/J mouse with a regulatable Simian Virus-40 gene to propagate the cell line. Cell line propagation occurred when we turned on the gene with temperature at 33°C and chemical IFN-γ. When cells were grown at 37°C in the absence of IFN-γ, they would behave like primary cells. Our cell line can differentiate to retain markers seen in the intact arteries. These endothelial markers include Pecam-1 (CD31), VE-cadherin (CD144), N-cadherin (CD325), eNOS, Klf2, and Klf4[34–36]. In addition to shear-induced nitric oxide production and release, functional characteristics of our endothelial cells were equivalent to an intact artery include cell migration (wound healing), functional tight junction and shear-induced activation of Tgfβ/Alk5 signaling[34,36,64].

Stable expression of SK channel subtypes

We first established stable cell lines of the SK channel subtypes. The SK channel subtype cDNAs were constructed by custom cloning services of Genscript Inc. as fusion proteins

with the GFP at the N-termini in the pIRES-puro3 vector (Clontech Laboratories, Inc.). The cultured endothelial cells were transfected with SK channel subtypes plasmids using a Lipofectamine® 3000 Transfection Kit (Invitrogen). Stable clones were obtained through selection by puromycin and enrichment using repeated GFP fluorescence-activated cell sorting. The cells were cultured at 37 ℃ and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin, streptomycin and puromycin. Microscopy images of cells were acquired using a Nikon Confocal Laser Microscope and analyzed with NIS-Elements imaging software (Nikon instruments Inc).

Electrophysiology

The apparent Ca^{2+} sensitivity of SK channels was investigated as previously described[20,37,38]. For experiments with HEK293 cells, channel activities were recorded 1–2 days after transfection with channel cDNAs, using an inside-out patch configuration. For the non-transfected cultured endothelial cells, the inside-out patches did not exhibit any induced current to 10 μM Ca^{2+}, compared to nominal 0 Ca^{2+}. For experiments with cultured endothelial cells stably expressing SK channel subtypes, channel activities were measured 1–2 days after seeding, using an inside-out patch configuration with an Axon200B amplifier (Molecular Devices) at room temperature.

pClamp 10.5 (Molecular Devices) was used for data acquisition and analysis. The resistance of the patch electrodes ranged from 3–4.5 MΩ. The extracellular solution contained (in mM): 140 KCl, 10 Hepes (pH 7.4), 1 $MgSO_4$. The bath solution containing (in mM): 140 KCl, 10 Hepes (pH 7.2), 1 EGTA, 0.1 Dibromo-BAPTA, and 1 HEDTA was mixed with Ca^{2+} to obtain the desired free Ca^{2+} concentrations, calculated using the software by Chris Patton of Stanford University ((https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcS.htm). The Ca^{2+} concentrations were verified using a Ca^{2+} calibration buffer kit (Thermo Fisher Scientific). Briefly, a standard curve was generated using the Ca^{2+} buffers from the kit and a fluorescence Ca^{2+} indicator. Then the Ca^{2+} concentrations of our bath solutions were determined through interpolation on the standard curve.

Currents were recorded using an inside-out patch configuration. Currents were recorded when the intracellular side of the patch was exposed to different free Ca^{2+} concentrations (0 – 30 μM). Currents were recorded by repetitive 1-s-voltage ramps from − 100 mV to + 100 mV from a holding potential of 0 mV. One minute after switching of bath solutions, ten sweeps with a 1-s interval were recorded. The integrity of the patch was examined by switching the bath solution back to the zero-Ca^{2+} buffer. As such, the recorded currents came from Ca^{2+} sensitive channels, rather than leak of the patch. Data from patches, which did not show significant changes in the seal resistance after solution changes, were used for further analysis. To construct the dose-dependent activation of channel activities, the current amplitudes at − 90 mV in response to various concentrations of Ca^{2+} were normalized to that obtained at maximal concentration of Ca^{2+}. The normalized currents were plotted as a function of the concentrations of Ca^{2+}. EC_{50} values and Hill coefficients were determined by fitting the data points to a standard concentration–response curve ($Y = 100/(1 + (X/EC50)^{\wedge}$

− Hill)). All data are presented in mean ± s.e.m. The data analysis was performed using pClamp 10.5 (Molecular Devices) in a blinded fashion.

Cell fractionation

We performed cell fractionation of the non-transfected cultured endothelial cells and cells stably expressing the SK channel subtypes to investigate the subcellular localization, using the technique previously described[12,45]. Briefly, confluent cultured endothelial cells from 30 dishes (100 mm) were harvested. The cell pellet was resuspended and homogenized in a buffer containing 225 mM mannitol, 75 mM sucrose and 30 mM Tris-HCl (pH 7.4) supplemented with mass spectrometry-compatible phosphatase inhibitor and protease inhibitor cocktail (Thermo Fisher Scientific). The homogenate was centrifuged at 800 × *g* for 5 minutes to remove unbroken cells and nuclei. The supernatant was further centrifuged at 10000 × *g* for 10 minutes to pellet the mitochondria. The resulting supernatant was centrifuged at 25000 × *g* for 20 minutes to pellet the plasma membrane. For separation of microsomes (vesicles reformed from the ER membrane) and cytosolic proteins the resulting supernatant was centrifuged at 95000 × *g* for 2.5 hours. The concentrations of plasma membrane and ER fractions were determined by BCA protein assay kit (Thermo Scientific). Equal amounts of protein (15 ug) were separated by SDS-PAGE gel (Bio-Rad). The proteins were transferred to PVDF membranes and incubated overnight at 4 ℃ with primary GFP antibody (1:2000; Invitrogen, Lot: 2015993), SK1 antibody (1:1000; RayBiotech, Lot: 907002018), SK2 antibody (1:200; Alomone Labs, Lot: APC028AN2325), SK3 antibody (1:200; Alomone Labs, Lot: APC025AN1125), IK antibody (1:1000; Santa Cruz, Lot: I2319), mitochondria marker Cytochrome C antibody (1:1000; Novus Biologicals, Lot: AB0115109A-2), ER marker GRP78/HSPA5 (1:4000; Novus Biologicals, Lot: H-1) antibody or plasma membrane marker sodium potassium ATPase alpha 1 (1:4000; Novus Biologicals, Lot: B-5) antibody. The PVDF membranes were washed with TBST and incubated with anti-rabbit (1:3000; Cell signaling technology) or anti-mouse (1:2000; Cell signaling technology) as secondary antibody for 1 hour at room temperature and then washed with TBST. The chemiluminescent signals were detected on a ChemiDoc XRS system (Bio-Rad Laboratories) after incubation with Luminol/Enhancer Solution (Thermo Scientific).

Isolation of SK2 channel proteins and LC-MS/MS analysis

The SK2 channel in complex with CaM was isolated from the plasma membrane prepared from cell fractionation described above. Briefly, the pellets were solubilized in extraction buffer (20 mM n-dodecyl-β-d-maltopyranoside (DDM), 4 mM cholesteryl hemisuccinate (CHS), 300 mM KCl, 2 mM $CaCl_2$ and 20 mM Tris pH 8, supplemented with mass spectrometry-compatible phosphatase inhibitor and protease inhibitor cocktail (Thermo Fisher Scientific)) for 1.5 hours. The binding between CaM and the SK channels is strong enough to be purified as a protein complex with these detergents[4]. Solubilized membranes were clarified by centrifugation and immunoprecipitated using GFP-Trap_A beads (Chromotek). The beads were washed with 10 column volumes of wash buffer (0.5 mM DDM, 0.1 mM CHS, 150 mM KCl, 2 mM $CaCl_2$ and 20 mM Tris pH 8), before the channel proteins were cleaved off the immunoprecipitates, separated on SDS-PAGE gels and stained with Coomassie blue. In-gel digestion of the protein bands was performed using

sequence-grade trypsin and then subjected to LC-MS/MS. The digested peptide mixtures were analyzed by nanospray LC-MS/MS on an Orbitrap Fusion Tribrid (Thermo Scientific) coupled to an EASY-nano-LC System (Thermo Scientific) with a preferred OT-HCD-OT workflow. All data processing was performed in Proteome Discoverer 2.2 (PD) (Thermo Scientific) using the SEQUEST search engine with a FDR <0.01 at peptide and protein levels.

Cell death assay

One day after seeding, cultured endothelial cells were treated with a series of palmitate concentrations for 24 hours. Palmitate causes protein misfolding in the ER and activates the unfolded protein response and eventually ER stress. The cells were harvested and the apoptotic cell death was measured with the Annexin V/propidium iodide (PI) double staining and flow cytometry, using a modified protocol that we previously reported[45]. Briefly, cells were incubated in binding buffer containing Annexin V-Alexa Fluor 647 and PI (Invitrogen) at room temperature for 20 minutes. Flow cytometric analysis was performed with excitation at 635 nm and emission at 660 nm for Annexin V, together with excitation at 535 nm and emission at 617 nm for PI using the FACSVerse flow cytometer (BD Biosciences). Dead cells were quantified as the sum of Annexin V and Annexin V/PI-positive cells. Data were collected from 10,000 cells per condition.

Statistical Analysis

Two-way ANOVA and post hoc tests were used for data comparison between channel subtypes and drug treatments. One-way ANOVA and post hoc tests were used for data comparison between data of more than three groups. The Student's *t*-test was used for data comparison if there were only two groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ca^{2+}	Calcium
SK	Small-conductance Ca^{2+}-activated K^+ channels
mAHP	medium after hyperpolarization
ER	endoplasmic reticulum
EDH	endothelium-dependent hyperpolarization
PKA	cyclic AMP-dependent protein kinase

CK2	casein kinase 2
CaM	Calmodulin
GFP	green fluorescent protein
DsRed2-ER	ER membrane targeted red fluorescence protein
PM	plasma membrane
LC-MS/MS	tandem mass spectrometry
HEK	HEK293 cells
ET	immortalized endothelial cell culture

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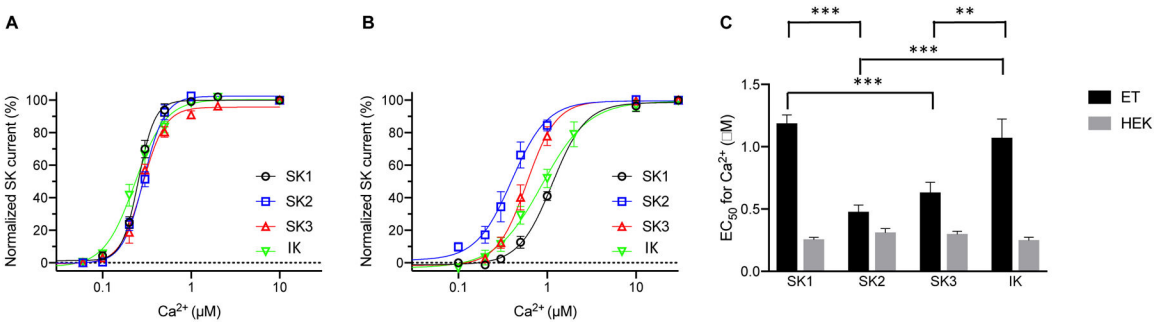

Fig. 1. The apparent Ca^{2+} sensitivity of SK1 and IK channel subtypes is more decreased than that of the SK3 and SK2 subtypes in immortalized endothelial cells.

Concentration-dependent activation by Ca^{2+} of the SK channel subtypes heterologously expressed in HEK293 cells (**A**) and immortalized endothelial cells (**B**). (**C**) EC_{50} values for the activation by Ca^{2+}, recorded from SK channel subtypes expressed in HEK293 cells (HEK) and immortalized endothelial cells (ET). All data are shown as mean ± S.E.M (n = 6–11, **P<0.01, ***P<0.001, Two-way ANOVA followed by Tukey's post hoc tests).

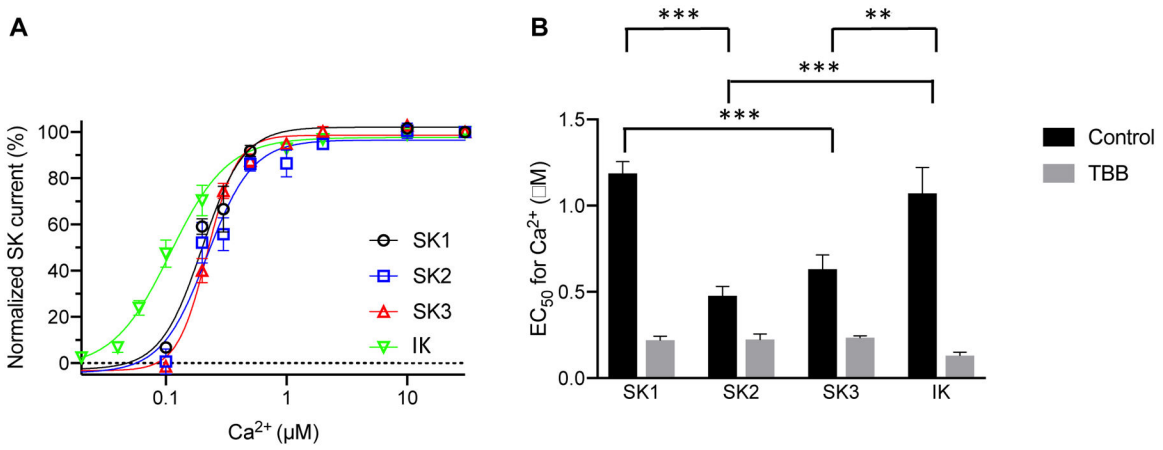

Fig. 2. The CK2 inhibitor TBB abolishes the differences between apparent Ca^{2+} sensitivity of SK channel subtypes in cultured endothelial cells.

(**A**) Concentration-dependent activation by Ca^{2+} of the SK channel subtypes heterologously expressed in immortalized endothelial cells in the presence of TBB (10 μM). (**B**) EC_{50} values for the activation by Ca^{2+}, recorded from SK channel subtypes expressed in the immortalized endothelial cells, in the absence (Control) and presence (TBB) of TBB. All data are shown as mean ± S.E.M (n = 6–9, **P<0.01, ***P<0.001, Two-way ANOVA followed by Tukey's post hoc tests).

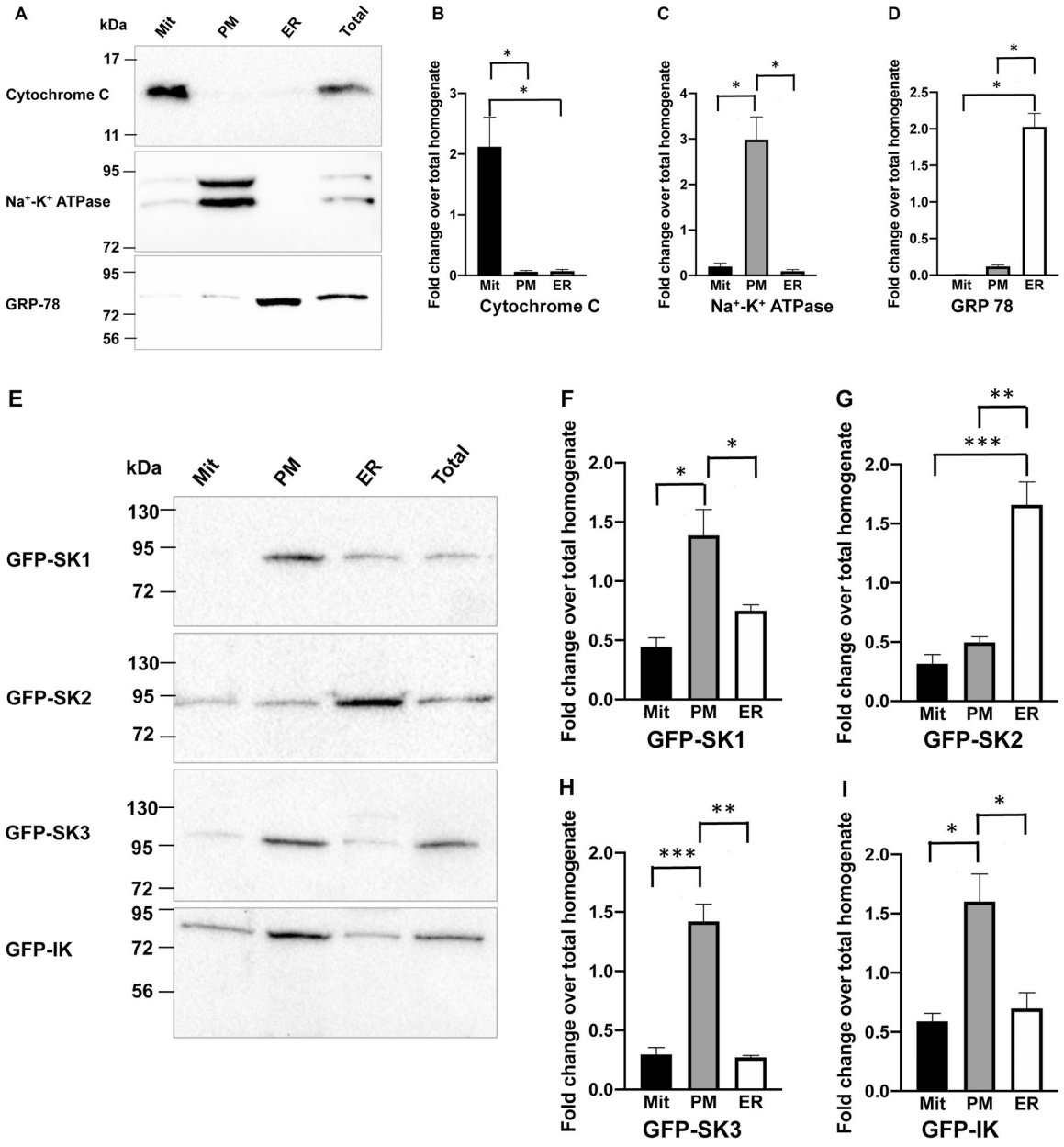

Fig. 3. Subcellular localization of the SK channel subtypes in immortalized endothelial cells.

(**A**) Cell fractions were verified by immunoblots with antibodies for mitochondria marker (Cytochrome C), ER marker (GRP-78) and plasma membrane marker (Na^+-K^+ ATPase). In (**B**), (**C**) and (**D**), densitometry results for the subcellular markers are summarized from 3–4 experiments. (**E**) In cell fractions, the SK2 channel subtype is abundantly expressed on the ER membrane, in contrast to other channel subtypes. In (**F**), (**G**), (**H**) and (**I**), densitometry results for the SK channel subtypes are summarized from 4–6 experiments. All data are shown as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, Two-way ANOVA followed by Tukey's post hoc tests.

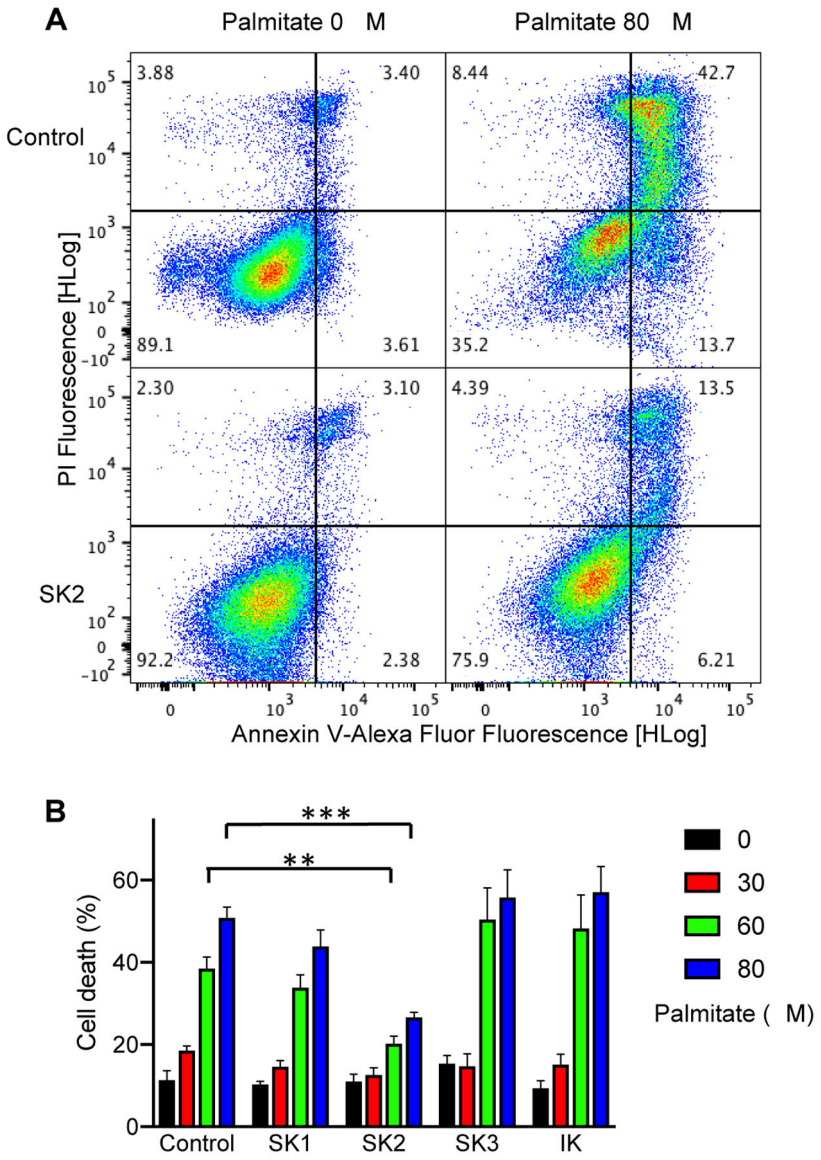

Fig. 4. The SK2 channel subtype protects the immortalized endothelial cells against apoptosis induced by palmitate.

(A) Representative flow cytometric analysis of non-treated cells and cells treated with 80 μM palmitate using double staining with Annexin V-Alexa Fluor 647/PI. Cells in the lower right quarter indicate AnnexinV-positive, early apoptotic cells. Cells in the upper right quarter indicate AnnexinV-positive/PI-positive, late apoptotic cells. (B) Quantification of cell death (the total of early and late apoptotic cells) after 24 h treatment with palmitate (mean ± S.E.M, n = 5; **P<0.01, ***P<0.001 compared with control parent cells, Two-way ANOVA followed by Tukey's post hoc tests).

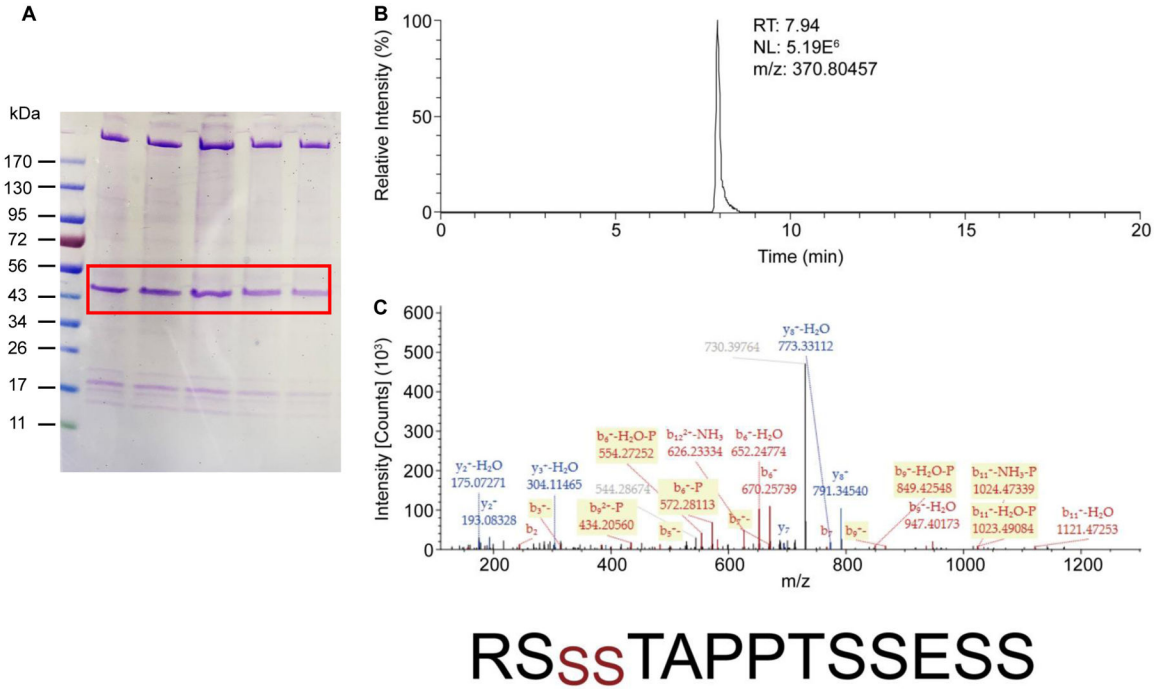

Fig. 5. Nano-LC/MS/MS analyses of SK2 protein isolated from the plasma membrane of the immortalized endothelial cells.

(**A**). The endothelial cells expressing SK2 channels were subjected to cell fractionation. SK2 channel proteins were purified from the plasma membrane fraction and then separated on SDS-PAGE gel. Protein bands at ~50 kDa and ~17 kDa correspond to SK2 channels (labeled in red box) and calmodulin, respectively. (**B**). The extracted ion-chromatograms of the peptide "^{567}RSSSTAPPTSSESS^{580}" of SK2 channels (doubly charged ion at m/z 370.80457). (**C**). The tandem mass spectrum of the peptide was acquired from the doubly charged precursor ion at m/z 370.80457. Fragment ion peaks as b- or y-type ions are also labeled in the spectrum. The peptide sequence including the phosphorylated sites at serines (red color) is indicated at the bottom.

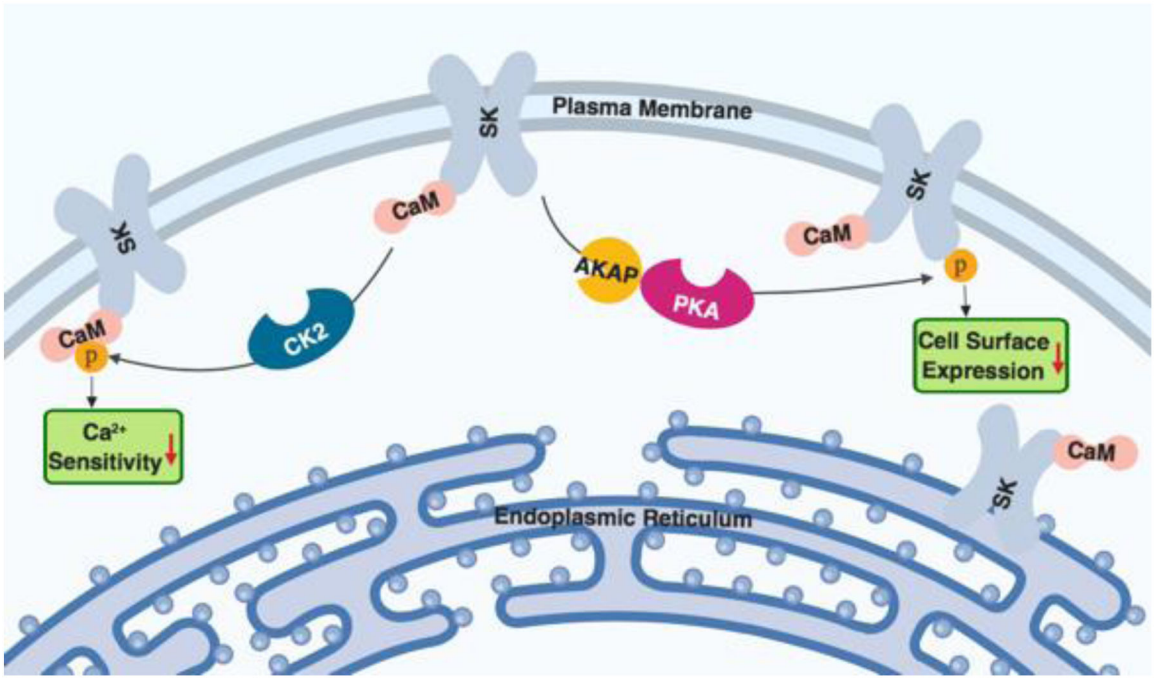

Fig. 6. SK channels are phosphorylated by kinases.

CK2 phosphorylates the CaM in complex with SK channels and leads to reduced apparent Ca^{2+} sensitivity. PKA phosphorylates the C-terminus of SK2 channels and reduces its cell surface expression. Counter-regulatory phosphatases are not shown for the purpose of clarity.