Aging-induced down-regulation of PKA/BK_{Ca} pathway in rat cerebral arteries

Na Li[1,#], Ruixiu Shi[2,#], Yang Ye[1], Yingying Zhang[1], Yumeng Zhang[3], Zhizhi Wang[1], Ying Gu[1], Yongxiang Yin[1], Daozhen Chen[1], Jiaqi Tang[3]

[#] These authors contributed equally to this work

[1]Perinatal Medicine Laboratory, The Affiliated Wuxi Maternity and Child Health Care Hospital of Jiangnan University, Wuxi, Jiangsu, China, [2]Department of Gynaecology and Obstetrics, Wuhan Fourth Hospital, Wuhan, Hubei, China, [3]Institute for Fetology, First Hospital of Soochow University, Suzhou, Jiangsu, China

*** Corresponding author**

Na Li, Perinatal Medicine Laboratory, The Affiliated Wuxi Maternity and Child Health Care Hospital of Jiangnan University, Wuxi, Jiangsu 214002, P.R. China. Email: 52smrehab@163.com. Daozhen Chen, Perinatal Medicine Laboratory, The Affiliated Wuxi Maternity and Child Health Care Hospital of Jiangnan University, Wuxi, Jiangsu 214002, P.R. China. Email: chendaozhen@163.com. Jiaqi Tang, Institute for Fetology, First Hospital of Soochow University, Suzhou, Jiangsu 215006, P. R. China. E-mail: tangjiaqi75@163.com

Summary

The incidence of cerebrovascular diseases increases significantly with aging. This study aimed to test the hypothesis that aging may influence the protein kinase A

(PKA)-dependent vasodilation via RyR/BK_{Ca} pathway in the middle cerebral arteries (MCA). Male Sprague-Dawley rats were randomly divided into control (4-6 month-old) and aged (24-month-old) groups. The functions of MCA and ion channel activities in smooth muscle cells were examined using myograph system and patch-clamp. Aging decreased the isoproterenol/forskolin-induced relaxation in the MCA. Large-conductance Ca^{2+}-activated-K^+ (BK_{Ca}) channel inhibitor, iberiotoxin, significantly attenuated the forskolin-induced vasodilatation and hyperpolarization in the young group, but not in the aged group. The amplitude and frequency of spontaneous transient outward currents (STOCs) were significantly decreased in the aged group. Single channel recording revealed that the mean open time of BK_{Ca} channels were decreased, while an increased mean closed time of BK_{Ca} channels were found in the aged group. The Ca^{2+}/voltage sensitivity of the channels was decreased accompanied by reduced BK_{Ca} α and β1-subunit, the expression of RyR2, PKA-Cα and PKA-Cβ subunits were also declined in the aged group. Aging induced down-regulation of PKA/BK_{Ca} pathway in cerebral artery in rats. The results provides new information on further understanding in cerebrovascular diseases resulted from age-related cerebral vascular dysfunction.

Keywords: Cerebral artery; Large-conductance Ca^{2+}-activated-K^+ channel; Aging; Vessel dilatation; Protein kinase A

Short title: Aging Damages Vasodilation/Membrane Hyperpolarization in MCA

Introduction

The world's population is ageing. About one fifth of the world's population will be aged 65 or older by 2030 [1]. According to the seventh national census in 2020, China's elderly population (aged 60 and above) had reached264.02 million, taking up 18.7% of total population. With the increasing number of elderly population, the

incidence of cardiovascular disease (CVD) which remains the leading cause of human death is also increased exponentially in many counties [1,2]. Stroke is one of the most common causes of death and is the main cause of persistent and acquired disability in adults worldwide [3]. Aging is known to induce an increase in cerebral vascular tone, therefore decreasing the cerebral blood flow and raising the risks of stroke [4]. The middle cerebral artery (MCA) is the largest artery of the cranial vessels and distributes 80% of the blood flow to the brain [5]. With age progresses, MCA became stiffer, thicker, and of higher spontaneous contractile activities [6]. Those changes increase vasospasm of MCA and risks of stroke [7,8]. Thus, knowledge involved in vascular ageing in cerebral circulation is required to develop strategies to preserve quality of life for an increasingly ageing population.

The large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels that are abundantly expressed in VSMCs act as critical regulators of membrane potential in adjusting cellular and vascular tone [9]. Since aging and vascular dysfunction seems to go hand in hand, it is a priority to identify ion channels in the cerebral myocytes that are involved in age-induced changes and to unravel the mechanisms of these changes. Local Ca^{2+} release through ryanodine-sensitive channels (RyRs) in the sarcoplasmic reticulum (SR), which is partially due to the phosphorylation of RyRs through the activation of protein kinase A (PKA) [10,11], activating the BK_{Ca} channels nearby in the membrane, producing large outward-currents, hyperpolarizing and relaxing vascular SMCs [12,13]. Previous studies showed that the number of BK_{Ca} channels were reduced with aging as well as the BK_{Ca} current density was decreased in the coronary [14] and mesenteric [15] arteries . However, researches on whether and how aging could impair the cerebral vascular tone remains limited.

Therefore, this study applied functional (pharmacomechanical and electrophysiological) experiments that were followed by immuno-fluorescence staining and PCR results to test the hypothesis that aging might influence cerebral artery vasodilatation via the PKA/BK_{Ca} signaling pathway. The knowledge gained from the experiments may potentially help to understand the effects of aging on the vasculature, and to preserve the quality of life, and alleviate cerebrovascular diseases

in the growing older population [16].

Material and methods

Animals

Sprague-Dawley rats (4-month-old) were obtained from Animal Center of Soochow University. The rats were housed in regular cages at 22±1°C in a 12-h light/dark cycle controlled environment with free access to a standard chow diet and water until 24 months old before testing. In this study, mature adult rats (8 month-old) served as the control group (20 rats/group) while old rats (24-month-old) served as the aged group (20 rats/group). All efforts were made to minimize animal suffering. All animal care and procedures were complied with the European Guidelines on Laboratory Animal Care, and were approved by the Institutional Animal Care and Use Committee of Jiangnan University.

Tissue preparation

Rats were euthanized with isoflurane inhalation (3%). Their brains were immediately removed from the skull and immersed in pre-oxygenated (95% O_2 , 5% CO_2 for 30 min) PSS solution containing (mM: 135 NaCl, 0.44 NaH_2PO_4 , 5.6 KCl, 4.2 $NaHCO_3$,1 $MgCl_2$, 10 HEPES, 0.42 Na_2HPO_4 , and 10 glucose, pH 7.35-7.45 with NaOH) at 4°C. The basilar artery, middle cerebral artery and circle of Willis artery were carefully removed from meningeal tissue under a stereomicroscope. Cerebral arteries were cut into cylindrical segments for *in vitro* pharmacological studies. The remaining were used for electrophysiological and cell immuno-fluorescence experiments, or frozen in liquid nitrogen and store at -80°C for real-time PCR.

Vascular ring myograph study

Cerebral arteries were isolated as previously described and placed in an ice-cold physiological solution (PSS) with continuously bubbled with 5% CO_2 in O_2 and

containing: (mM: NaCl, 125; $NaHCO_3$, 13.5; KCl, 4.6; $CaCl_2$, 2.5; EDTA, 0.025; NaH_2PO_4, 1.2; and glucose, 10.0; pH:7.4 with NaOH). Immediately, the MCA were cut into 3 mm rings and mounted on a myograph system (Danish Myo Technology, Denmark) [17], filled with oxygenated PSS at 37℃ and continuously equilibrated with 95% O_2-5% CO_2. The rings were adjusted to maintain a suitable passive force and equilibrated for 60 minutes. Then, they were repeatedly exposed to KCl (120 mM) to confirm the viability and integrity of MCA rings [18]. Subsequently, vessels were first contracted by 5-hydroxytryptamine (5-HT, 10^{-6} M), which allowed to reach a stable maximal level of contraction. Then, MCA rings were relaxed with cumulative concentrations of isoproterenol (ISO,10^{-14}-10^{-4} M) or forskolin (FK, 10^{-11}-10^{-5} M). Rp-cAMP (5×10^{-5} M) or iberiotoxin (IBTX, 10^{-7} M) or ryanodine [Ry(-), 10^{-5} M] was used to pretreat the segments for 40 minutes before application of ISO or FK. Signals were recorded by Power-Lab system with Chart 7 software (AD Instruments, Castle Hill, New South Wales, Australia).

Cell isolation

Single smooth muscle cells were isolated from cerebral arteries using an enzymatic dissociation procedure as previously described [18,19]. Cerebral arteries were evenly cut into small pieces (1mm length), and incubated in mixture enzyme solutions containing papain (5mg/ml, Solarbio, China), ABV (2mg/ml, BioSHARP, China) and dithiothreitol (1g/ml, BioSHARP, China) for 37 min at 37° C. Then, digested solutions were replaced by Ca^{2+}-free PSS buffer for 3 times and fragments were blended gently using a pipette to release single muscle cells. All cells were stored in Ca^{2+}-free PSS buffer at 4° C for electrophysiological experiments within 6 hours after isolation.

Electrophysiological experiments

Single channel recording

The inside-out and cell-attached configurations were recorded under symmetrical K^+ (145 mM) at room temperature [20]. The pipettes (15~20 MΩ) and bath solutions contained (mM): 145.0 KCl, 1.0 EGTA, 10.0 HEPES, and 5.0 glucose, with pH

adjusted to 7.2 and 7.4 with KOH , respectively. Ca^{2+} ($CaCl_2$) was added to achieve the desired free-Ca^{2+} concentrations in the bath solution [determined by using Max Chelator software (Chris Patton, Stanford University; http://maxchelator.stanford.edu/webmaxc/webmaxcS.htm) [21]. Currents were sampled at 20 kHz and filtered at 2 kHz with a Bessel filter (8-pole). Continuous recordings of no less than 15,000 ms were used for open probability (Po) and kinetics analysis. We used the channel number per patch (N) and open probability (Po) as an index of BK_{Ca} steady-state activity. The normalized open probability (NPo) represents BK_{Ca} activity, calculated using the following equation: NPo=Σ (t1 + t2…ti), where ti is the relative open time (time open/total time) for each channel level. The number of BK_{Ca} per patch was determined while patches were held at +80 mV in the presence of 10 μmol/L Ca^{2+} in the bath solution. Po=NPo/N. The voltage sensitivity data were fitted with Boltzmann equation: $Po=1/\{1+ \exp [-ZF/RT (V-V_{1/2})]\}$,

Po=1/{1+exp[(-ZF/RT)(V-V1/2)]}

where $V_{1/2}$ is the voltage of half-maximal channel activation. The NPo was normalized to the max probability. Here, the values of NPo were normalized to the NPo value at 100mV. Data acquisition and analysis were carried out using pCLAMP 10.2 and Clampfit 10.2 software (Axon Instruments, Foster City, CA).

Whole-cell recording

Spontaneous transient outward currents (STOCs) were measured at gap-free mode using perforated whole-cell patch-clamp technique. The bath solution was (mM): 135 NaCl, 5 KCl,1 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). Patch pipettes (3-5MΩ) were filled with an internal solution containing (mM) 110 K-Asp,30 KCl, 1 $MgCl_2$, 10 HEPES, 0.5 EGTA, 3 Na_2ATP and 0.2 amphotericin B (pH 7.2 with KOH). STOCs were recorded over a range of holding potential (HP) from -50 mV to 0 mV. Signals were sampled at 10 kHz, filtered at 2 kHz. Data were captured on-line using a Digidata 1440 interface run under the pClamp 10.2program (Axon Instruments, Foster City, CA). STOCs were analyzed and plotted using MiniAnalysis Program (Synaptosoft, Inc., Decatur, GA).

Membrane potential measurements

The current patch-clamp configuration was used to investigate the membrane potential at gap-free mode, where the membrane current was clamped at 0 pA [23]. The bath solution contained (mM): 135 NaCl, 10 glucose, 1 $MgCl_2$, 10 HEPES, 5 KCl and 1.8 $CaCl_2$, (pH 7.4 with NaOH). Patch pipettes (3-5MΩ) were filled with an internal solution containing (mM) 110 K-Asp, 30 KCl, 1 $MgCl_2$, 0.5 EGTA, 10 HEPES, 3 Na_2ATP and 0.2 amphotericin B (pH 7.2 with KOH). Data were captured on-line using a Digidata 1440 interface run under the Axon Multiclamp 700B with Clampex 10.2 program (Axon Instruments, Foster City, CA). Forskolin (10^{-5} M) was added once the basis of potential achieved equilibrium. Iberiotoxin (IBTX, 10^{-7} M) was added once maximal hyperpolarization was obtained.

Cell immuno-fluorescence staining

Single vascular SMCs were isolated from cerebral arteries using an enzymatic dissociation procedure [22]. Cells were plated on fibronetin-coated glass coverslips for approximately 30 min at room temperatur. Then, SMCs were treated with fixer solution made of 4% paraformaldehyde in phosphate buffered solution (PBS, Na_2HPO_4 8mM, NaCl 136mM, NaH_2PO_4 2mM, pH7.2-7.4,) for 20 min, following a permeabilized treatment with 0.2% Triton X-100 for 10 mins. After 3 times' washing, cells were incubated in 5% bovine serum albumin in PBST [Na_2HPO_4 8mM, NaCl 136mM, KH_2PO_4 2mM, KCL 2.6 mM, Tween-20 0.05% (V/V), pH7.2-7.4] for 1 hour at 37℃ before addition of an antibody at appropriate dilution (1:400 for $K_{Ca}1.1$; 1:300 for sloβ1; 1:500 for RyR2, Alomone Labs) and further incubated for 4℃ overnight. The cells were then washed with PBST and incubated with a fluor-conjugated secondary antibodies and 4',6'-diamidino2-phenylindole (Invitrogen) for 1 hour at 37℃. Following PBST washing, the images were captured with a Leica SP5 system.

Real-time PCR

The mRNA abundance in MCA were tested using Trizol reagent, and then reversed transcribed using the first-strand cDNA Synthesis Kit (Invitrogen). Primer sequences were listed in Table 1. Real-time PCR was performed in 20 μl system

including 10 μl of SYBR Premix (Takara), 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer(10 μM), 1 μl cDNA, and 8 μl of nuclease free water. The information of primer pairs was shown in Table1 . The PCR was carried out using iCycler, MyiQ two Color Real-Time PCR Detection System (Bio-Rad) according to the following program: one cycle at 95°C for 5 min; 40 cycles at 95°C and 60 °C for 15 seconds each. Each assay was repeated three times, and the relative gene expression (RGE) was calculated as $RGE = 2^{-\Delta\Delta Ct}$, and the relative expression level was determined on the basis of the comparative ΔΔCt method using the reverse transcription products from the control group as the calibrator.

Drugs

All reagents were obtained from Sigma-Aldrich unless otherwise stated.

Statistical analysis

The statistical analysis was carried out using GraphPad Prism 5 (GraphPad software). Data are expressed as mean SEM. Repeated measures two-way ANOVA and Unpaired Student t test were used to determine P values. $P<0.05$ was considered significant.

Results

PKA contributed to the impaired ISO-induced relaxation in aged middle cerebral arteries

The body weight was significant increased in aged group, however, there was no significant differences in the brain weight between the control and aged groups (Fig. 1A). ISO produced dose-dependent relaxations following 5-HT-caused contraction in both young and aged group (Fig. 1B,C). However, such dilation was greatly weakened in the aged group (Fig. 1C, pD_2:8.49±0.11 versus 8.96±0.07, control vs. aged, $p<0.05$). Since the ISO-induced vasodilatation could be mediated by cAMP/PKA pathway [23], we then evaluated the effect of cAMP/PKA pathway on

ISO-induced relaxation in the presence of PKA inhibitor, Rp-cAMP. Pretreatment with Rp-cAMP (5×10^{-5} M) significantly decreased the vasodilatations induced by ISO in both groups. Moreover, it seemed that the inhibitor effect by blockade of PKA was greater than that in the aged group, indicating that the PKA pathway could be impaired by aging (Fig. 1D,E).

Aging-decreased vascular dilation was correlated with PKA/BK_{Ca} pathway

We then used the cAMP/PKA activator, forskolin, to confirm our hypothesis. Obviously, the forskolin-induced vasodilation was more pronounced in the control group (Fig. 2A,B). The MCA from aged group were significantly insensitive to cumulative doses of FK with lower pD2 (-log[50% effective concentration]) as compared with the control (Fig. 2C). It is reported that the activation of BK_{Ca} channels plays an important role in the regulation of PKA-mediated relaxation [24]. We then examined the contribution of BK_{Ca} channels to forskolin-induced relaxation in the presence of BK_{Ca} inhibitor. IBTX(10^{-6} M) partially inhibited the relaxation induced by forskolin in both groups. However, the inhibitory effect was weaker in the aged group as compared with the control (Fig. 2D,E). These results suggest that the ISO-induced vasodilatation was decreased in the age group via PKA/BK_{Ca} pathway.

Aging decreased the FK and IBTX-caused membrane potential modulation and single channel currents in cerebral SMCs

To test the effect of PKA/BK_{Ca} pathway on the modulation of membrane hyperpolarization in the aged MCA, current-clamp were used to measure the changes of membrane potential under the stimulation of PKA activator and the inhibitor of BK_{Ca} channels. Figure 3A illustrates the IBTX-caused depolarization following FK induced hyperpolarization in isolated single cerebral myocytes. The FK-induced hyperpolarization was much weaker in the aged group (Fig. 3B), and the depolarization by IBTX was also blunted (Fig. 3C). These results together with those *in vitro* vascular experimental data, indicated that the aging-reduced sensitivity of MCA to PKA could be related to BK_{Ca} channel functions. Subsequently, the effect of

PKA activation on single BK_{Ca} channel was tested using cell-attached patch (Fig. 3D). Agonist of PKA (8-Br-cAMP) markedly increase the NPo of BK_{Ca} channels in both groups. Application of 10^{-6} M 8-Br-cAMP evoked approximately 3.41±0.50-fold of increase in NPo in the control, while only 1.89±0.38-fold of increase was observed in the aged group (Fig.3E). Thus, the activation of BK_{Ca} by PKA agonist was descended in the aged group. We further detected the mRNA expression of PKA-Cα and Cβ subunits in the MCA. The expression of both subunits were significantly reduced (Fig. 3F), which may contribute to the down-regulation of PKA to BK_{Ca} currents in the aged group.

Biophysical properties of the BK_{Ca} channels in aged cerebral SMCs

The dysfunction of PKA/BK_{Ca} pathway could be due to the intrinsic alteration of the channels. Therefore, the influence of aging on the biophysical properties of BK_{Ca} channels was examined using inside-out patch-clamp. Ca^{2+}-sensitivity of BK_{Ca} channels was assessed with $[Ca^{2+}]_i$ at two physiological concentrations of intracellular free Ca^{2+} (1 and 10 μM, Fig. 4A). The NPo of BK_{Ca} channels at both Ca^{2+} concentrations was lower in the aged group than that in the control group (Fig. 4B). The Po-voltage relationships were fitted with the Boltzmann distribution to determine the voltage for half activation of the channels ($V_{1/2}$). The half activation voltage was right-ward shifted from 40.96±3.49 mV (control) to 62.97±2.05 mV (aged) in the presence of 1 μM $[Ca^{2+}]_i$ (Fig. 4C). We further investigated the effects of aging on the gating properties of the BK_{Ca} channels. The mean open time was profoundly reduced in the aged group (Fig. 4D) while the mean close time was significantly increased (Fig. 4E).

To investigate molecular changes that caused the functional modifications of BK_{Ca} channels, we examined the protein and mRNA expression of BK_{Ca} channel α and β1-subunits in cerebral SMCs. As shown in Figure 4, the mean fluorescence density and the mRNA of the two subunits were distinctly reduced in the aged group (Fig. 4F-H). Taken together, the BK_{Ca} biophysical properties were impaired resulting from the down-regulation of both α and β1-subunits in the aged group.

Aging reduced spontaneous transient outward currents (STOCs)

Transient release of Ca^{2+} from the endoplasmic reticulum, which activates BK_{Ca} channels, leading to STOCs was reported to be one of the key mechanisms in the regulation of vessel relaxation [24]. Since PKA could dilate arteries through activation of STOCs [11], we tested activities of functional STOCs in the forskolin-caused dilation. *In vitro* experiments showed that the inhibition caused by ryanodine (an inhibitor of RyRs) was much weaker in the aged group than that in the control (Fig. 5 A,B), indicating that the function of RyRs could probably be impaired with aging. STOCs were recorded from the isolated MCA myocytes using perforated patch-clamp. As shown in Figure 5, both amplitude and frequency of STOCs in MCAs were reduced with aging at the same HP (Fig. 5C-E). To test the effect of PKA activation on the activity of STOCs, cells were treated with the PKA agonist 8-Br-cAMP. In both groups, 8-Br-cAMP triggered an immediate increase in STOCs activity, however, it was weaker in the aged group (Fig. 5F,G). Therefore, we suspected that the function of the RyR channels activated by PKA phosphorylation in the sarcoplasmic reticulum might be impaired, leading to reduced sensitivity to upstream PKA regulation and decreased STOCs frequency in the aged group.

Since the RyR2 is the predominant molecular and functional RyR subtype that is expressed in the SR membrane of cerebral arteries [25], we examined the expression of RyR2 in cerebral SMCs in two groups. The intensity of the fluorescence comfocal signal and the mRNA expression of the RyR2 were both reduced in the aged group (Fig. 6). These results suggest that the down-regulation of BK_{Ca} channels by PKA via RyR2 was involved into the PKA/BK_{Ca} dysfunctions in the aged MCA.

Discussion

This study used rat models to reveal the effects of aging on MCA vascular functions and clarify its underlying mechanisms. The data gained in this study suggest that aging disrupted the relaxation mechanism in MCA myocytes and manifested the process of impaired regulation of PKA/BK_{Ca} pathway during relaxation. The major

novel findings are summarized as followings: (1) MCA relaxation was decreased in response to isoproterenol and forskolin in the aged rats. (2) Such vascular insensitivity was linked to dysfunctions of PKA and its downstream BK_{Ca} channels. Together, this study provides direct evidence showing that ageing could damage the structure and functions of PKA related to a down-regulation of RyR and BK_{Ca} channels in SMCs, resulting in attenuated vasodilatation in cerebral arteries. Figure 7 summarized the working model.

Aging is known to influence cellular excitability, causing impaired cell functions. During the process of aging, the vascular system undergoes a series of deleterious adaptations that may cause vascular diseases [6]. Aging-related vascular changes may occur at the endothelium or smooth muscle cells. In SMCs, the activation of BK_{Ca} channels can be promoted by activation of the cAMP/PKA signaling pathway. We evaluated the activation of BK_{Ca} channels by stimulating the cAMP/PKA at organ and cell levels. The ISO-induced dilation was partly insensitive to PKA inhibitor, suggesting the existence of other pathways involved in the relaxation mechanisms, eg., the NO dependent mechanism [26,27]. Besides, the forskolin-induced dilation was partially insensitive to IBTX or ryanodine, especially at higher concentrations of forskolin, suggesting that except the effects on BK_{Ca} channels, other mechanisms may also contribute to the forskolin mediated vasodilation, eg. the ATP-sensitive potassium (K_{ATP}) channels [28] or delayed rectifier K^+ channels [29]. How these channels are involved into the process of vascular aging deserve further investigation.

BK_{Ca} channels are key regulators of arterial tone in many regions of the vascular tree and in various smooth muscles due to its abundant expression and high conductance [9,30]. As it is known, the BK_{Ca} channels consist of four pore-forming α subunits which is responsible for the basic ion flux functions of BK_{Ca} channels, and four regulatory β1-subunits which modulate the kinetics voltage /Ca^{2+} sensitivity of the channels [31]. Previous studies indicated that aging reduced BK_{Ca} channel expression and functions in the coronary and mesenteric arteries [15,32] as well as reduced the functions and molecular expression of the β1 but not pore-forming α subunit in the cerebrovascular myocytes [33]. Due to the sample size was usually

small (most of them were less than 10 animals), variations were noted among reports. This study used larger sample size (n>20 animals) as well as defined "adult/control group" as the age when skeletal growth tapers off (7-8 months) not just sexual maturity, which is more closely related to human development and physiology. The results showed that the mRNA expression of both subunits were decreased, and the functional β1 (Ca^{2+}/voltage sensitivity) was impaired, affecting vasodilation in central arteries by aging. This also provides novel information that targeting certain ion channel subunits may be helpful in exploring strategies to prevent or delay aging-mediated vascular impairment.

PKA holoenzyme is an inactitive tetramer consisting of two regulatory subunits and two catalytic subunits (Cα, Cβ). cAMP binds to PKA regulatory subunits, thereby releasing catalytic subunits, permitting phosphorylase activities of PKA [34]. The decreased mRNA expression of Cα and Cβ in the aged group suggests that ageing process is based upon the influence of down-regulated PKA on BK_{Ca} channels in MCA. PKA could activate the ryanodine-sensitive calcium-release channels (RyRs), leading to ryanodine-sensitive Ca^{2+} release from the SR, which activates BK_{Ca} channels to induce STOCs [35]. Thus, the RyRs-BK_{Ca} coupling in the SR of SMCs should be assessed. Our experiments investigated the activation of RyRs-BK_{Ca} in the forskolin-induced relaxation. *In vitro* experiments showed that the inhibitory effect of sparks inhibitor, ryanodine (10^{-5}mM), was weaker in the aged group than that in the control, suggesting that the functional RyR channels were probably impaired. Moreover, the perforated-patch configuration was used to measure STOCs that represents the coupling between local Ca^{2+} signaling and the BK_{Ca} channels in mediating smooth muscle relaxation [24]. The perforated whole-cell recording demonstrated a significant decrease in amplitude of STOCs in cerebral arterial myocytes of the aged group at the same HP. At the holding potential of -10mV, a distinct decrease in both the amplitude and frequency of STOCs was observed in the aged group, suggesting aging weakened the negative-feedback regulation of RyRs-BK_{Ca} coupling in membrane depolarization. Further addition of PKA agonist (8-Br-cAMP) increased the activity of STOCs, however, the sensitivity of STOCs to

8-Br-cAMP was reduced in the aged group. In consideration of the correlated relationship between STOCs frequency/amplitude and spark [24], we suspected that the function of RyRs was altered by aging. RyR2 is the predominant ryanodine receptor expressed in the SR membrane of cerebral SMCs [25]. A molecular experiment showed a decreased mRNA and protein expression of RyR2, further confirming a dysfunction of RyRs-BK_{Ca} in mediating PKA-induced cerebrovascular relaxation.

What are the mechanisms involved in age-induced down-regulation of BKα and β1 subunit expression? Previous study reported that the BK_{Ca} channels could be altered with aging via β1 subunit promoter methylation [36,37], it is reasonable to consider DNA methylation is part of an epigenetic regulatory mechanism involved in aging. Further research would focus on the aged cerebral vessels in the presence and absence of DNA methylation treatments to test whether DNA methylation plays a causative role in age-induced down-regulation of BK_{Ca} subunits.

Taken together, the present study provided new functional and molecular evidence showing that aging would depress the vasorelaxation via damaging the PKA/BK_{Ca} functions in cerebral SMCs. The novel findings offer important information on the mechanism underlying age-induced vascular pathophysiological alteration, holding new insight for developing strategies in reducing incidence of the cerebral vascular diseases in aged population.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Wuxi Comminssion of Health and Family Planning (Q201204), the Natural Science Foundation of China (81500322), the Wuxi Taihu Rencai Project Fund and the Wuxi Taihu Talent Program (BJ2020072)

References

Costantino S, Paneni F, Cosentino F. Ageing, metabolism and cardiovascular disease. *J Physiol*. 2016;594(8):2061-2073.

[2] Fleg JL, Strait J. Age-associated changes in cardiovascular structure and function: a fertile milieu for future disease. *Heart Fail Rev*. 2012;17(4-5):545-554.

Sarikaya H, Ferro J, Arnold M. Stroke prevention--medical and lifestyle measures. *Eur Neurol*. 2015;73(3-4):150-157.

[4] Kalaria RN. The role of cerebral ischemia in Alzheimer's disease. *Neurobiol Aging*. 2000;21(2):321-330.

Nagata K, Yamazaki T, Takano D, Maeda T, Fujimaki Y, Nakase T, Sato Y. Cerebral circulation in aging. *Ageing Res Rev*. 2016;30:49-60.

Toro L, Marijic J, Nishimaru K, Tanaka Y, Song M, Stefani E. Aging, ion channel expression, and vascular function. *Vascul Pharmacol*. 2002;38(1):73-80.

Faraci FM, Heistad DD. Regulation of large cerebral arteries and cerebral microvascular pressure. *Circ Res*. 1990;66(1):8-17.

[8] Foulquier S, Lartaud I, Dupuis F. Impact of short-term treatment with telmisartan on cerebral arterial remodeling in SHR. *PLoS One*. 2014;9(10):e110766.

[9] Hu XQ, Zhang L. Function and regulation of large conductance Ca(2+)-activated K^+ channel in vascular smooth muscle cells. *Drug Discov Today*. 2012;17(17-18):974-987.

[10] Song Y, Simard JM. beta-Adrenoceptor stimulation activates large-conductance Ca^{2+}-activated K^+ channels in smooth muscle cells from basilar artery of guinea pig. *Pflugers Arch*. 1995;430(6):984-993.

[11] Porter VA, Bonev AD, Knot HJ, Heppner TJ, Stevenson AS, Kleppisch T, Lederer WJ, Nelson MT. Frequency modulation of Ca^{2+} sparks is involved in regulation of arterial diameter by cyclic nucleotides. *Am J Physiol*. 1998;274(5):C1346-C1355.

[12] Standen NB, Quayle JM. K^+ channel modulation in arterial smooth muscle. *Acta Physiol Scand*. 1998;164(4):549-557.

[13] Barman SA, Zhu S, White RE. Hypoxia modulates cyclic AMP activation of

BK_{Ca} channels in rat pulmonary arterial smooth muscle. *Lung*. 2005;183(5):353-361.

[14] Nishimaru K, Eghbali M, Lu R, Marijic J, Stefani E, Toro L. Functional and molecular evidence of MaxiK channel beta1 subunit decrease with coronary artery ageing in the rat. *J Physiol*. 2004;559(Pt 3):849-862.

[15] Shi L, Liu X, Li N, Liu B, Liu Y. Aging decreases the contribution of MaxiK channel in regulating vascular tone in mesenteric artery by unparallel downregulation of α- and β1-subunit expression. *Mech Ageing Dev*. 2013;134(9):416-425.

[16] Martin GM. Frontiers of aging. *Science*. 2001;294(5540):13.

[17] Li N, Li Y, Gao Q, Li D, Tang J, Sun M, Zhang P, Liu B, Mao C, Xu Z. Chronic fetal exposure to caffeine altered resistance vessel functions via RyRs-BK_{Ca} down-regulation in rat offspring. *Sci Rep*. 2015;5:13225. Published 2015 Aug 17.

[18] Xu T, Fan X, Zhao M, et al. DNA Methylation-Reprogrammed Ang II (Angiotensin II) Type 1 Receptor-Early Growth Response Gene 1-Protein Kinase C ε Axis Underlies Vascular Hypercontractility in Antenatal Hypoxic Offspring. *Hypertension*. 2021;77(2):491-506.

[19] Li N, Liu B, Xiang S, Shi L. Similar enhancement of BK(Ca) channel function despite different aerobic exercise frequency in aging cerebrovascular myocytes. *Physiol Res*. 2016;65(3):447-459

[20] Li N, Shi Y, Shi L, Liu Y, Zhang Y. Effects of aerobic exercise training on large-conductance Ca(2+)-activated K (+) channels in rat cerebral artery smooth muscle cells. *Eur J Appl Physiol*. 2013;113(10):2553-2563.

[21] Bers DM, Patton CW, Nuccitelli R. A practical guide to the preparation of Ca(2+) buffers. *Methods Cell Biol*. 2010;99:1-26. doi:10.1016/B978-0-12-374841-6.00001-3.

[22] Pucovský V, Bolton TB. Localisation, function and composition of primary Ca(2+) spark discharge region in isolated smooth muscle cells from guinea-pig mesenteric arteries. *Cell Calcium*. 2006;39(2):113-129.

[23] Satake N, Shibata M, Shibata S. The inhibitory effects of iberiotoxin and 4-aminopyridine on the relaxation induced by beta 1- and beta 2-adrenoceptor activation in rat aortic rings. *Br J Pharmacol*. 1996;119(3):505-510.

[24] Manoury B, Idres S, Leblais V, Fischmeister R. Ion channels as effectors of

cyclic nucleotide pathways: Functional relevance for arterial tone regulation. *Pharmacol Ther*. 2020;209:107499.

[25] Vaithianathan T, Narayanan D, Asuncion-Chin MT, Jeyakumar LH, Liu J, Fleischer S, Jaggar JH, Dopico AM. Subtype identification and functional characterization of ryanodine receptors in rat cerebral artery myocytes. *Am J Physiol Cell Physiol*. 2010;299(2):C264-C278.

[26] Akimoto Y, Horinouchi T, Shibano M, Matsushita M, Yamashita Y, Okamoto T, Yamaki F, Tanaka Y, Koike K.. Nitric oxide (NO) primarily accounts for endothelium-dependent component of beta-adrenoceptor-activated smooth muscle relaxation of mouse aorta in response to isoprenaline. *J Smooth Muscle Res*. 2002;38(4-5):87-99.

[27] Flacco N, Segura V, Perez-Aso M, Estrada S, Seller JF, Jiménez-Altayó F, Noguera MA, D'Ocon P, Vila E, Ivorra MD. Different β-adrenoceptor subtypes coupling to cAMP or NO/cGMP pathways: implications in the relaxant response of rat conductance and resistance vessels. *Br J Pharmacol*. 2013;169(2):413-425.

[28] Zhang L, Bonev AD, Mawe GM, Nelson MT. Protein kinase A mediates activation of ATP-sensitive K+ currents by CGRP in gallbladder smooth muscle. *Am J Physiol*. 1994;267(3 Pt 1):G494-G499.

[29] Cole WC, Clément-Chomienne O, Aiello EA. Regulation of 4-aminopyridine-sensitive, delayed rectifier K^+ channels in vascular smooth muscle by phosphorylation. *Biochem Cell Biol*. 1996;74(4):439-447.

[30] Fan G, Cui Y, Gollasch M, Kassmann M. Elementary calcium signaling in arterial smooth muscle. *Channels (Austin)*. 2019;13(1):505-519.

[31] Szteyn K, Singh H. BK_{Ca} Channels as Targets for Cardioprotection. *Antioxidants (Basel)*. 2020;9(8):760. Published 2020 Aug 17.

[32] Nishimaru K, Eghbali M, Lu R, Marijic J, Stefani E, Toro L. Functional and molecular evidence of MaxiK channel beta1 subunit decrease with coronary artery ageing in the rat. *J Physiol*. 2004;559(Pt 3):849-862.

[33] Reed JT, Pareek T, Sriramula S, Pabbidi MR. Aging influences cerebrovascular myogenic reactivity and BK channel function in a sex-specific manner. *Cardiovasc*

Res. 2020;116(7):1372-1385.

[34] Baro Graf C, Ritagliati C, Stival C, Luque GM, Gentile I, Buffone MG, Krapf D. Everything you ever wanted to know about PKA regulation and its involvement in mammalian sperm capacitation. *Mol Cell Endocrinol*. 2020;518:110992.

[35] Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N, Marks AR. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell*. 2000;101(4):365-376.

[36] Zhang Y, Liao J, Zhang L, Li S, Wu Y, Shi L. BK_{Ca} channel activity and vascular contractility alterations with hypertension and aging via β1 subunit promoter methylation in mesenteric arteries. *Hypertens Res*. 2018;41(2):96-103.

[37] Xu T, Zhao M, Li H, Zhou X, Liu B, Sun M, Xu Z, Gao Q. Antenatal Dexamethasone Exposure Impairs the High-Conductance Ca^{2+}-Activated K^+ Channels via Epigenetic Alteration at Gene Promoter in Male Offspring. *Arterioscler Thromb Vasc Biol*. 2020;40(11):e284-e295.

Figure legends

Figure 1. The effect of PKA on isoproterenol (ISO)-induced relaxation in middle cerebral arteries. A, The body (a) and brain (b) weight of the two groups (n=20 form 5

litters for each group). B, Typical experimental tracings showing: effect of ISO on 5-hydroxytryptamine (5-HT)-induced vessel contraction (upper); effect of preincubation with PKA inhibitors Rp-cAMP ($5x10^{-5}$ M) for 40 mins (arrows) on the ISO-induced vasodilation (lower). Black triangles in B mark the time of addition of ISO (10^{-14}, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M). C, Dose-response curves of ISO-induced relaxation in the two groups. D and E, Dose-response curves of ISO-induced relaxation in the presence or absence of Rp-cAMP in young (D) and aged (E) groups. *P<0.05, **P<0.01, ***P<0.001, n=8-10/group.,

Figure 2. The effect of PKA/BK_{Ca} pathway on isoproterenol (ISO) and forskolin (FK)-induced relaxation in middle cerebral arteries (MCA). A, Typical experimental tracings showing: effect of FK on 5-hydroxytryptamine (5-HT)-induced vessel contraction in each group (upper); effect of preincubation with BK_{Ca} blockers IBTX (10^{-7} M) for 40 mins (arrows) on the FK-induced vasodilation (lower). Black triangles in A mark the time of addition of FK (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M). B, Dose-response curves of FK-induced vessel relaxation in the two groups. C, EC_{50} of FK-induced vasodilation in each group. D and E, Dose-response curves of FK-induced vasodilation in the presence or absence of IBTX in young (D) and aged (E) group. *P<0.05, **P<0.01, ***P<0.001, n=8-10/group.

Figure 3. The effect of protein kinase A activation on large-conductance Ca^{2+}-activated K^+ channel currents from membrane potential recordings and cell-attached patches in two groups. A, Representative recordings of iberiotoxin (IBTX, 10^{-7} M)-induced depolarization following forskolin (FK, 10^{-5} M)-induced hyperpolarizations in control and aged group. Arows: drugs were applied. B and C, Average of the FK-caused hyperpolarization (B) and IBTX-caused depolarization (C). ***P,<0.001; n=5 animals, 20 cells. D, Representative cell-attached recordings of BK_{Ca} channels in the absence and presence of 8-Br-cAMP in cerebral arterial myocytes (HP= +60 mV). E, Summarized data of normalized P_O(NP_O) of BK_{Ca} channels at the membrane potential of +60 mV. Bar plots summarize the fold changes in the NP_O after the application of PKA activator. *p < 0.05, control vs. aged, n =7-8 animals, 30 cell. F, The mRNA expressions of PKA C-α and C-β subunits in cerebral arteries from two groups. *p<0.05, ***p<0.001, young vs. aged, n=5/group.

Figure 4. Aging induced changes in the large-conductance Ca^{2+}-activated K^+(BK_{Ca}) channel properties. A, Inside-out recordings at +40 mV in the presence of 1 and 10 μM $[Ca^{2+}]_i$ in two groups. B, Bar plots summarize the normalized open probability (*N*Po) of BK_{Ca} channel at 1 and 10 μM $[Ca^{2+}]_i$ (HP = +40 mV). C, Voltage dependence of BK_{Ca} at $[Ca^{2+}]_i$=1 μM. D and E, Summary of the Dwell time of open and close state at 1μM $[Ca^{2+}]_i$ at +40 mV. *P<0.05, **P<0.01, ***P<0.001,control vs. aged, n= 30 cells from 7-8 animals, . F and G, Representative immuno-fluorescence staining images (F) and the summarized fluorescence intensity (G) of BK_{Ca} subunits. **P<0.005, ***P<0.001, n= 30 cells from 5 animals, . H, The relative level of BK_{Ca} α and β1-subunit mRNA expression in cerebral arteries from two groups.*P<0.05, ***P<0.001,control vs. aged, n=8.

Figure 5. The functional and molecular alteration of ryanodine sensitive calcium release channel induced by ageing. A and B, Cumulative concentration of forskolin-induced vasodilatation curves in the presence or absence of ryanodine (10^{-5} M) in the middle cerebral arteries (MCA) form control (A) and aged (B) groups. *P<0.05, **P<0.005, ***P<0.001,control vs. aged, n=8. C, Representative currents of STOCs with or without 8-Br-cAMP (PKA+) at HP=-30mV and -10mV in the two groups. D-G, Summarized data showing STOCs amplitude and frequency with or without PKA (+) at membrane potential of -30 mV and -10 mV,*P<0.05, **P<0.005, ***P<0.001,control vs. aged, n=20 cells from 6 animals.

Figure 6. The expression of RyR2 in the two groups. A, Representative images of RyR2in the MCA myocytes detected by immuno-fluorescence staining. B, Bar plots summarizes the fluorescence intensity of RyR2. *P<0.05, control vs. aged, n=6. C, Summarized data showing the relative mRNA levels of RyR2 in the two groups.**P<0.005, control vs. aged, n=8.

Figure 7. This figure illustrates a possible mechanism for how aging affects vascular relaxation in the middle cerebral artery and leads to impairments of vasoactivity. β-AR, β-adrenergic receptors; Gs, G protein; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; BK_{Ca} channels, large-conductance Ca^{2+}-activated K^+ channels; RyRs, ryanodine receptor Ca^{2+} release channels; STOCs, spontaneous transient outward currents; Ca^{2+} sparks, local Ca^{2+} release events; β1, large-conductance Ca^{2+}-activated K^+ α subunit and β1 subunit. “(-)” means molecular/functional down-regulation.

Table 1. Gene-specific primers used for RT-PCR

Genes	Forward primer	Reverse primer
β-Actin	CCTAAGGCCAACCGTGAAAAG	GCTCGAAGTCTAGGGCAACATAG
BKα	ACTTCGCTTCAGGACAAGGA	ATGGGAATGTTGACTCCGGT
BKβ1	TCAAGGACCAGGAAGAGCTG	AGGCTGTCTGGTAGTTGTCC
RyR_2	ACAACCCAAATGCTGGTCTC	TCCGGTTCAGACTTGGTTTC
PKA-cα	TTG GGA GGT TCA GGT GAC AGA	TAG ATC TGG ATA GGC TGG TCA G
PKA-cβ	ATC CTC AGC AAG GGT TAC	TGC AGC AGA TTC CGC AGA AGG T

Figure 1

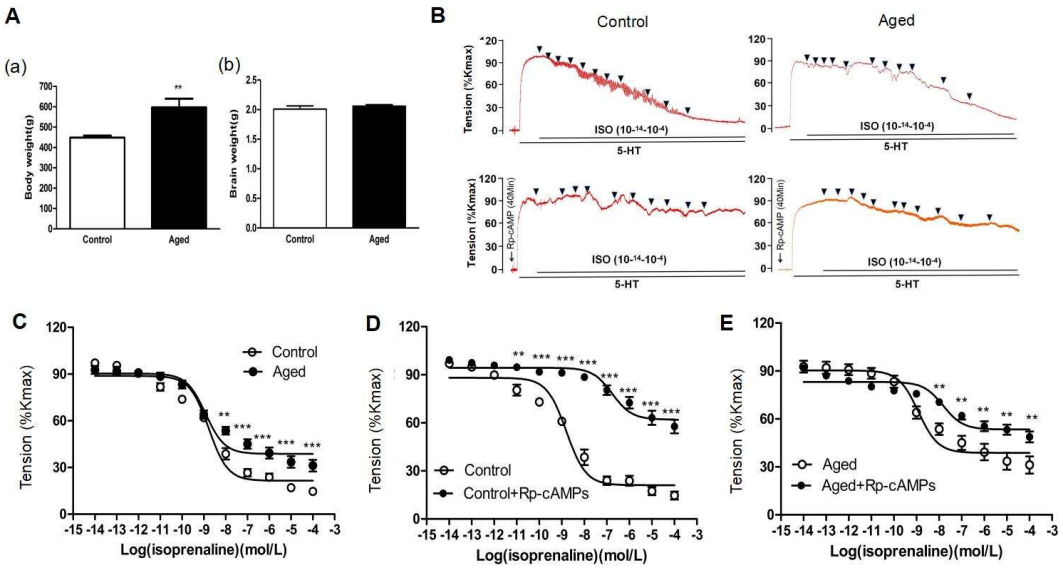


Figure 2

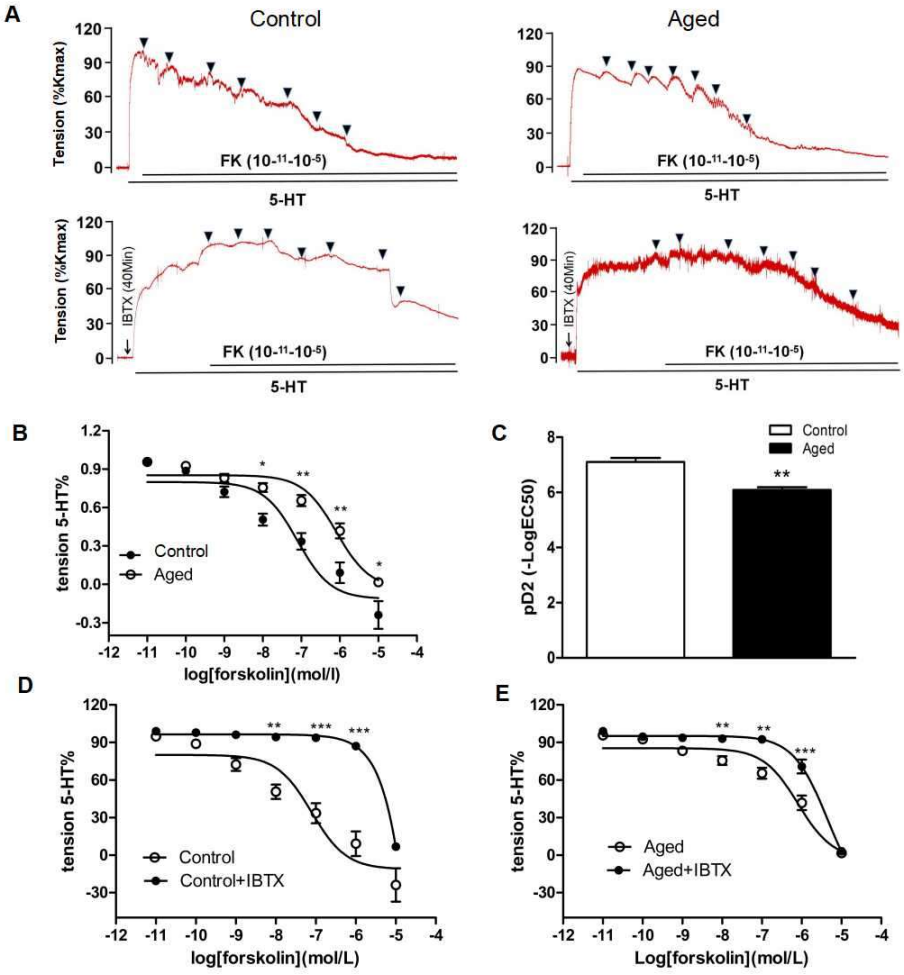


Figure 3

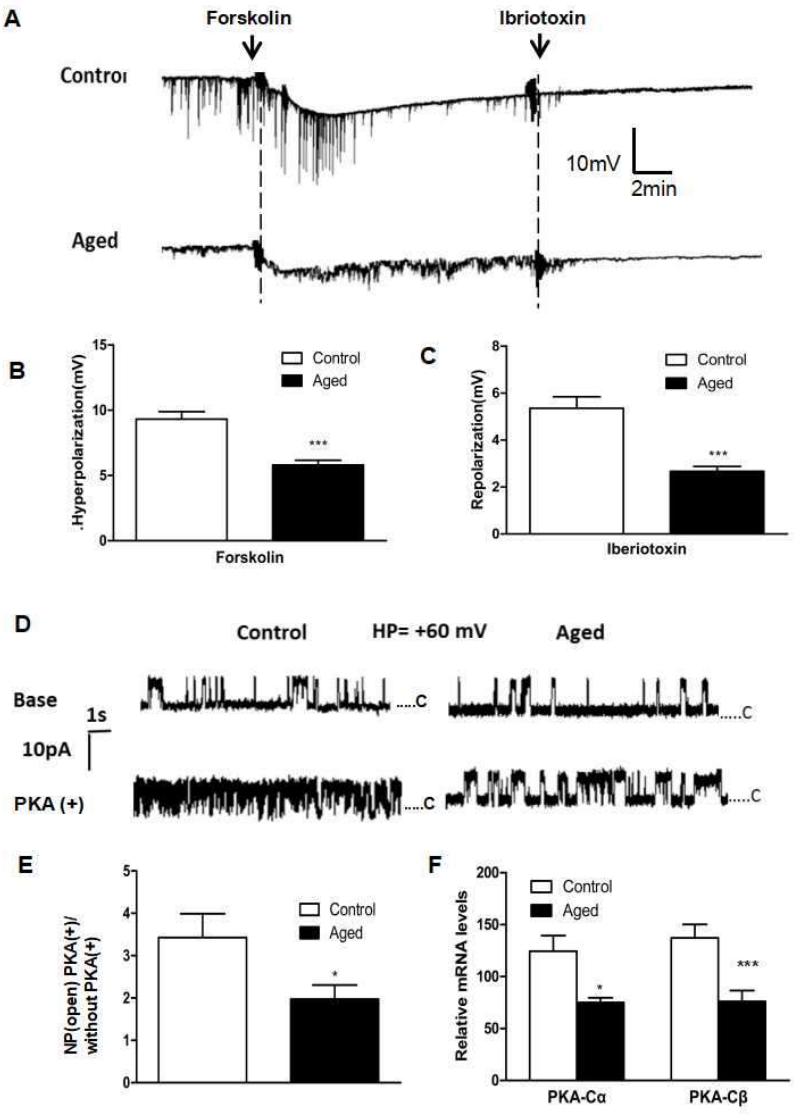

A
Forskolin
Ibriotoxin
Control
Aged
10mV
2min
B
Hyperpolarization(mV)
Control
Aged
***
Forskolin
C
Repolarization(mV)
Control
Aged
***
Iberiotoxin
D
Control
HP= +60 mV
Aged
Base
1s
10pA
PKA (+)
E
NP(open) PKA(+)/ without PKA(+)
Control
Aged
*
F
Relative mRNA levels
Control
Aged
*
***
PKA-Cα
PKA-Cβ

Figure 4

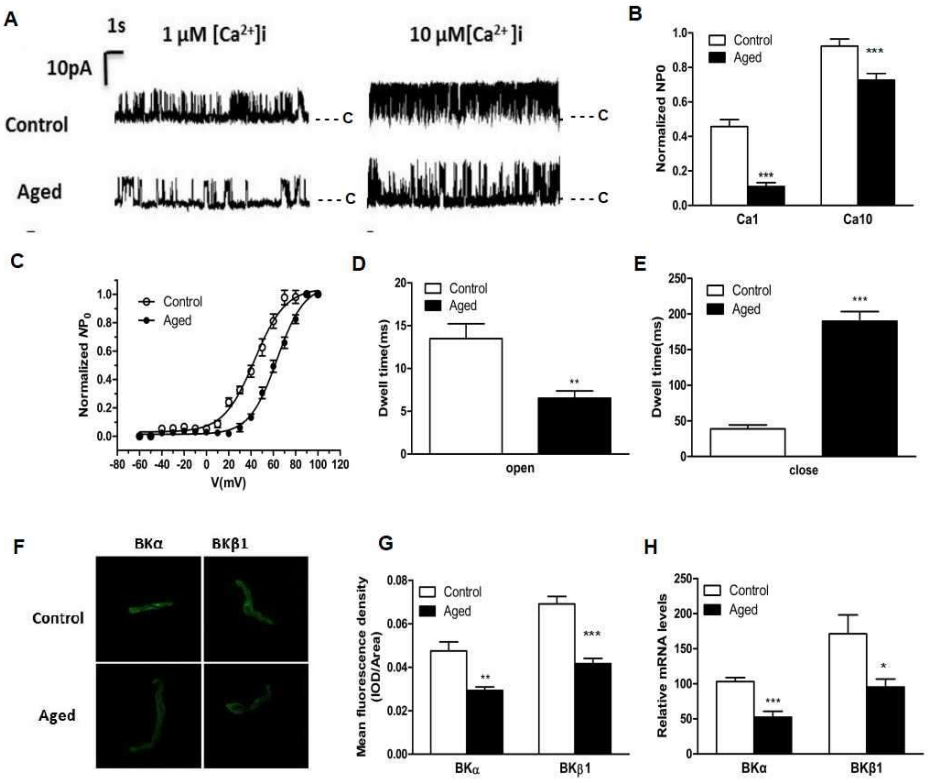

A
1s
1 μM [Ca2+]i
10 μM[Ca2+]i
10pA
Control
Aged
C
B
Control
Aged
Normalized NP0
Ca1
Ca10
C
Normalized NP0
V(mV)
D
Dwell time(ms)
open
E
Dwell time(ms)
close
F
BKα
BKβ1
Control
Aged
G
Mean fluorescence density (IOD/Area)
H
Relative mRNA levels

Figure 5

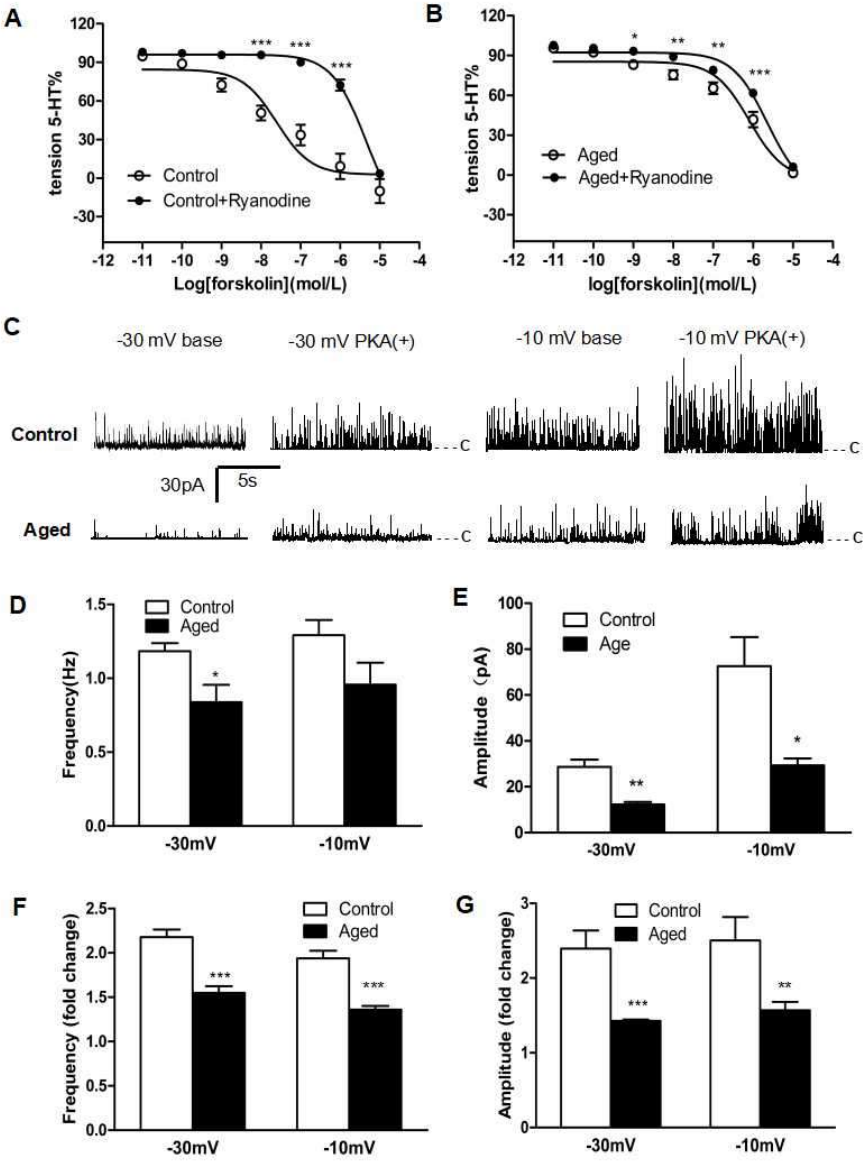


Figure 6

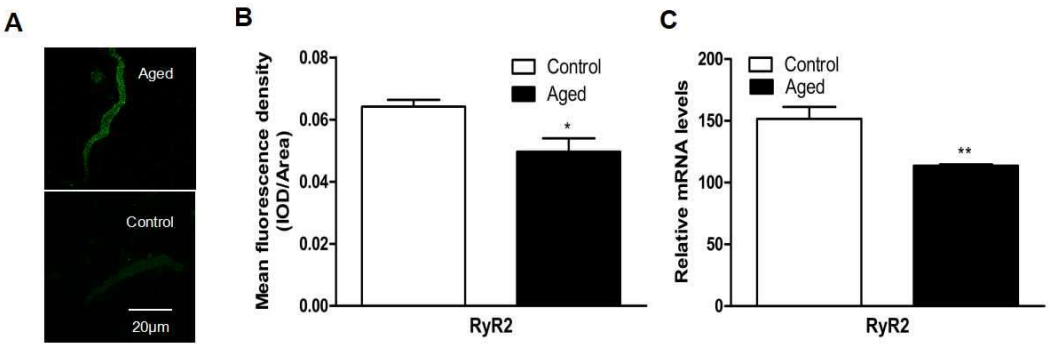
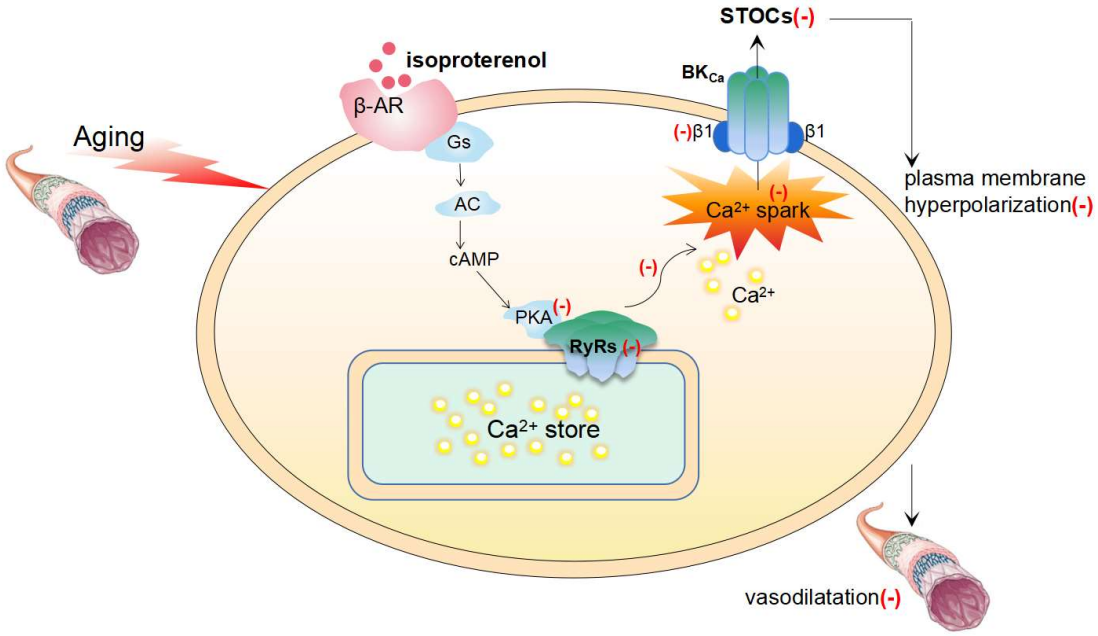


Figure 7



Podle reviewera (Dr Krůšek) je rovnice takto:

$Po=1/\{1+\exp[(-ZF/RT)(V-V_{1/2})]\}$