An increase in opening of BK_{Ca} channels in smooth muscle cells in streptozotocin-induced diabetic mice[1]

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ABSTRACT

AIM: To investigate the changes of function of large conductance of calcium-activated potassium channels (BK_{Ca} channels) in thoracic aortic smooth muscle cells in early stage of streptozotocin (STZ)-induced diabetic C57BL/6J mice. **METHODS:** Vascular muscle tension in the isolated thoracic aortic rings of mice was compared, and the role of BK_{Ca} channels in relaxation of isolated mice thoracic aortic rings induced by acetylcholine (ACh) was determined. Meanwhile, single vascular smooth muscle cells (VSMCs) were isolated by collagenase, and BK_{Ca} currents were recorded by patch-clamp single channel recording technique in symmetric high potassium solution. **RESULTS:** Tetraethylammonium (TEA) 1 mmol/L, a selective calcium-activated potassium channel blocker, caused significant rightward shift in the concentration-response curves of ACh in the isolated thoracic aortic rings of diabetic mice and pD_2 value of ACh-induced relaxation was decreased notably after TEA treatment [(6.3±0.4) *vs* (6.9±0.5), *n*=10 rings from 7 mice, *P*<0.01]. But pD_2 value of ACh-induced relaxation in age-matched control mice did not change in presence and absence of TEA 1 mmol/L [(6.4±0.15) *vs* (6.5±0.5), *n*=7 rings from 6 mice, *P*>0.05]. Furthermore, conductance of BK_{Ca} channels in single thoracic aortic smooth muscle cells was decreased [(199±15) pS, *n*=10 cells from 7 mice *vs* (266±11) pS, *n*=12 cells from 6 mice, *P*<0.01], but probability of open of BK_{Ca} channels was increased [(0.51±0.28) *vs* (0.11±0.06), *n*=6 cells from 6 mice, *P*<0.01], and the mean closed time in diabetic mice was reduced [(15±15) *vs* (132±98), *n*=6 cells from 6 mice, *P*<0.05]. **CONCLUSION:** The opening of BK_{Ca} channels was increased in thoracic aortic smooth muscle cells in the early stage of STZ-induced diabetic C57BL/6J mice by reducing mean closed time, but the conductance of BK_{Ca} channels was decreased.

INTRODUCTION

Diabetes mellitus (DM) is a kind of disease that metabolism decompensates with hyperglycemia and re-

sults in multi-organ damage[1]. Thus, the risk of coronary disease, cerebrovascular disease, and other cardiovascular complications increase. These changes, at least partially, due to diabetes functional changes in blood vessels including endothelial cell dysfunction. Simultaneously, altered ion channel function in vascular smooth muscle are also involved[2,3].

In vascular smooth muscle cells (VSMCs), potassium channels (K^+ channels) are the dominant ion conductive pathway contributing to regulation of mem-

brane potential and vascular tension, and cells express at least 4 types of K$^+$ channels such as voltage-dependent K$^+$ channels (K$_V$ channels), calcium-activated K$^+$ channels (K$_{Ca}$ channels), ATP-sensitive K$^+$ channels (K$_{ATP}$ channels), and inward rectifier K$^+$ channels (K$_{IR}$ channels)[4,5]. Furthermore, as for different conductance, K$_{Ca}$ channels are classed as 3 types again: large conductance calcium-activated K$^+$ channels (BK$_{Ca}$), intermediate calcium-activated K$^+$ channels (IK$_{Ca}$), and small calcium-activated K$^+$ channels (SK$_{Ca}$). Because the current of BK$_{Ca}$ channel is very large, the role of BK$_{Ca}$ channels in K$_{Ca}$ channels in regulating membrane potential are very important. Tetraethylammonium (TEA) is a relatively non-specific K$^+$ channel blocker, but at 1 mmol/L TEA shows selectivity to block K$_{Ca}$ channels in VSMCs[6]. During diabetes, a lot of reports about changes of K$^+$ channels function in VSMCs mainly focus on K$_{ATP}$ channels, and get many conflicting results[7,8]. But about other types of K$^+$ channels, information is still generally lacking.

The aim of the present study was to investigate the changes of BK$_{Ca}$ channels in VSMCs in early stage of STZ-induced diabetic C57BL/6J mice.

MATERIALS AND METHODS

Drugs Phenylephrine (PE), acetylcholine (ACh), TEA, type IA collagenase, aspartic acid, ovalbumin, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), 1.4-dithiothreitol (DTT), ethylene glycol-*bis* (2-aminoethyl)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA) were from Sigma Chemical, St Louis, MO, USA. Papain (Sino-American Biotechnology Co, China) was dissolved in Krebs-Henseleit solution or Ca^{2+}-free physiological salt solution (PSS). STZ (Sigma Chemical, St Louis, MO, USA) was dissolved in distilled water. Similar dilutions of the solvents into Krebs-Henseleit solution were used as controls and had no effect on neither the basal tension nor the evoked tension of thoracic aortic rings. All concentrations given are final molar concentrations in the organ chambers.

Induction of diabetes mellitus Fifty male C57BL/6J mice were obtained at the age of 4 weeks from Experimental Animal Center of Peking University Health Science Center (SPF, N̲o̲ SCXK11-00-0004).

As previous report, diabetic mice were made by five-day ip injections of STZ (40 mg/kg) which was dissolved in a citrate buffer (pH 4.2) just before injection[10]. Age-matched control mice were injected with

buffer alone. Each blood sample was taken from the tail vein only once after an 8-h fast at every four weeks. Up to 17-18 weeks after making model successfully, the two group mice were investigated with next protocol. The plasma glucose level was determined with One Touch Blood Glucose Monitoring System (Lifescan Inc, USA), and the mice that fasting plasma glucose level was higher than 11.1 mmol/L were selected to study.

Isolated thoracic aortic strip segment and tension measurement The isolated thoracic aortic strip segment and tension measurement was the same as that described previously[11]. Briefly, after the mice were killed by cervical dislocation, at room temperature (22-23 °C) thoracic aorta of mouse was quickly dissected free and placed in Krebs-Henseleit solution containing (in mmol/L) NaCl 118, KCl 4.7, CaCl$_2$ 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$(7H$_2$O) 1.2, NaHCO$_3$ 25.2, and glucose 11.1 (pH 7.4 with NaOH). By a dissecting microscope, adhering perivascular tissue was carefully removed, and the descending thoracic aorta was cut into 2-mm long rings. The vessels were mounted onto two thin stainless steel holders in 2-mL organ baths containing Krebs-Henseleit solution at 37 °C, and continuously bubbled with a gas mixture of 95 % O$_2$ and 5 % CO$_2$ to maintain a pH of 7.4. A movable device allowed the application of a passive tension of 500-550 mg, which were determined to be the optimal resting tension for obtaining the maximal active tension induced by K$^+$ solution 60 mmol/L containing (in mmol/L) NaCl 58, KCl 64.7, CaCl$_2$ 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$(7H$_2$O) 1.2, NaHCO$_3$ 25.2, and glucose 11.1 (pH 7.4 with NaOH). The isometric tension was recorded on a polygraph (Biolap 310). After an equilibration period of 1 h, the contractile function of vessel was tested twice after replacing the Krebs-Henseleit solution with K$^+$ solution 60 mmol/L. After washout, the vessel was contracted once with PE 10 µmol/L for 10 min and then relaxed with ACh 10 µmol/L for 4 min[11]. After another washout period, cumulative dose-response curves for ACh were created. ACh-induced vasorelaxation was tested after precontractions evoked by PE 10 µmol/L to determine endothelium-dependent relaxation.

Cell isolation The thoracic aortic segments were obtained like above method, and placed in ice-cold Ca^{2+}-free PSS containing (in mmol/L) NaCl 55, sodium glutamate 80, KCl 5.6, HEPES 10, MgCl$_2$ 2, glucose 10 (pH 7.4 with NaOH), and washed twice in this solution. The vessel was opened longitudinally, and the smooth muscle layer was obtained by tearing out adherent ad-

ventitia under a dissection microscope. Then this tissue was cut into 2-3 mm in length and 0.5-2 mm in width strip, and incubated in plastic tube with 1 mL Ca^{2+}-free PSS containing type IA collagenase 2 g/L, papain 9 g/L, ovalbumin 5 g/L, DTT 1.75 g/L at 37 ℃ for 56-60 min. After digestion, tissue masses were transferred to another plastic tube containing Ca^{2+}-free PSS with a wide-pore Pasteur pipette, and washed twice in this solution for 10 min. Then VSMCs were dispersed by gentle trituration with a slender Pasteur pipette, and the cell suspension was transferred to the cell champer and well-attached VSMCs were selected for experiments.

Electrophysiological measurements Single channel K^+ currents were measured by the cell-attached and inside-out patch configuration of the patch-clamp technique. Patch pipettes (resistance, 5-8 MΩ) were filled with an internal pipette solution containing (in mmol/L): potassium aspartate 40, KCl 100, $CaCl_2$ 1, HEPES 10 (pH 7.2 with KOH). Micropipettes were got from Shanghai Institute of Brain Research,Chinese Academy of Sciences, and pulled by micropipette puller (P-97, Sutter, America). When the seal resistance reached up to 10 GΩ, single channel currents were recorded with EPC-9 patch clamp amplifier (HEKA, Germany) in voltaged-clamp mode. Pipette and membrane capacitance and series resistance were electronically compensated. Voltage-clamp protocol was applied with Pulse 8.5 (HEKA, Germany). Data were filtered at 5 kHz and analyzed with TAC+TACFit 4.0.9 (Bruxton Corporation, America). The bath solution contained the following (in mmol/L): potassium aspartate 100, KCl 40, $CaCl_2$ 0.55, EGTA 1, HEPES 10 (pH 7.2 with KOH), and all experiments were performed at room temperature (22-25 ℃).

Data analysis Individual pD_2 values for treated and untreated curves were calculated using linear regression analysis. Results were expressed as mean±SD. The two-tailed paired-samples t-test was used to compare results in treated and untreated aortas from each strain, and two-tailed independent samples t-test was used to compare results in different groups. When more than two groups are compared, one way ANOVA followed by Least-significant difference test was used. Differences were considered significant when $P<0.05$. In electrophsiological experiments n represents cells number.

RESULTS

General characteristics of mice Weight and fasting plasma glucose levels of all groups were measured at every four weeks after the fasting plasma glucose level was higher than 11.1 mmol/L. By comparison with the age-matched controls, fasting plasma glucose level was markedly increased in STZ-induced diabetic mice (Fig 1A) but weight was greatly decreased (Fig 1B).
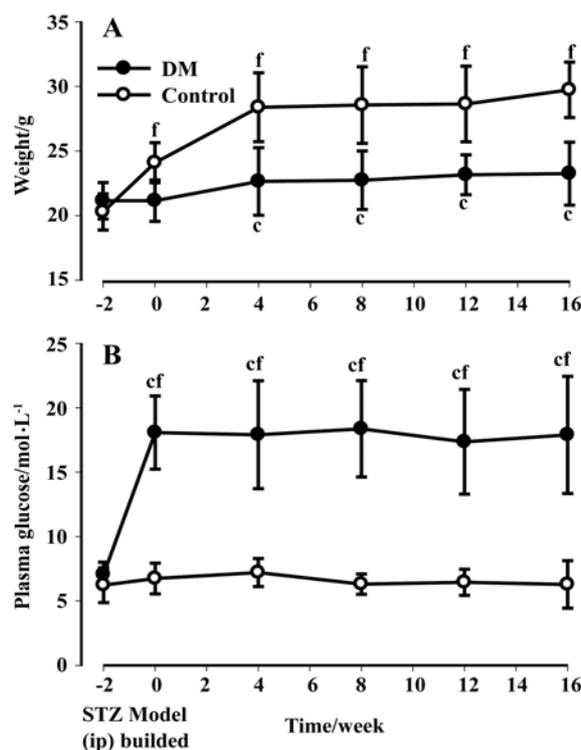
Fig 1. Weight (A) and fasting plasma glucose levels (B) in diabetic (DM, $n=24$) and age-matched control group (Control, $n=25$). Mean±SD. $^cP<0.01$ *vs* Control. $^fP<0.01$ *vs* -2 Week.

Effects of TEA on ACh-induced relaxation In the presence of TEA 1 mmol/L for 10 min, cumulative concentration-response curves to ACh in thoracic aortic rings of diabetic mice were shifted to the right with decreased pD_2 value [(6.3±0.4) *vs* (6.9±0.5), $n=10$ rings from 7 mice, $P<0.01$, Fig 2A]. But in age-matched control mice, TEA 1 mmol/L did not affect concentration-response curves to ACh and pD_2 value [(6.4±0.2) *vs* (6.5±0.4), $n=7$ rings from 6 mice, $P>0.05$, Fig 2B]. TEA did not affect basal tension in isolated thoracic aortic rings of two groups.
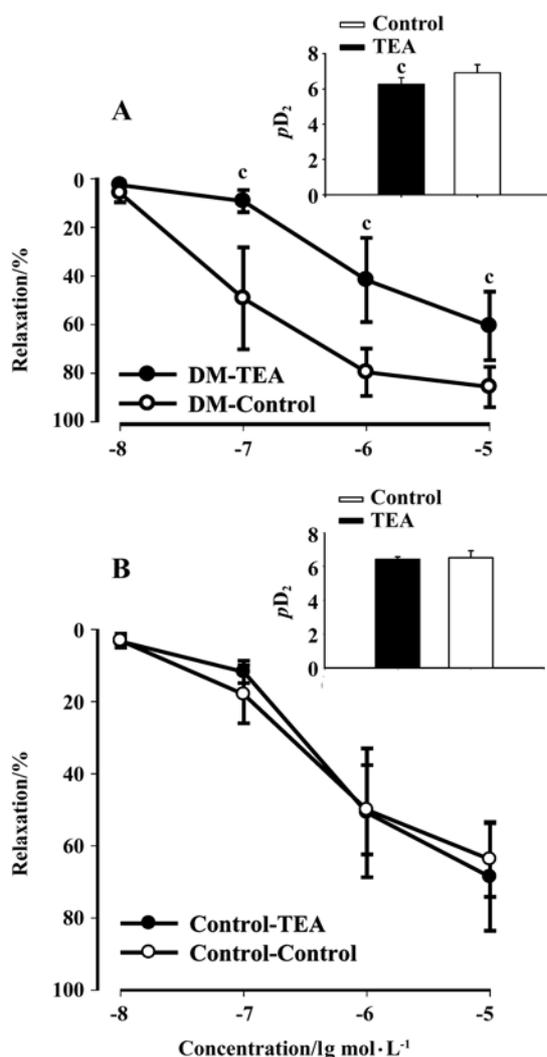
Fig 2. **Cumulative concentration-response curves to ACh and pD_2 value in thoracic aortic rings of diabetic mice (A, DM-TEA, n=10 rings from 7 mice. Mean±SD. $^c P<0.01$ *vs* DM-Control) and age-matched control mice (B, Control-TEA, n=7 rings from 6 mice. Mean±SD).**

Characterization of BK_{Ca} single channel currents in mice VSMCs After the seal resistance reached up to 10 GΩ, in cell attached model, step depolarizations from 0 mV to +80 mV induced voltaged-dependent outward currents in symmetric high potassium solution (Fig 3A), and the conductance of channels gained from current-voltage relationship (*I-V*) curve was very high [(266±11) pS, n=12 cells from 6 mice]. Simultaneously, voltaged-dependent outward currents were significantly suppressed by TEA 1 mmol/L in cell attached model [at +40 mV, from (9.89±0.11) to (1.42± 0.44) pA, n=10 cells from 5 mice, $P<0.01$ Fig 3B]. It was noninactivating and characterized by great noise. Furthermore, in inside-out model, probability of open
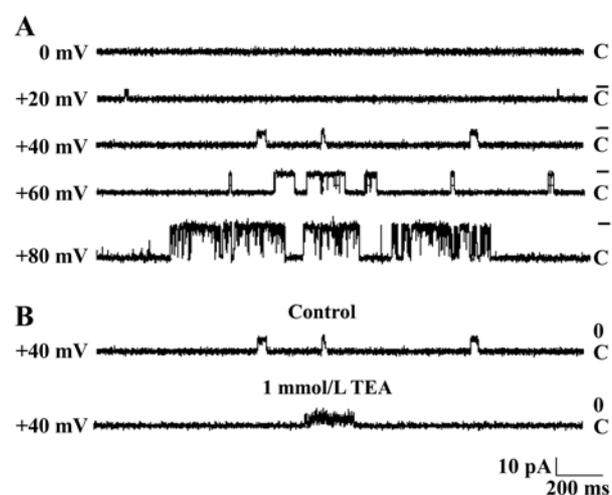
Fig 3. **(A) In cell attached model, step depolarizations from 0 mV to +80 mV induced voltaged-dependent outward currents in symmetric high potassium solution; (B) in cell attached mode and at +40 mV voltaged-dependent outward currents.**

(Po) of these channels was enhanced gradually with increasing $[Ca^{2+}]_i$ from 0.1 mol/L to 100 μmol/L (at +50 mV, n=5 cells from 5 mice, Fig 4). These characteristics are consistent with previous reports about BK_{Ca} channels[12].
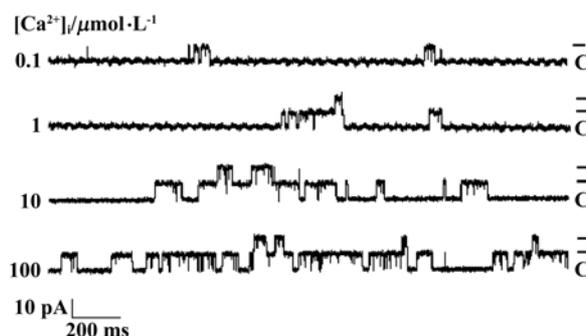
Fig 4. **In inside-out model and at +50 mV, cumulative increase in $[Ca^{2+}]_i$ enhanced probability of open of BK_{Ca} channels.**

Changes of conductance, Po, average open time (To), average closed time (Tc) of BK_{Ca} channels in diabetic mouse VSMCs In cell attached model, step depolarizations from 0 mV to +80 mV elicited BK_{Ca} single channel currents, and the *I-V* curve exhibited a reduced conductance in diabetic mouse VSMCs [(199±15) pS, n=10 cells from 7 mice *vs* (266±11) pS, n=12 cells from 6 mice, $P<0.01$, Fig 5]. At +40 mV, BK_{Ca} currents were recorded in cell attached model in

diabetic and age-matched control mice, respectively. Po of BK_{Ca} channels was increased markedly (Fig 6, 7, Tab 1), but the amplitude of BK_{Ca} channels was reduced in diabetic VSMCs (Fig 8). Tc of BK_{Ca} channels was
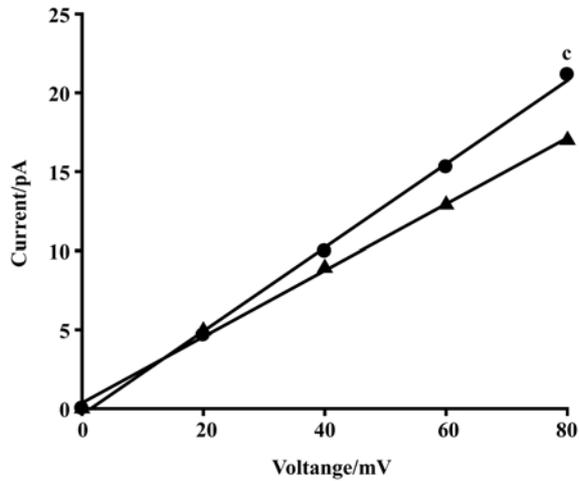
Fig 5. Current-voltage relationship (*I-V*) curve in cell-attached model. (▲) diabetic mice VSMCs; (●) age-matched control mice VSMCs.
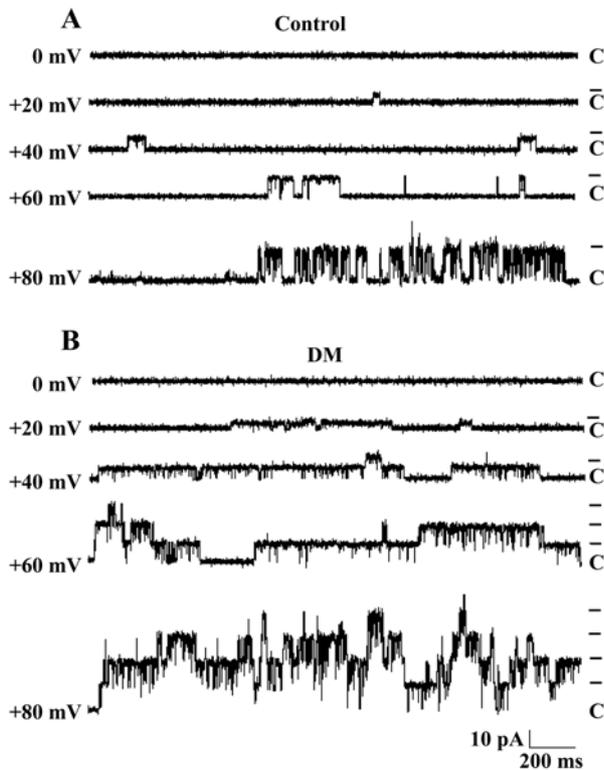
Fig 6. In cell attached model, step depolarizations from 0 mV to +80 mV induced voltaged-dependent outward currents in symmetric high potassium solution: (A) age-matched control mice VSMCs (Control); (B) diabetic mice VSMCs (DM).
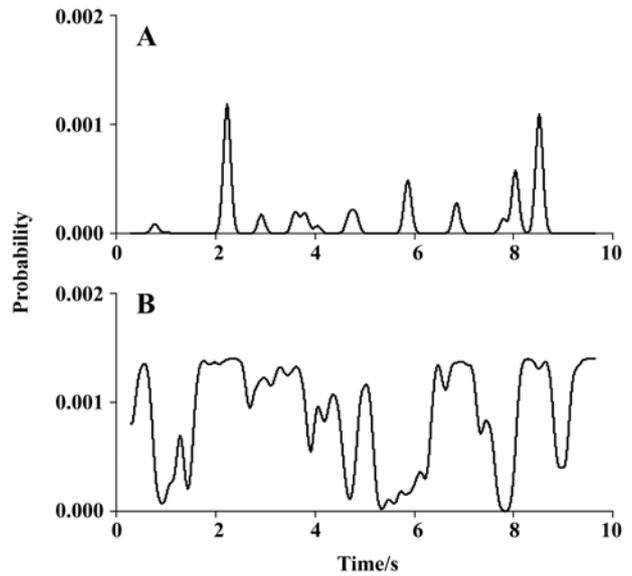
Fig 7. In cell attached model and at +40 mV, probability of open and time relation curve of BK_{Ca} channels: (A) age-matched control mice VSMC; (B) diabetic mice VSMC.

greatly reduced, but To did not change in diabetic VSMCs (Tab 1).

Tab 1. At +40 mV, probability of open (Po), average open time (To), average closed time (Tc) of BK_{Ca} channels in diabetic mice (DM) and age-matched control (Control) VSMCs in cell-attached model. *n*=6. Mean±SD. [b]P<0.05, [c]P<0.01 *vs* Control.

Group	Po	Tc (ms)	To (ms)
Control	0.11±0.06	132±98	11±7
DM	0.51±0.28[c]	15±15[b]	10.5±1.6

DISCUSSION

The present study demonstrated that the opening of BK_{Ca} channels in VSMCs of STZ-induced 17-18 week diabetic mice was enhanced. We have found that an enhanced response to ACh in isolated thoracic aortic rings contracted by alpha-adrenoceptor agonist in early stage of diabetic mice involved increased production and/or secretion of PGI_2 and EDHF[9]. In this study, the concentration-response effect to ACh in the presence and absence of TEA, a BK_{Ca} channel blocker, were also observed. Pretreatment with TEA in diabetic mice, ACh-induced relaxation was significantly decreased, but
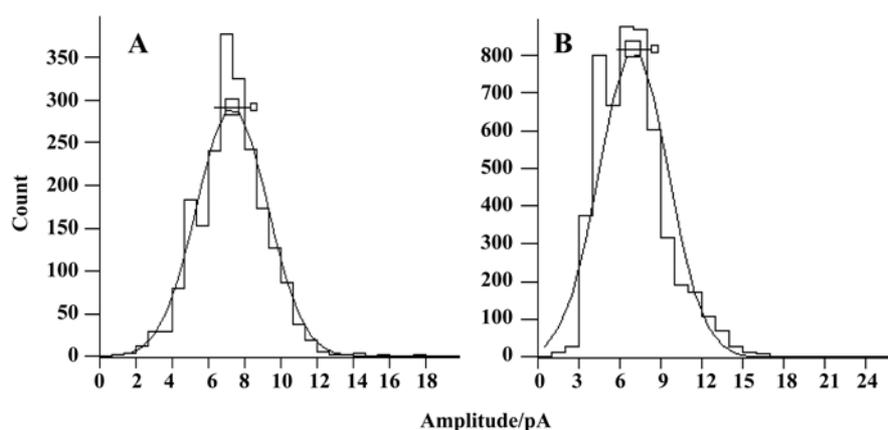
Fig 8. In cell attached model and at +40 mV, amplitude histogram of BK$_{Ca}$ channels: (A) age-matched control mice VSMC; (B) diabetic mice VSMC.

ACh-induced relaxation was not changed in age-matched controls in the presence or absence of TEA. The findings indicate that increased ACh-induced relaxation is due to, at least partly, an increase of opening of BK$_{Ca}$ channels.

Furthermore, for getting more direct evidences, patch-clamp single channel recording was expropriated to investigate the changes of BK$_{Ca}$ channel current and functional responses in isolated single thoracic aortic smooth muscle cells in diabetic mice and age-matched controls, respectively. Single patch-clamping showed an increase in probability of open of BK$_{Ca}$ channels by decreasing mean closed time in VSMCs of diabetic mice, but the conductance of BK$_{Ca}$ channels was decreased. As we know, hyperglycaemia is a diabetic characteristic, and causes various economy impairs[13]. In this status, the production of oxygen-derived radical is increased, and nitric oxide (NO), a principal mediator of endothelium-dependent relaxation, is destroyed easily causing a reduction of NO bioavailability[14]. Simultaneously, ACh is an agonist of endothelium, and combining with its receptor causes endothelium to release endothelium-derived relaxing factors (EDRFs): NO, PGI$_2$, and EDHF[15]. So, combined with our previous correlated study, these results probably indicate that in this stage of DM increased the opening of BK$_{Ca}$ channels maybe compensated for diminished NO bioactivity induced by increased oxygen-derived radical. This opinion is also consistent with Misurski & Gopalakrishnan study with STZ-induced diabetic rats[16]. However, in this study, BK$_{Ca}$ channel conductance was reduced. This may suggest that hyperglycaemia impaired electric kinetics of BK$_{Ca}$ channels.

Concerning transformation of K$^+$ channels in VSMCs of DM animal models, lots of reports are conflicting. Some researchers found the function of K$_{ATP}$, K$_{Ca}$, or K$_V$ channels was reduced directly or indirectly, but some got hold of the reverse[7,8,16]. In our opinion, these may be the different exhibitions in different stages of disease. In early stage of DM, economy can compensate the damage of hypergly-caemia by various pathways. However, in later stage, compensation is maladjusted and then reduced K$^+$ channel function is emerged. These may be dependent on the duration of disease. In our DM animal model, STZ-induced diabetic C57BL/6J mice in early stage have been discussed detailedly in our previous study[9]. Thus, the function of BK$_{Ca}$ channels is enhanced to compensate the damage of hyperglycaemia.

In conclusion, the opening of BK$_{Ca}$ channels is enhanced in thoracic aortic smooth muscle cells of the early stage STZ-induced diabetic mice due to reduction of mean closed time, but the conductance of BK$_{Ca}$ channels is decreased. This phenomenon may be only observed in early stage of DM.

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