

Published in final edited form as:

*Circ Res.* 2007 March 16; 100(5): 703–711. doi:10.1161/01.RES.0000260182.36481.c9.

## Diabetes downregulates BK $\beta$ 1 potassium channel subunit in retinal arteriolar smooth muscle

MK McGahon<sup>1</sup>, DP Dash<sup>1</sup>, A Arora<sup>1</sup>, N Wall<sup>1</sup>, J Dawicki<sup>1</sup>, DA Simpson<sup>1</sup>, CN Scholfield<sup>2</sup>, JG McGeown<sup>2</sup>, and TM Curtis<sup>1</sup>

<sup>1</sup>Centre for Vision Sciences, The Queen's University of Belfast, Institute of Clinical Sciences, The Royal Victoria Hospital, Grosvenor Road, Belfast BT12 6BA, Northern Ireland <sup>2</sup>Cell and Metabolic Signalling Group, School of Medicine and Dentistry, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland

### Abstract

Retinal vasoconstriction and reduced retinal blood flow precede the onset of diabetic retinopathy. The pathophysiological mechanisms that underlie increased retinal arteriolar tone during diabetes remain unclear. Normally, local Ca<sup>2+</sup> release events (Ca<sup>2+</sup>-sparks), trigger the activation of large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup>(BK)-channels which hyperpolarize and relax vascular smooth muscle cells (VSMCs), thereby causing vasodilatation. In the present study, we examined BK channel function in retinal VSMCs from streptozotocin(STZ)-induced diabetic rats. The BK channel inhibitor, Penitrem A, constricted non-diabetic retinal arterioles (pressurized to 70mmHg) by 28%. The BK current evoked by caffeine was dramatically reduced in retinal arterioles from diabetic animals even though caffeine-evoked [Ca<sup>2+</sup>]<sub>i</sub> release was unaffected. Spontaneous BK currents were smaller in diabetic cells, but the amplitude of Ca<sup>2+</sup>-sparks was larger. The amplitudes of BK currents elicited by depolarizing voltage steps were similar in control and diabetic arterioles and mRNA expression of the pore-forming BK $\alpha$  subunit was unchanged. The Ca<sup>2+</sup>-sensitivity of single BK channels from diabetic retinal VSMCs was markedly reduced. The BK $\beta$ 1 subunit confers Ca<sup>2+</sup>-sensitivity to BK channel complexes and both transcript and protein levels for BK $\beta$ 1 were appreciably lower in diabetic retinal arterioles. The mean open times and the sensitivity of BK channels to tamoxifen were decreased in diabetic cells, consistent with a downregulation of BK $\beta$ 1 subunits. The potency of blockade by Pen A was lower for BK channels from diabetic animals. Thus, changes in the molecular composition of BK channels could account for retinal hypoperfusion in early diabetes, an idea having wider implications for the pathogenesis of diabetic hypertension.

### Introduction

Diabetes causes changes to the structure and function of blood vessels in the retina leading to visual impairment and blindness<sup>1</sup>. The cellular and molecular basis of diabetic retinopathy is not wholly understood although large prospective clinical trials have established the importance of hyperglycaemia in precipitating this disease in both type 1 and type 2 diabetic patients<sup>2,3</sup>. A major pathway through which hyperglycaemia is believed to contribute to retinal microangiopathy is the disruption of retinal blood flow. Patient-based studies have shown that retinal haemodynamic abnormalities occur prior to the onset of

Corresponding Author: Dr Tim M Curtis, Centre for Vision Sciences, School of Biomedical Sciences, The Queen's University of Belfast, Institute of Clinical Sciences, The Royal Victoria Hospital, Grosvenor Road, Belfast BT12 6BA, Northern Ireland, Tel: +44 (0)28 90635027, Email: t.curtis@qub.ac.uk.

clinical diabetic retinopathy<sup>4</sup> and that the development and progression of diabetic retinopathy correlates with the extent of the blood flow changes observed<sup>5-8</sup>. In diabetic patients without retinopathy, retinal arteriolar vasoconstriction<sup>9, 10</sup> and decreased total retinal blood flow has been reported<sup>4, 11</sup>. However, as the disease progresses, the arterioles begin to dilate<sup>12</sup> and bulk retinal blood flow increases in proportion to the severity of retinopathy, and thus the degree of retinal ischemia<sup>11</sup>. Presently, the molecular mechanisms underlying retinal vasoconstriction during early diabetes are unknown, yet an improved understanding of the pathophysiology involved could be crucial to the development of better therapies for the treatment of diabetic retinopathy.

A major factor controlling the contractile state of arterioles is the activity of ion channels on the plasma membranes of the VSMCs<sup>13</sup>. BK channels are known to play a crucial role in the regulation of arterial smooth muscle tone since blockade of these channels using the specific inhibitor iberiotoxin, causes membrane depolarisation and vasoconstriction in pressurized isolated vessels<sup>14</sup>. BK channels are activated by local  $\text{Ca}^{2+}$  release events, termed  $\text{Ca}^{2+}$  sparks, resulting from opening of ryanodine receptor (RyR) channels in the sarcoplasmic reticulum (SR;<sup>15</sup>). The activation of BK channels results in outward  $\text{K}^+$  current that opposes VSMC contraction by causing membrane hyperpolarisation. This reduces  $\text{Ca}^{2+}$  influx by reducing activation of voltage-dependent  $\text{Ca}^{2+}$  channels<sup>15</sup>. BK channels are composed of  $\alpha$ -subunits and accessory  $\beta$ -subunits<sup>16</sup>. The  $\alpha$ -subunit forms the  $\text{K}^+$  selective pore, while the  $\beta$  subunits influence the kinetics, pharmacology and  $\text{Ca}^{2+}$  sensitivity of BK currents<sup>17, 18</sup>. Four members of the BK  $\beta$ -subunit family have been identified to date ( $\beta 1$ - $\beta 4$ ) and the  $\beta 1$ -subunit is expressed predominantly in VSMCs<sup>19</sup>. Targeted deletion of the  $\beta 1$ -subunit gene reduces the  $\text{Ca}^{2+}$  sensitivity of BK channels and the coupling of  $\text{Ca}^{2+}$  sparks to BK channel activity in VSMCs from cerebral arteries<sup>19, 20</sup>. The functional significance of the  $\beta 1$ -subunit of VSMC BK channels is underlined by the observation that knockout mice are hypertensive and display enhanced vascular reactivity to application of vasoconstrictors<sup>19, 20</sup>.

In the present study we have examined the effects of STZ-induced diabetes on the properties of BK channels in VSMCs of the rat retinal microcirculation. A clear advantage of using STZ-diabetic rats is that it is well documented that retinal arteriolar vasoconstriction and decreased retinal blood flow occur in this animal model following several weeks of diabetes<sup>21</sup>. These animals subsequently exhibit many of the vasodegenerative changes associated with human diabetic retinopathy<sup>22</sup>. We hypothesised that diabetes causes a downregulation of the  $\alpha$ - and/or  $\beta 1$ -subunit, thereby reducing the capacity of the BK channels to hyperpolarise retinal VSMCs and resist vasoconstriction. We show that diabetes reduces coupling between  $\text{Ca}^{2+}$  release from RyR sensitive  $\text{Ca}^{2+}$  stores and BK channel activation. No alteration in the expression of the pore-forming  $\alpha$ -subunit was evident, but  $\beta 1$ -subunit expression was reduced at both the mRNA and protein level. Consistent with this, we found that BK channels in retinal VSMCs from STZ-diabetic animals exhibit a diminished sensitivity to  $\text{Ca}^{2+}$  and we provide pharmacological evidence supporting the idea that the expression of functional  $\alpha$ - $\beta 1$  subunit complexes is reduced in diabetes. These results suggest that changes in the molecular composition of BK channels in retinal VSMCs during diabetes might contribute to the onset and early progression of diabetic retinopathy.

## Methods

All procedures with animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the United Kingdom Animals (Scientific Procedures) Act, 1986. Full details of the methods and materials used are in the online data supplement available at <http://circres.ahajournals.org>.

## Results

### Characteristics of experimental animals

Diabetic animals had higher mean plasma glucose levels ( $27.8 \pm 1.3$  mmol/L;  $n=41$ ) than non-diabetic animals ( $7.6 \pm 0.3$  mmol/L;  $n=37$   $p<0.001$ ). Mean glycosylated haemoglobin values were  $5.9 \pm 0.2\%$  and  $17.6 \pm 0.8\%$  in the non-diabetic and diabetic groups, respectively ( $p<0.001$ ). Both sets of animals gained weight during the 3-month experimental period, but the increase was greater in non-diabetic than diabetic rats ( $304 \pm 17$  g vs  $141 \pm 9.7$  g, respectively;  $p<0.001$ ).

### Functional Significance of BK channels in retinal VSMCs

We examined the contribution of BK channels to the regulation of retinal arteriolar tone by pressurizing freshly isolated retinal arterioles from non-diabetic animals to 70 mmHg and measuring the change in internal diameter induced by 100 nmol/L Penitrem A (Pen A), a potent inhibitor of BK channels<sup>23</sup>. Pen A caused a 28% decrease in the diameter of pressurized retinal vessels (Fig 1). These results suggest that BK activity plays an important vasodilatory role in retinal blood vessels.

### Caffeine-induced BK currents, but not $Ca^{2+}$ activated $Cl^-$ currents, are smaller in retinal VSMCs from diabetic animals

To investigate BK channel activity during diabetes we tested activation following  $Ca^{2+}$  release from caffeine-sensitive  $Ca^{2+}$  stores. Retinal arterioles were bathed in low  $Cl^-$  Hanks' solution containing 1 mmol/L 9-anthracene carboxylic acid (9AC) to block  $Ca^{2+}$  activated  $Cl^-$  channels ( $Cl_{Ca}$  channels). Application of 10 mmol/L caffeine for 5 seconds evoked large, noisy transient outward currents at positive membrane potentials (Fig 2A,C). These currents were fully abolished by addition of 100 nmol/L Pen A (Fig 2A;  $n=6$ ). Fig 2B shows typical caffeine-induced BK currents in retinal VSMCs from non-diabetic and diabetic animals. Fig 2C shows the average peak current density plotted as a function of voltage for the caffeine-induced BK currents. BK currents evoked by  $Ca^{2+}$  release from caffeine-sensitive  $Ca^{2+}$  stores were dramatically reduced in VSMCs from diabetic animals.

The attenuated BK currents in arterioles from diabetic rats could be explained by decreased  $Ca^{2+}$  release from the caffeine-sensitive stores. To test this, global  $[Ca^{2+}]_i$  responses were measured in retinal VSMCs from non-diabetic and diabetic animals using fura 2 based  $Ca^{2+}$  microfluorimetry (Fig 3A). No differences in the peak amplitude of  $[Ca^{2+}]_i$  transients evoked by 10 mmol/L caffeine were observed. We also compared the size of caffeine-evoked  $Cl_{Ca}$  currents at a range of voltages in myocytes from non-diabetic and diabetic arterioles bathed in normal Hanks' solution containing 100 nmol/L Pen A to block BK channels. Application of 10 mmol/L caffeine evoked currents that reversed close to  $E_{Cl}$  (0 mV; Fig 3B,D). These were completely abolished in low  $Cl^-$  Hanks' solution containing the  $Cl^-$  channel inhibitor, 9AC (Fig 3B;  $n=6$ ). Isolation of the caffeine-evoked  $Cl^-$  currents in diabetic arterioles required longer pre-incubation times with Pen A ( $\sim 20$  min as compared to  $\sim 5$ -min for non-diabetic vessels). Fig 3C,D show representative traces and summary data for the caffeine-evoked  $Cl^-$  currents in non-diabetic and diabetic retinal VSMCs. The mean peak current densities were similar for normal and diabetic cells at all voltages tested. Thus, it appears that the smaller caffeine-induced BK currents in diabetic retinal VSMCs reflect reduced coupling efficiency between  $Ca^{2+}$  release and BK channel activation rather than defective  $Ca^{2+}$  release.

### $Ca^{2+}$ sparks are larger, but STOCs are smaller in diabetic retinal VSMCs

From a physiological perspective it is well established that  $Ca^{2+}$  sparks activate BK channels generating spontaneous transient outward  $K^+$  currents (STOCs), modulating

vascular tone<sup>15, 24</sup>. STOCs were recorded from retinal VSMCs of non-diabetic and diabetic animals at test potentials between -80mV to +80mV (increased in 40mV increments) using the perforated patch clamp technique. STOCs were evident at membrane potentials positive to -40mV and were completely abolished in the presence of 100nmol/L Pen A (n=6, non-diabetic VSMCs). Individual STOC events were superimposed (Fig 4A) precluding an accurate assessment of STOC amplitudes and frequencies, so we calculated the integral of the STOC densities for recordings lasting 5-10 minutes. Visual inspection of the original traces suggested that STOC amplitudes and frequencies were greatly reduced in diabetic retinal VSMCs, and integrated current densities were considerably smaller (Fig 4A). These data confirm that retinal VSMCs from diabetic animals demonstrate less spontaneous BK current activity than cells from non-diabetic animals.

Using confocal imaging techniques, we have recently described the presence of two distinct populations of spontaneous Ca<sup>2+</sup> sparks in retinal VSMCs: “basal” sparks that arise from resting fluorescence levels (i.e. from  $F/F_0 = 0.95-1.05$ ) and “sparks on oscillations” that overlay global Ca<sup>2+</sup> transients<sup>25</sup>. Fig 4B shows representative images of basal Ca<sup>2+</sup> sparks, Ca<sup>2+</sup> sparks on oscillations and global Ca<sup>2+</sup> oscillations in non-diabetic arteriolar myocytes. We considered the possibility that the lower STOC activity in diabetic retinal VSMCs may reflect reduced spontaneous sub-cellular [Ca<sup>2+</sup>]<sub>i</sub> signaling in these cells. Fig 4C shows typical images of basal Ca<sup>2+</sup> sparks in non-diabetic and diabetic arteriolar myocytes. Below the image panels, the time course of the normalized fluorescence for each event has been plotted and the traces superimposed. It is evident that the peak amplitude of the Ca<sup>2+</sup> spark in the diabetic cell is around twice that of the non-diabetic. Quantitative data for basal Ca<sup>2+</sup> sparks, Ca<sup>2+</sup> sparks on oscillations and global oscillations in non-diabetic and diabetic VSMCs are summarized in Table 1 of the online data supplement. The peak amplitude of both populations of Ca<sup>2+</sup> sparks was substantially larger in diabetic than in non-diabetic VSMCs ( $\Delta F/F_0$  basal sparks,  $0.92 \pm 0.06$  and  $0.42 \pm 0.03$ , respectively;  $\Delta F/F_0$  sparks on oscillations,  $1.56 \pm 0.21$  and  $0.36 \pm 0.04$ , respectively;  $p < 0.001$  in both cases), while the frequency and duration (FDHM) of these events remained unchanged. No differences were observed in amplitude, frequency or duration of global Ca<sup>2+</sup> oscillations between non-diabetic and diabetic VSMCs. These results show that decreased sub-cellular Ca<sup>2+</sup> signaling activity cannot explain the decreased STOC activity observed in diabetic VSMCs.

### Diabetes reduces the Ca<sup>2+</sup> sensitivity of BK channels in diabetic retinal VSMCs

Both the comparisons of caffeine-evoked currents and Ca<sup>2+</sup> transients, and of STOC and spark activity, suggest decreased coupling between Ca<sup>2+</sup> release and BK channel activation in retinal VSMCs after short-term diabetes. This might be explained by a reduced number of functional BK channels in diabetic myocytes. Quantitative RT-PCR, however, failed to reveal any differences in BK $\alpha$  transcript expression in non-diabetic and diabetic retinal arterioles (Fig 5A). To assess BK channel density, we also compared depolarization dependent whole-cell BK currents (Fig 5Bi,ii). Vessels were bathed in low Cl<sup>-</sup> Hanks' solution containing 1mmol/L 9AC and 10mmol/L 4-aminopyridine (4AP) to block Cl<sub>Ca</sub> and K<sub>v</sub> channels<sup>26</sup>, respectively. 10mmol/L 4AP has no effect on BK channels in retinal arterioles; caffeine-evoked BK currents at +40mV in the absence and presence of 10mmol/L 4AP were  $115 \pm 29.8$  and  $114 \pm 26$ pA/pF, respectively (n=4; p=0.84; paired t-test). Voltage-activated BK currents in control VSMCs were unaffected by the removal of extracellular Ca<sup>2+</sup>, inhibition of voltage-dependent Ca<sup>2+</sup> channels with 10 $\mu$ mol/L nifedipine or blockade of RyR receptors with 100 $\mu$ mol/L tetracaine (Fig 5Biii-v). This demonstrates that BK currents evoked by depolarisation are independent of Ca<sup>2+</sup> influx and Ca<sup>2+</sup> store release in these cells i.e. they appear to be entirely dependent on voltage gating of the BK channels. As such, the peak current density should be proportional to the number of functional BK channels, assuming that the single-channel conductance and the voltage dependence of

activation remain constant. Single channel conductance's of BK channels were similar for non-diabetic and diabetic VSMCs ( $160 \pm 2\text{pS}$  and  $160 \pm 4\text{pS}$ , respectively;  $n=21$  and  $15$  patches,  $p>0.05$ ). Fig 5C shows the average peak current density as a function of voltage for the voltage-activated BK currents in retinal VSMCs from non-diabetic and diabetic animals. No differences were observed, suggesting that BK channel density is unaltered in retinal VSMCs during diabetes. Consistent with this, in single channel recordings we found no difference in the number of BK channels per membrane patch in non-diabetic ( $2.4 \pm 0.2$  channels) and diabetic ( $2.1 \pm 0.2$  channels) retinal VSMCs ( $p>0.05$ ,  $n=40$  patches).

Another possible explanation for the reduced coupling between  $\text{Ca}^{2+}$  release and BK channel activation is a decrease in the sensitivity of the BK channels to activation by  $\text{Ca}^{2+}$ . We examined the  $\text{Ca}^{2+}$ -sensitivity of BK channels using inside-out membrane patches from retinal VSMCs (Fig 6). The open probability ( $P_o$ ) at  $+80\text{mV}$  was determined for a range of  $\text{Ca}^{2+}$  concentrations between  $0.01$ - $100\mu\text{mol/L}$  (Fig 6A,B). The activity of BK channels from both non-diabetic and diabetic animals increased with increasing  $\text{Ca}^{2+}$  concentrations, but the  $P_o$  vs  $\text{Ca}^{2+}$  curve was shifted to the right and the Hill slope was reduced for diabetics (Fig 6B). No differences in  $P_o$ 's were observed at  $\text{Ca}^{2+}$  concentrations of  $0.01$  and  $0.1\mu\text{mol/L}$ , and this may explain the similarity in voltage-activated, whole cell BK-currents described above.  $P_o$  vs voltage relations were also determined at a single  $\text{Ca}^{2+}$  concentration,  $10\mu\text{mol/L}$  (Fig 6C). There was a strong rightward shift along the voltage axis ( $>100\text{mV}$ ) for BK channels from diabetic animals.

### **$\beta 1$ expression and function is lower in diabetic retinal VSMCs**

The results above suggest that BK channels in diabetic VSMCs have a reduced  $\text{Ca}^{2+}$  sensitivity. Since the  $\text{Ca}^{2+}$  sensitivity of BK channels is known to be dependent on the presence of  $\beta 1$  accessory subunits<sup>17, 18</sup>, a downregulation of the  $\beta 1$  subunit could explain the changes observed in diabetes. Expression of the BK $\beta 1$  subunit in retinal arterioles from non-diabetic and diabetic animals was investigated at the mRNA level.  $\beta 1$  transcripts were approximately 60% less abundant in diabetic than in non-diabetic arterioles (Fig 7A). We also estimated changes in expression of the  $\beta 1$  subunit by immunohistochemistry. A punctuate distribution of BK $\beta 1$ -associated fluorescence was apparent throughout the VSMC layer of retinal arterioles from non-diabetic animals (Fig 7B). Consistent with the RT-PCR analysis, BK $\beta 1$  immunostaining decreased dramatically in retinal arterioles of diabetic rats (Fig 7B) i.e. BK $\beta 1$  expression is reduced in retinal VSMC in diabetes.

Besides increasing the  $\text{Ca}^{2+}$  sensitivity of the BK $\alpha$ -subunit, the BK $\beta 1$ -subunit also modifies the kinetics and pharmacological properties of BK channels<sup>18</sup>. The  $\beta 1$  subunit increases the stability of BK channel open states<sup>27</sup>. If there is a decrease in the coupling ratio of  $\alpha/\beta 1$  subunits in diabetes then BK channel open times should be reduced. We compared the open times of BK channels from control and diabetic retinal VSMCs by constructing open time histograms at  $+80\text{mV}$  with  $10\mu\text{mol/L}$   $\text{Ca}^{2+}$ . Histograms were fitted with a single exponential function. BK channels from diabetic myocytes ( $\tau_{\text{open}} = 2.6 \pm 1.5\text{ms}$ ) had shorter open times than those from control VSMCs ( $\tau_{\text{open}} = 6.36 \pm 0.54\text{ms}$ ;  $p<0.05$ ), supporting the view that  $\beta 1$  subunit function is decreased during diabetes. Recently it has been shown that the xenoestrogen, tamoxifen, markedly increases the  $P_o$  of BK channels but only when they are associated with a  $\beta 1$  subunit<sup>28</sup>. Exposure of BK channels from non-diabetic cells to  $1\mu\text{mol/L}$  tamoxifen increased the  $P_o$  by approximately 1.5-fold but had no significant effect on BK channels from diabetic retinal VSMCs (Fig 7C). From these data, we conclude that the reduced coupling efficiency between  $\text{Ca}^{2+}$  release and BK channel activation in diabetic retinal VSMCs results from reduced  $\text{Ca}^{2+}$  sensitivity of the BK channels, reflecting a decrease in the functional expression of the BK $\beta 1$  accessory subunit.

It has also previously been reported that BK $\alpha$ + $\beta$  channels are markedly less sensitive to blockade by iberiotoxin when compared to BK $\alpha$  channels alone<sup>17</sup>. To determine whether the BK $\beta$ 1 subunit might affect the sensitivity of BK channels to Pen A, we compared the effects of this inhibitor on the  $P_o$  of BK channels from non-diabetic and diabetic retinal VSMCs (Fig 7D). Application of 100nmol/L Pen A to BK channels from non-diabetic cells caused close to 100% block of channel activity. By contrast, the  $P_o$  of BK channels from diabetic cells was reduced by only 27%. These results are consistent with the possibility that BK $\beta$ 1 increases the sensitivity of BK channels to Pen A, although further studies utilizing heterologous expression systems are required to confirm this.

## Discussion

It has been recognised for the past 25 years that abnormal blood flow to the retina occurs during early diabetes and that this may contribute to the pathogenesis of diabetic retinopathy<sup>12</sup>. Despite this, surprisingly little is known about the precise mechanisms linking chronic hyperglycaemia to retinal arteriolar vasoconstriction and reduced retinal blood flow prior to the onset of overt retinopathy. In the present study, we have identified a major pathophysiological mechanism that may play a specific role in the development of retinal perfusion abnormalities in diabetes. We have obtained molecular and functional data to suggest that during diabetes, the BK $\beta$ 1 subunit is downregulated in retinal VSMCs, while the expression of the pore-forming  $\alpha$ -subunit remains unaltered. Consistent with this, we observed a marked reduction in the Ca<sup>2+</sup> sensitivity of BK channels and an uncoupling of BK channel activation from Ca<sup>2+</sup> release in diabetic retinal VSMCs. Of particular note, the decreased expression of the  $\beta$ 1 subunit drastically reduced the ability of spontaneous Ca<sup>2+</sup> sparks to activate BK channel-mediated STOCs. Because STOCs act to hyperpolarise and relax VSMCs<sup>15, 29</sup>, loss of STOC activity could well underlie the observed arteriolar vasoconstriction seen in the development of diabetic retinopathy.

Information is generally lacking regarding the effects of diabetes on vascular BK channel function. Coronary microvessels from diabetic dyslipidemic swine exhibit an uncoupling in the relationship between Ca<sup>2+</sup> sparks and STOC activation<sup>30</sup> and whole-cell BK current density is reduced in microvascular smooth muscle cells of mesenteric arteries from Zucker diabetic fatty (ZDF) rats<sup>31</sup> and fructose-fed, insulin resistant rats<sup>32</sup>. The precise mechanisms underlying the changes in vascular BK channel activity were not resolved in these studies, although it is interesting that an alteration in BK $\beta$ 1 expression was not apparent in ZDF rats<sup>31</sup>. This discrepancy with the present study could possibly be attributed to the different animal models used and the origins of the microvessels studied. In general terms, however, the findings from the current work do strengthen the view that BK channel function is impaired in VSMCs of the microcirculation during diabetes. Intriguingly, impaired BK channel function may be limited to the microvasculature since the  $P_o$  of BK channels is increased in thoracic aortic VSMCs of STZ-induced diabetic mice<sup>33</sup>.

Hypertension is approximately twice as frequent in patients with diabetes compared to patients without the disease<sup>34</sup>. Furthermore work linked to the United Kingdom Prospective Diabetes Study (UKPDS) showed that the incidence of micro- and macrovascular diabetic complications was strongly associated with elevated blood pressure (BP)<sup>35</sup>. Presently the etiology of hypertension in diabetic patients is not fully understood, although loss of the normal vascular relaxation to insulin in both type 1 (insulin-deficiency) and type 2 (insulin-resistance) diabetes may contribute<sup>36</sup>. The present study is the first to raise the possibility that there is a selective downregulation of BK $\beta$ 1 resulting in an impairment of RyR-BK channel signaling which could be an important component in the pathogenesis of hypertension in diabetic patients. However, detailed profiling of BK $\beta$ 1 expression in a range of vascular tissues during diabetes, particularly in small arteries and arterioles that are

central to BP regulation<sup>37</sup>, is required before any firm conclusions can be made. It is likely that a widespread downregulation of BK $\beta$ 1 in diabetes could be sufficient to induce hypertension since BK $\beta$ 1 knockout mice are indeed hypertensive<sup>19, 20</sup>. Furthermore, downregulation of BK $\beta$ 1 has been reported in animal models of both acquired and genetic hypertension<sup>38, 39</sup>. Also, a recent population-based epidemiological study has reported that a gain-of-function polymorphism (E65K) in the BK $\beta$ 1 subunit, which increased the apparent Ca<sup>2+</sup>- and voltage-sensitivity of the pore-forming BK $\alpha$  subunit, is associated with a low prevalence of diastolic hypertension in this population<sup>40</sup>. Future work should be directed towards a clearer understanding of the role BK $\beta$ 1 downregulation in the development of hypertension in diabetic patients as well as elucidating the nature of the signaling mechanisms that regulate BK $\beta$ 1 expression during diabetes.

Another novel observation in the current study was that Ca<sup>2+</sup> spark amplitudes were greater in retinal VSMCs from diabetic animals, even though the frequency and duration of these events were unaltered. It seems unlikely that this represents a compensatory response to the downregulation of BK $\beta$ 1 and loss of Ca<sup>2+</sup> spark/BK channel coupling since the spatiotemporal properties of Ca<sup>2+</sup> sparks were unaltered in VSMCs of BK $\beta$ 1 knockout animals<sup>19, 20</sup>. Ca<sup>2+</sup> spark amplitude should depend on the number and activity (open times) of RyR channels in each spark unit and the driving force for Ca<sup>2+</sup> efflux from the SR. It seems unlikely that the larger Ca<sup>2+</sup> spark amplitudes can be attributed to changes in the SR Ca<sup>2+</sup> load, since caffeine-evoked [Ca<sup>2+</sup>]<sub>i</sub> transients and caffeine-activated Cl<sub>Ca</sub> currents were similar in retinal VSMCs from non-diabetic and diabetic animals. Diabetic retinal vessels are known to accumulate increased levels of advanced glycation end-products<sup>41</sup> and these adducts are known to accumulate heavily on RyR channels during diabetes<sup>42</sup>. The possible contribution of AGE crosslinking of RyRs to alterations in Ca<sup>2+</sup> spark activity in diabetic retinal VSMCs deserves further investigation.

In summary, we have presented data that strongly supports the hypothesis that diabetes downregulates the expression of the BK $\beta$ 1 subunit and consequently decreases Ca<sup>2+</sup> dependent activity of BK channels in retinal VSMCs. Our findings may have important implications with respect to the early pathogenesis of diabetic retinopathy. It is also fascinating to speculate that this mechanism might contribute to the development of hypertension in diabetic patients, although more extensive studies are needed to fully evaluate this possibility.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by grants from The Juvenile Diabetes Research Foundation, USA; Fight for Sight, UK; and The Wellcome Trust.

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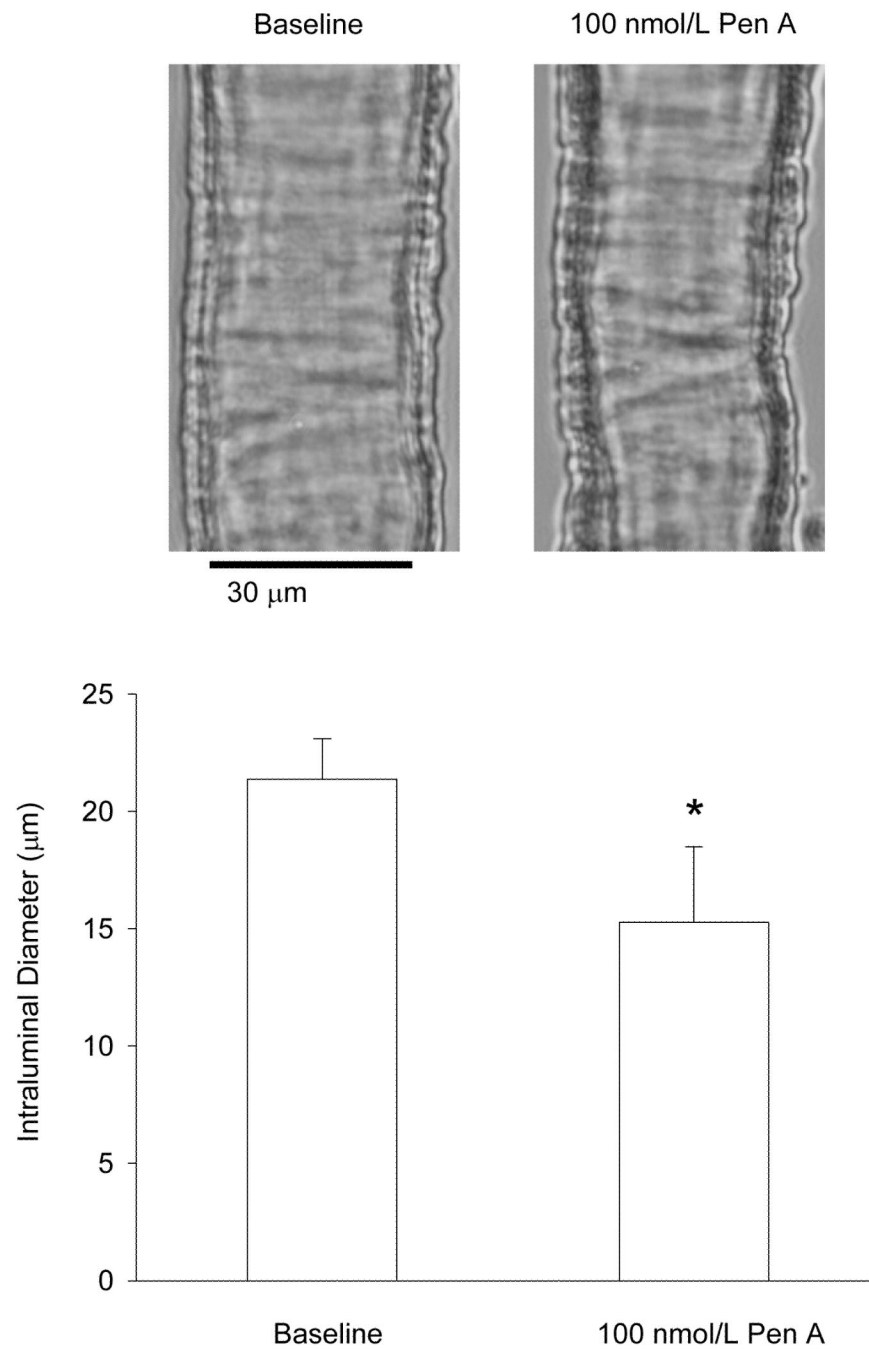
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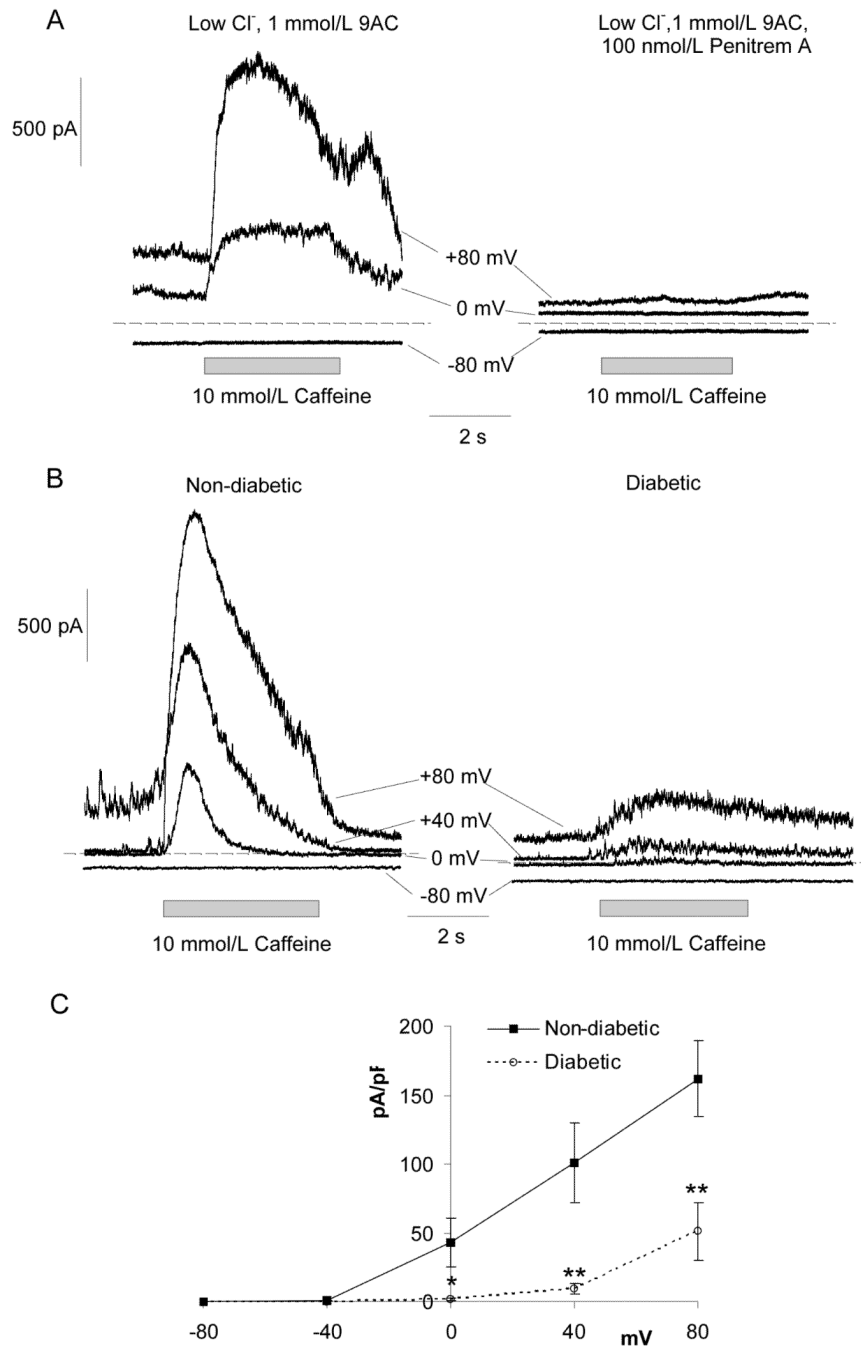
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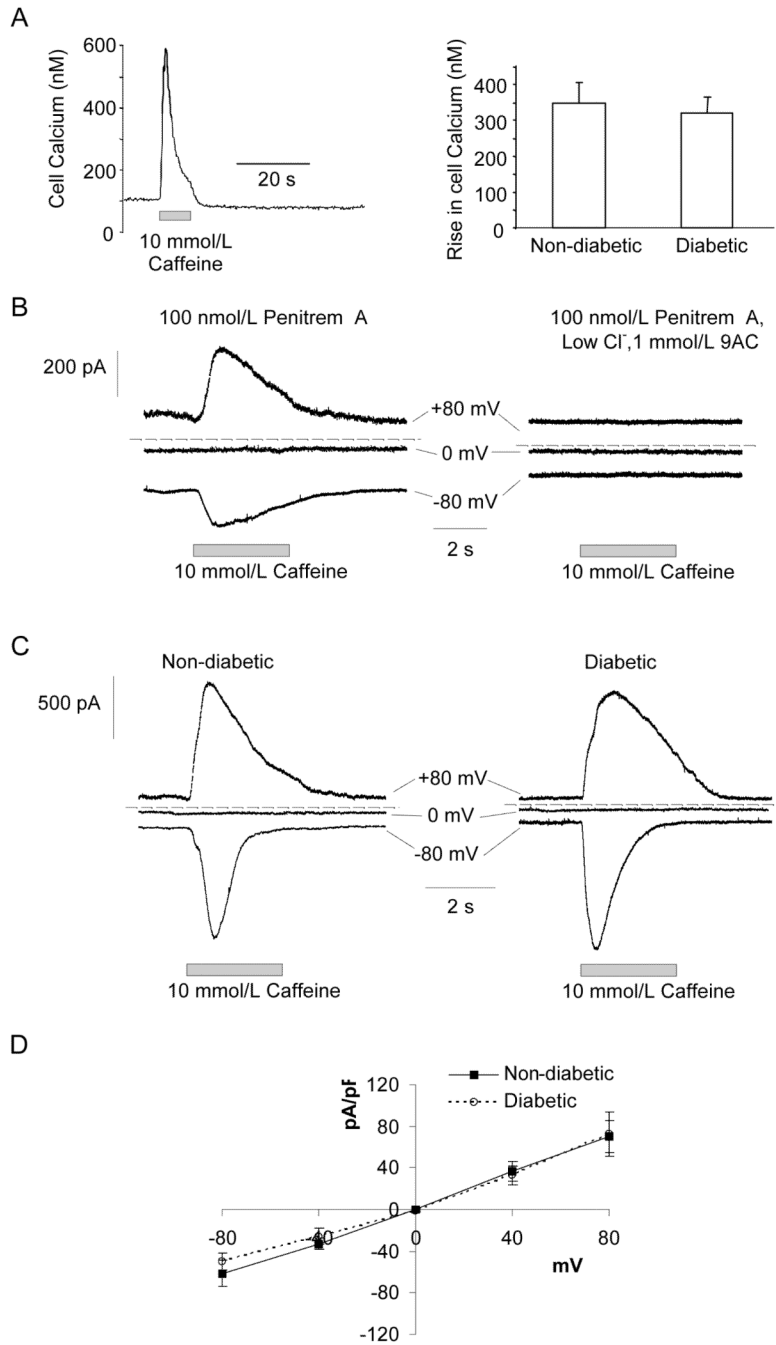
**Figure 1. Physiological significance of BK channels in retinal arterioles**

Top panel, representative photomicrographs of a pressurized retinal arteriole (70mmHg) before and 10-min after the application of 100nmol/L Pen A. Bottom panel, mean  $\pm$  SEM of the Pen A-induced constriction in non-diabetic retinal arterioles (n=8 vessels, p<0.05; paired t-test).



**Figure 2. Caffeine-induced BK currents recorded from retinal VSMCs of non-diabetic and diabetic animals**

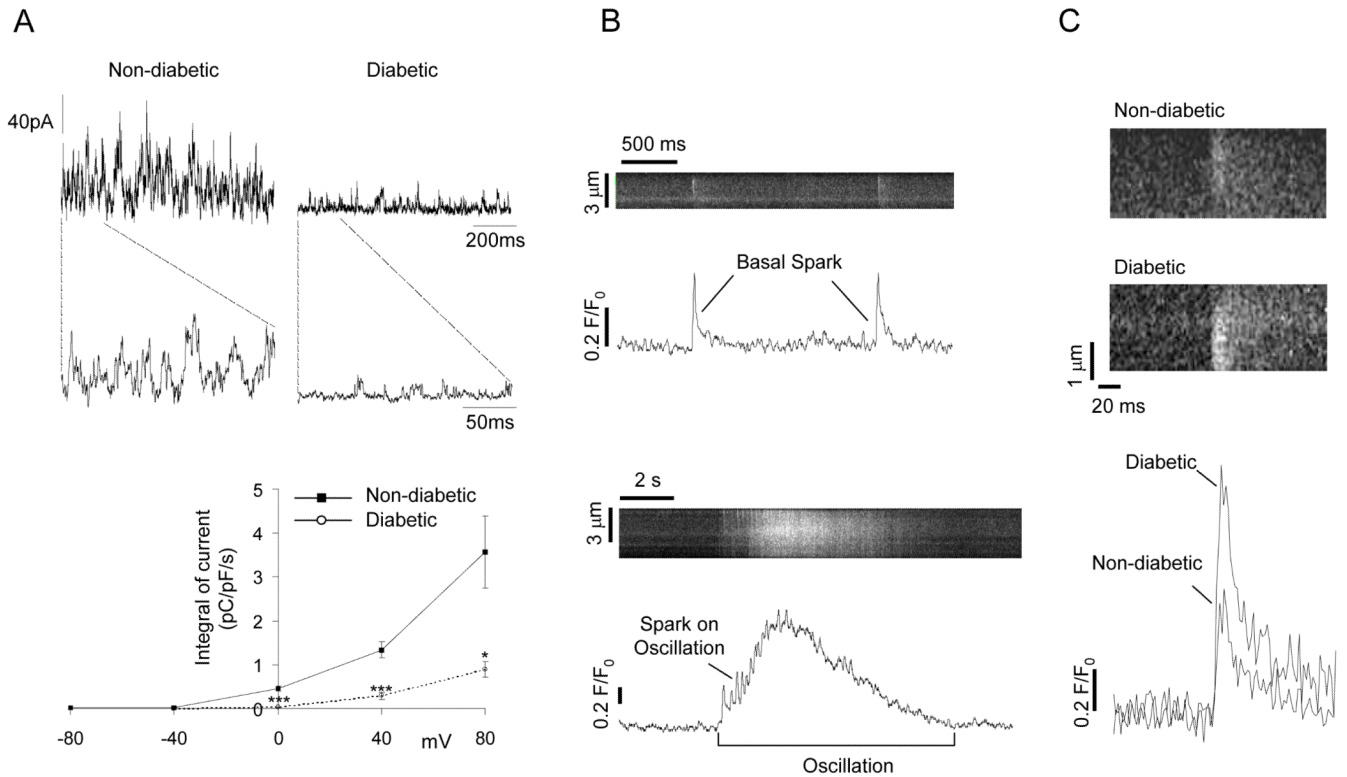
(A) Left, whole-cell records showing caffeine-induced outward currents in a non-diabetic vessel held at a range of potentials. The vessel was bathed in low Cl<sup>-</sup> Hanks' solution with 1mmol/L 9AC to block Cl<sub>Ca</sub> currents. Right, the BK channel inhibitor, Pen A (100nmol/L), completely abolished the caffeine-induced currents. (B) Typical traces showing caffeine-induced BK currents at -80, 0, +40 and +80 mV in non-diabetic and diabetic retinal arterioles. (C) Summary current-voltage relationships for the caffeine-induced BK currents in non-diabetic (for each point, n=5-13) and diabetic (n=7-19) vessels.



**Figure 3. Caffeine-evoked global  $[Ca^{2+}]_i$  transients and  $Cl_{Ca}$  currents in non-diabetic and diabetic retinal VSMCs**

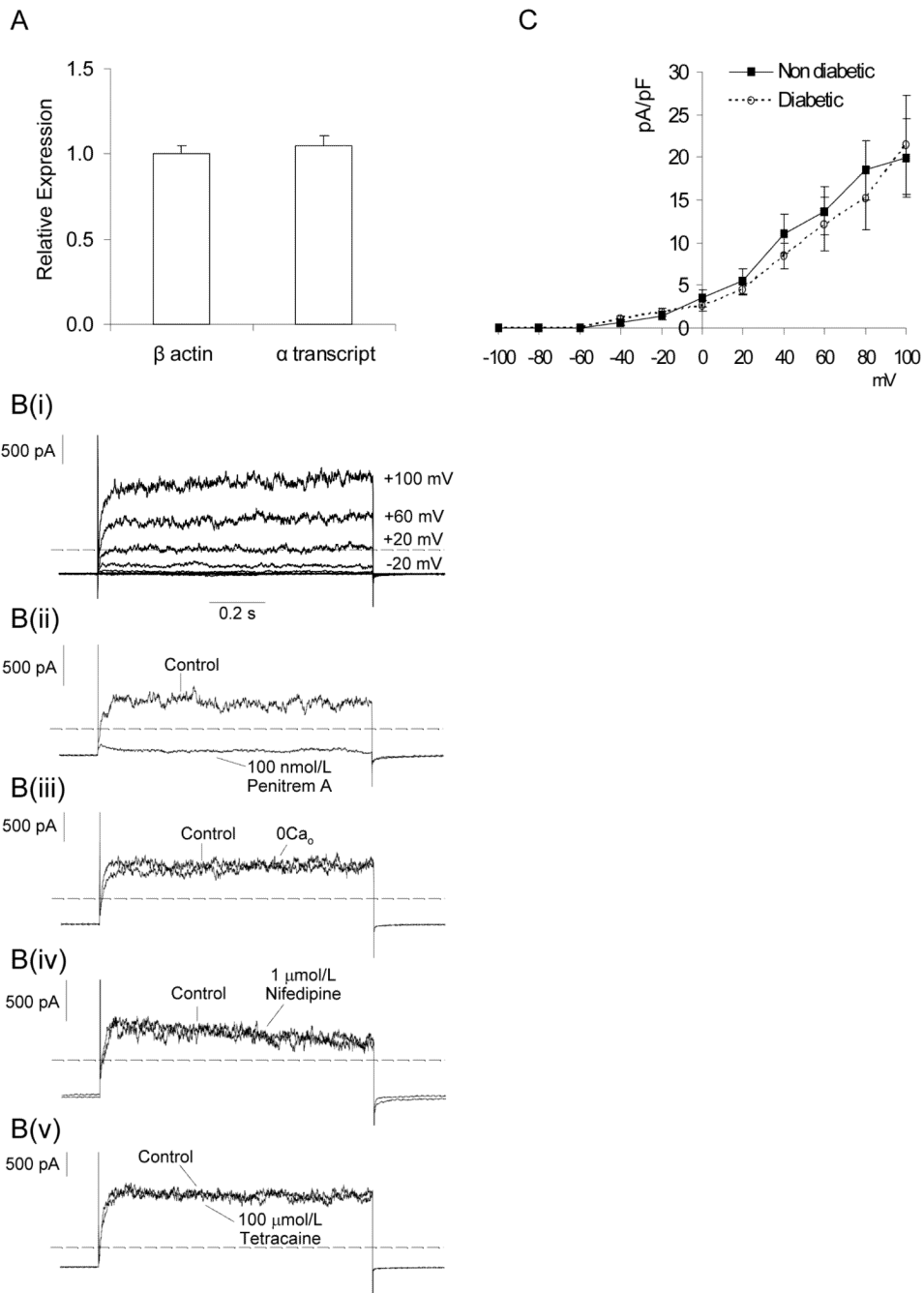
(A) Left, time-course record showing the effects of 10mmol/L caffeine on global  $[Ca^{2+}]_i$  in a non-diabetic retinal arteriole segment. Right, mean data showing that caffeine-induced  $[Ca^{2+}]_i$  transients were unaffected by diabetes. (B) Left, whole-cell currents elicited by 10 mmol/L caffeine in a non-diabetic vessel held at a range of test potentials and bathed in normal Hanks' solution containing the BK channel antagonist, Pen A (100nmol/L). Right, the  $Cl_{Ca}$  channel inhibitor, 9AC (1mmol/L), completely blocked the caffeine-evoked transient inward and outward currents. (C) Original records showing caffeine-induced  $Cl_{Ca}$  currents at -80, 0 and +80mV in non-diabetic and diabetic vessels. (D) Plot showing the

mean peak current density against voltage for the caffeine-evoked  $Cl_{Ca}$  currents in non-diabetic (n=12) and diabetic (n=10) retinal VSMCs.



**Figure 4. STOC activity is reduced but Ca<sup>2+</sup> sparks are greater in retinal VSMCs from diabetic animals**

(A) Top panel, whole-cell recordings of STOC activity in a non-diabetic and diabetic vessel at a holding potential of +40mV. Bottom panel, graph showing the mean integrated current density versus voltage for STOCs from non-diabetic (n=8) and diabetic (n=8) retinal arterioles. (B) Top panel, line-scan image recorded from a non-diabetic retinal VSMC showing two consecutive Ca<sup>2+</sup> sparks originating from the same Ca<sup>2+</sup> spark site. The graph below plots the fractional fluorescence change (F/F<sub>0</sub>) for this panel. Bottom panel, line scan image and graph on slower time scales from another non-diabetic cell in which Ca<sup>2+</sup> sparks amalgamate to produce a cell-wide global Ca<sup>2+</sup> oscillation. (C) Representative line-scan images of basal Ca<sup>2+</sup> sparks in non-diabetic and diabetic retinal VSMCs. Bottom, average temporal profile for each spark has been plotted and the traces superimposed.

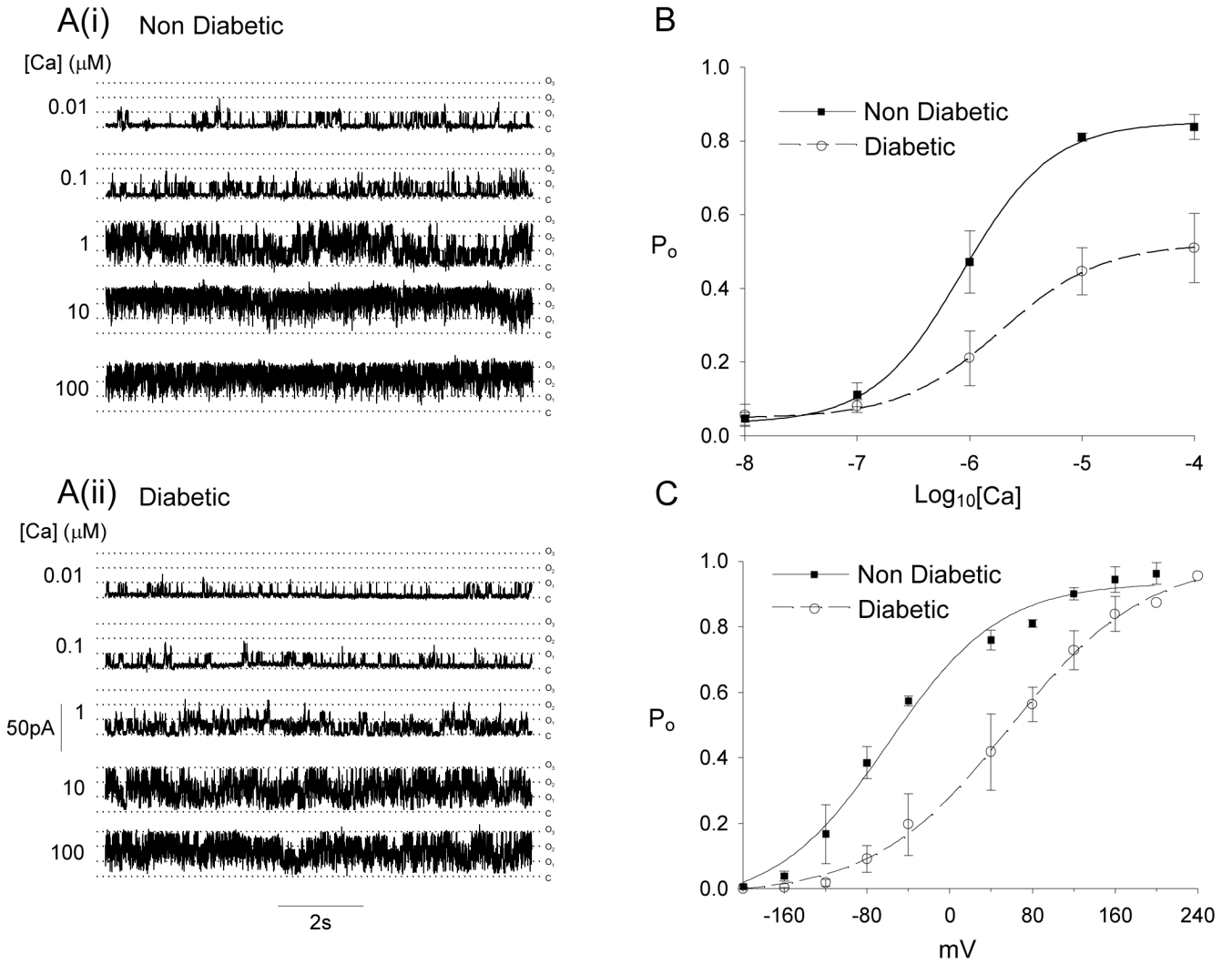


### Figure 5. Diabetes does not affect BK channel density

(A) Histogram showing relative BK $\alpha$  transcript expression in non-diabetic and diabetic retinal arterioles as determined by quantitative PCR. Amplifications were performed in triplicate. 9 non-diabetic and 9 diabetic rats were used in total and RNA was isolated from 15-25 retinal arterioles collected from 3 non-diabetic and 3 diabetic animals per replicate. BK $\alpha$  transcript expression was normalized to  $\beta$ -actin. (Bi) Family of whole-cell voltage clamp currents evoked in a non-diabetic retinal VSMC by voltage steps ranging between -100 mV to +100mV from an initial holding potential of -80mV in the presence of 9AC (1 mmol/L) and 4AP (10mmol/L). (Bii) The voltage-dependent current was abolished by the BK channel inhibitor, Pen A (100nmol/L; % change,  $-99.3 \pm 1.1\%$ ;  $p < 0.05$ ;  $n = 4$ ), but was

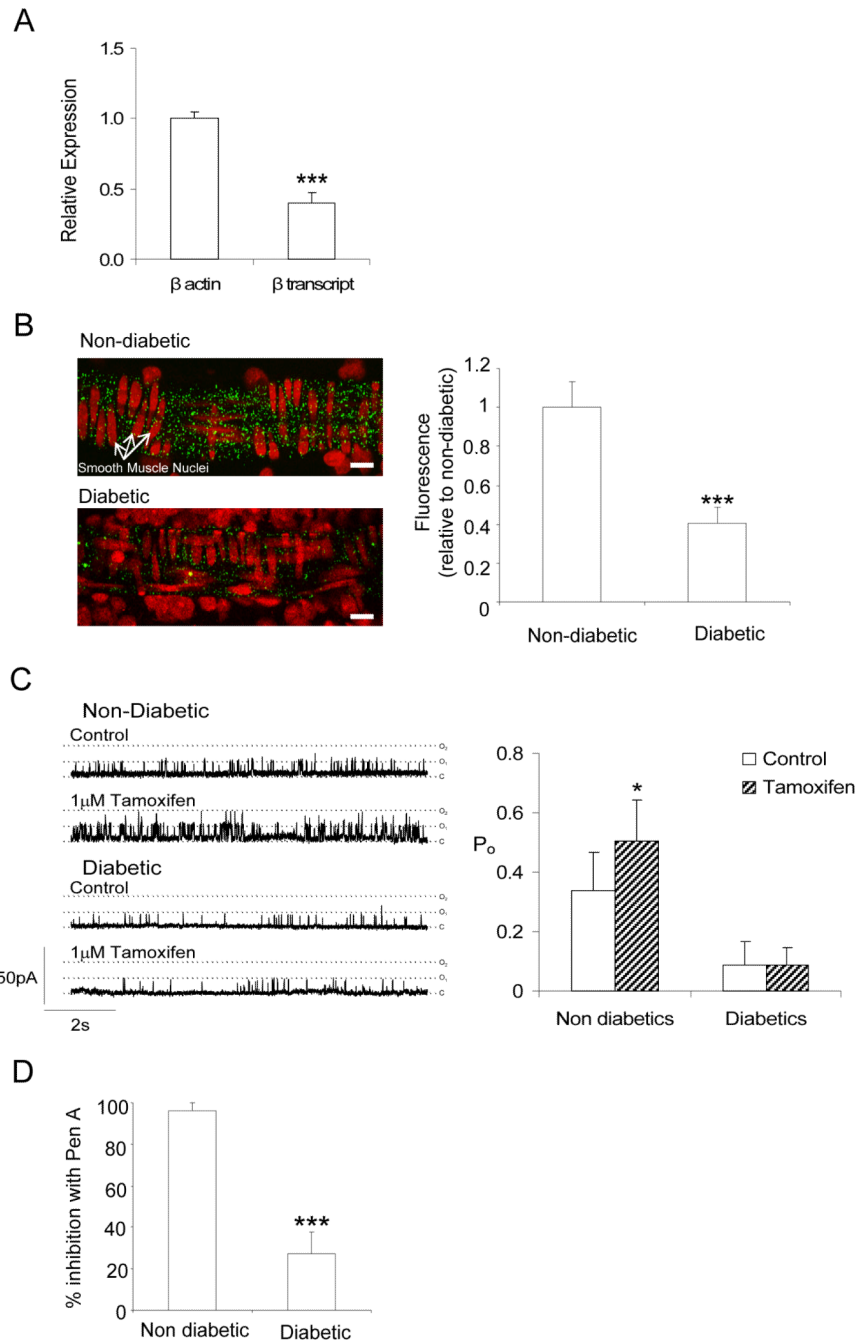


unaffected by (Biii) the removal of extracellular  $\text{Ca}^{2+}$  ( $2.5 \pm 8.5\%$ ;  $p > 0.05$ ;  $n=4$ ), (Biv) the L-type  $\text{Ca}^{2+}$  channel inhibitor, nifedipine ( $10 \mu\text{mol/L}$ ;  $-1.6 \pm 17.2\%$ ;  $p > 0.05$   $n=5$ ) and (Bv) the RyR antagonist tetracaine ( $100 \mu\text{mol/L}$ ;  $3.4 \pm 15.2\%$ ;  $p > 0.05$ ;  $n=5$ ). Responses were constant across the full voltage range. For clarity current records are presented for single steps between  $-80$  to  $+80\text{mV}$ . *Dashed lines*: zero current (C) Average peak current density as a function of voltage for the voltage-activated BK current in non-diabetic ( $n=13$ ) and diabetic ( $n=13$ ) vessels.



**Figure 6.  $\text{Ca}^{2+}$  sensitivity of single BK channels is reduced in diabetes**

(A) Representative single BK channel records in inside-out patches (holding potential +80mV) from non-diabetic and diabetic VSMCs exposed to increasing  $[\text{Ca}^{2+}]$ . (B) Summary data of the mean  $\pm$  SEM  $P_o$  at the five  $\text{Ca}^{2+}$  concentrations tested. Non-diabetic,  $n=8-11$ ; diabetics,  $n=8-11$ . Curves are fitted with the Hill equation as described in the supplementary methods. Fit parameters are as follows: non-diabetic ( $Kd = 0.86 \mu\text{mol/L}$ , Hill slope 1.1), diabetic ( $Kd = 1.9 \mu\text{mol/L}$ , Hill slope 0.97). (C)  $P_o$ -V relations determined at  $10 \mu\text{mol/L}$   $\text{Ca}^{2+}$ . Non-diabetic,  $n=2-11$ ; diabetics,  $n=1-11$ . Curves are Boltzmann fits with the following parameters: non-diabetic ( $V_{1/2} = -59.7 \text{ mV}$ ); diabetic ( $V_{1/2} = 57.9 \text{ mV}$ ).



**Figure 7. BK $\beta$ 1 subunit expression and function**

(A) Downregulation of BK $\beta$ 1 mRNA in retinal VSMC cells from diabetic arterioles. BK $\beta$ 1 expression in diabetic arterioles is presented relative to non-diabetic vessels. Amplifications were performed in triplicate (same samples as for Fig 5A) and normalized as described for BK $\alpha$  transcripts (B) Left, confocal images of non-diabetic and diabetic retinal arterioles embedded within retinal flatmount preparations and labeled with anti-BK $\beta$ 1 Ab (green) and propidium iodide (red: nuclear label). Labeling of the circular smooth muscle is reduced in the tissue from the diabetic animal. Right, summary data showing statistically significant reduction in anti-BK $\beta$ 1 fluorescence for diabetic samples (n=6 retinas, 30 vessels) relative to non-diabetics (n=6 retinas, 25 vessels). (C) Sensitivity of single BK channels in inside out

patches to 1 $\mu$ mol/L tamoxifen (holding potential +80mV; 1 $\mu$ mol/L free [Ca<sup>2+</sup>]) from non-diabetic and diabetic retinal VSMCs. Right, summary data showing the differential effects of tamoxifen on the  $P_o$  of single BK channels from non-diabetic (n=7) and diabetic (n=8) vessels. (D) Pharmacology of single BK channels from non-diabetic (n=5) and diabetic (n=9) retinal VSMCs exposed to Pen A. Mean data is expressed as the % inhibition of  $P_o$  after 5-min of exposure to 100nmol/L Pen A.