Diabetes Downregulates Large-Conductance Ca\(^{2+}\)-Activated Potassium \(\beta1\) Channel Subunit in Retinal Arteriolar Smooth Muscle

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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\(^{2+}\) release events (Ca\(^{2+}\)-sparks), trigger the activation of large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK)-channels which hyperpolarize and relax vascular smooth muscle cells, thereby causing vasodilatation. In the present study, we examined BK channel function in retinal vascular smooth muscle cells from streptozotocin-induced diabetic rats. The BK channel inhibitor, Penitrem A, constricted nondiabetic 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\(^{2+}\)]\(_{i}\), release was unaffected. Spontaneous BK currents were smaller in diabetic cells, but the amplitude of Ca\(^{2+}\)-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\(^{2+}\)-sensitivity of single BK channels from diabetic retinal vascular smooth muscle cells was markedly reduced. The BK\(\beta1\) subunit confers Ca\(^{2+}\)-sensitivity to BK channel complexes and both transcript and protein levels for BK\(\beta1\) 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\(\beta1\) 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 hyperperfusion in early diabetes, an idea having wider implications for the pathogenesis of diabetic hypertension. (Circ Res. 2007;100:703-711.)

Key Words: Ca\(^{2+}\) sparks ■ diabetes mellitus ■ microcirculation ■ potassium channels ■ vascular smooth muscle cells

Diabetes causes changes to the structure and function of blood vessels in the retina leading to visual impairment and blindness.\(^1\) 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.\(^2,3\) 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 hemodynamic abnormalities occur before the onset of clinical diabetic retinopathy\(^4\) and that the development and progression of diabetic retinopathy correlates with the extent of the blood flow changes observed.\(^5-8\) In diabetic patients without retinopathy, retinal arteriolar vasoconstriction\(^9,10\) and decreased total retinal blood flow has been reported.\(^4,11\) However, as the disease progresses, the arterioles begin to dilate\(^12\) and bulk retinal blood flow increases in proportion to the severity of retinopathy, and thus the degree of retinal ischemia.\(^11\) 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 vascular smooth muscle cells (VSMCs).\(^13\) Large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels are known to play a crucial role in the regulation of arterial smooth muscle tone because blockade of these channels using the specific inhibitor ivabradine, causes membrane depolarisation and vasconstriction in pressurized isolated vessels.\(^14\) BK channels are acti-

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vated by local Ca\(^{2+}\) release events, termed Ca\(^{2+}\) sparks, resulting from opening of ryanodine receptor (RyR) channels in the sarcoplasmic reticulum (SR). The activation of BK channels results in outward K\(^+\) current that opposes VSMC contraction by causing membrane hyperpolarisation. This reduces Ca\(^{2+}\) influx by reducing activation of voltage-dependent Ca\(^{2+}\) channels. BK channels are composed of α-subunits and accessory β-subunits. The α-subunit forms the K\(^+\) selective pore, whereas the β subunits influence the kinetics, pharmacology and Ca\(^{2+}\) sensitivity of BK currents. Four members of the BK β-subunit family have been identified to date (β1-β4) and the β1-subunit is expressed predominantly in VSMCs. Targeted deletion of the β1-subunit gene reduces the Ca\(^{2+}\) sensitivity of BK channels and the coupling of Ca\(^{2+}\) sparks to BK channel activity in VSMCs from cerebral arteries. The functional significance of the β1-subunit of VSMC BK channels is underlined by the observation that knockout mice are hypertensive and display enhanced vascular reactivity to application of vasoconstrictors.

In the present study we have examined the effects of streptozotocin (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. These animals subsequently exhibit many of the vasodegenerative changes associated with human diabetic retinopathy. We hypothesized that diabetes causes a downregulation of the α- and/or β1-subunit, thereby reducing the capacity of the BK channels to hyperpolarise retinal VSMCs and resist vasoconstriction. We show that diabetes reduces coupling between Ca\(^{2+}\) release from RyR sensitive Ca\(^{2+}\) stores and BK channel activation. No alteration in the expression of the pore-forming α-subunit was evident, but β1-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 Ca\(^{2+}\) and we provide pharmacological evidence supporting the idea that the expression of functional α+β1 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.

**Materials and 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 to 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](http://circres.ahajournals.org).

**Results**

**Characteristics of Experimental Animals**

Diabetic animals had higher mean plasma glucose levels (27.8±1.3mmol/L; n=41) than nondiabetic animals (7.6±0.3mmol/L; n=37 P<0.001). Mean glycosylated hemoglobin values were 5.9±0.2% and 17.6±0.8% in the nondiabetic 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 nondiabetic than diabetic rats (304±17g versus 141±9.7g, 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 nondiabetic animals to 70mmHg and measuring the change in internal diameter induced by 100nmol/L Penitrem A (Pen A), a potent inhibitor of BK channels. Pen A caused a 28% decrease in the diameter of pressurized retinal vessels (Figure 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 1mmol/L 9-anthracene carboxylic acid (9AC) to block Ca\textsuperscript{2+} activated Cl\textsubscript{Ca} channels. Application of 10mmol/L caffeine for 5 seconds evoked large, noisy transient outward currents at positive membrane potentials (Figure 2A and 2C). These currents were fully abolished by addition of 100nmol/L Pen A (Figure 2A; n=6).

Figure 2B shows typical caffeine-induced BK currents in retinal VSMCs from nondiabetic and diabetic animals. Figure 2C shows the average peak current density plotted as a function of voltage for the caffeine-induced BK currents. BK currents evoked by Ca\textsuperscript{2+} release from caffeine-sensitive Ca\textsuperscript{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\textsuperscript{2+} release from the caffeine-sensitive stores. To test this, global [Ca\textsuperscript{2+}] responses were measured in retinal VSMCs from nondiabetic and diabetic animals using fura 2 based Ca\textsuperscript{2+} microfluorimetry (Figure 3A). No differences in the peak amplitude of [Ca\textsuperscript{2+}]\textsubscript{i} transients evoked by 10mmol/L caffeine were observed. We also compared the size of caffeine-evoked Cl\textsubscript{Ca} currents at a range of voltages in myocytes from nondiabetic and diabetic arterioles bathed in normal Hanks’ solution containing 100nmol/L Pen A to block BK channels. Application of 10mmol/L caffeine evoked currents that reversed close to E\textsubscript{Cl} (0mV; Figure 3B and 3D). These were completely abolished
in low Cl⁻ Hanks’ solution containing the Cl⁻ channel inhibitor, 9AC (Figure 3B; n=6). Isolation of the caffeine-evoked Cl⁻ currents in diabetic arterioles required longer preincubation times with Pen A (∼20 minutes as compared with ∼5-minute for nondiabetic vessels). Figure 3C and 3D show representative traces and summary data for the caffeine-evoked Cl⁻ currents in nondiabetic and diabetic retinal VSMCs. The mean peak current densities were similar for nondiabetic and diabetic cells at all voltages tested. Thus, it appears that the smaller caffeine-induced BK currents in the nondiabetic. Quantitative data for basal Ca²⁺ sparks on oscillations and global Ca²⁺ oscillations in nondiabetic 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 Ca²⁺ spark in the diabetic cell is around twice that of the nondiabetic. Quantitative data for basal Ca²⁺ sparks, Ca²⁺ sparks on oscillations and global oscillations in nondiabetic

**Figure 4.** STOC activity is reduced but Ca²⁺ sparks are greater in retinal VSMCs from diabetic animals. A, top panel, Whole-cell recordings of STOC activity in a nondiabetic and diabetic vessel at a holding potential of +40mV. Bottom panel, Graph showing the mean integrated current density versus voltage for STOCs from nondiabetic (n=8) and diabetic (n=8) retinal arterioles. B, top panel, Line-scan image recorded from a nondiabetic retinal VSMC showing 2 consecutive Ca²⁺ sparks originating from the same Ca²⁺ spark site. The graph below plots the fractional fluorescence change (F/F₀) for this panel. Bottom panel, Line scan image and graph on slower time scales from another nondiabetic cell in which Ca²⁺ sparks amalgamate to produce a cell-wide global Ca²⁺ oscillation. C, Representative line-scan images of basal Ca²⁺ sparks in nondiabetic and diabetic retinal VSMCs. Bottom, average temporal profile for each spark has been plotted and the traces superimposed.

**Ca²⁺ Spars Are Larger, but STOCs Are Smaller in Diabetic Retinal VSMCs**

From a physiological perspective it is well established that Ca²⁺ sparks activate BK channels generating spontaneous transient outward K⁺ currents (STOCs), modulating vascular tone. STOCs were recorded from retinal VSMCs of nondiabetic 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, nondiabetic VSMCs). Individual STOC events were superimposed (Figure 4A) precluding an accurate assessment of STOC amplitudes and frequencies, so we calculated the integral of the STOC densities for recordings lasting 5 to 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 (Figure 4A). These data confirm that retinal VSMCs from diabetic animals demonstrate less spontaneous BK current activity than cells from nondiabetic animals.

Using confocal imaging techniques, we have recently described the presence of 2 distinct populations of spontaneous Ca²⁺ sparks in retinal VSMCs: “basal” sparks that arise from resting fluorescence levels (ie, from F/F₀=0.95 to 1.05) and “sparks on oscillations” that overlay global Ca²⁺ transients. Figure 4B shows representative images of basal Ca²⁺ sparks, Ca²⁺ sparks on oscillations and global Ca²⁺ oscillations in nondiabetic arteriolar myocytes. We considered the possibility that the lower STOC activity in diabetic retinal VSMCs may reflect reduced spontaneous subcellular [Ca²⁺], signaling in these cells. Figure 4C shows typical images of basal Ca²⁺ sparks in nondiabetic 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 Ca²⁺ spark in the diabetic cell is around twice that of the nondiabetic. Quantitative data for basal Ca²⁺ sparks, Ca²⁺ sparks on oscillations and global oscillations in nondiabetic...
and diabetic VSMCs are summarized in supplemental Table I of the online data supplement. The peak amplitude of both populations of Ca\(^{2+}\) sparks was substantially larger in diabetic than in nondiabetic VSMCs (ΔF/F₀, basal sparks, 0.92±0.06 and 0.42±0.03, respectively; ΔF/F₀ sparks on oscillations, 1.56±0.21 and 0.36±0.04, respectively; P<0.001 in both cases), whereas the frequency and duration (FDHM) of these events remained unchanged. No differences were observed in amplitude, frequency or duration of global Ca\(^{2+}\) oscillations between nondiabetic and diabetic VSMCs. These results show that decreased subcellular Ca\(^{2+}\) signaling activity cannot explain the decreased STOC activity observed in diabetic VSMCs.

### Diabetes Reduces the Ca\(^{2+}\) Sensitivity of BK Channels in Diabetic Retinal VSMCs

Both the comparisons of caffeine-evoked currents and Ca\(^{2+}\) transients, and of STOC and spark activity, suggest decreased coupling between Ca\(^{2+}\) 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 BKα transcript expression in diabetic and diabetic retinal arterioles (Figure 5A). To assess BK channel density, we also compared depolarization dependent whole-cell BK currents (Figure 5B; and 5Bii). Vessels were bathed in low Cl\(^{-}\) Hanks’ solution containing 1mmol/L H11006, 10 H1006 and 10 H9262 mmol/L Ca\(^{2+}\) and 26 respectively. 10 mmol/L 4AP has no effect on BK currents in retinal arterioles; caffeine-evoked BK currents at +40mV in the absence and presence of 10 mmol/L 4AP were 115±29.8 and 114±26pA/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\(^{2+}\), inhibition of voltage-dependent Ca\(^{2+}\) channels with 10 μmol/L nifedipine or blockade of RyR receptors with 100 μmol/L tetracaine (Figure 5Biii-v). This demonstrates that BK currents evoked by depolarisation are independent of Ca\(^{2+}\) influx and Ca\(^{2+}\) 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 nondiabetic and diabetic VSMCs (160±2pS and 160±4pS, respectively; n=21 and 15 patches, P>0.05). Figure 5C shows the average peak current density as a function of voltage for the voltage-activated BK currents in retinal VSMCs from nondiabetic 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 nondiabetic (2.4±0.2 channels) and diabetic (2.1±0.2 channels) retinal VSMCs (P>0.05, n=40 patches).

Another possible explanation for the reduced coupling between Ca\(^{2+}\) release and BK channel activation is a decrease in the sensitivity of the BK channels to activation by Ca\(^{2+}\). We examined the Ca\(^{2+}\)-sensitivity of BK channels using inside-out membrane patches from retinal VSMCs (Figure 6). The open probability (P₀) at +80mV was determined for a range of Ca\(^{2+}\) concentrations between 0.01 to 100μmol/L (Figure 6A and 6B). The activity of BK channels from both nondiabetic and diabetic animals increased with increasing Ca\(^{2+}\) concentrations, but the P₀ versus Ca\(^{2+}\) curve was shifted to the right and the Hill slope was reduced for diabetics (Figure 6B). No differences in P₀’s were observed at Ca\(^{2+}\) concentrations of 0.01 and 0.1μmol/L, and this may explain the similarity in voltage-activated, whole cell BK-currents described above. P₀ versus voltage relations were also determined at a single Ca\(^{2+}\) concentration, 10μmol/L (Figure 6C). There was a strong rightward shift along the voltage axis (>100mV) for BK channels from diabetic animals.

### β1 Expression and Function is Lower in Diabetic Retinal VSMCs

The results above suggest that BK channels in diabetic VSMCs have a reduced Ca\(^{2+}\) sensitivity. Because the Ca\(^{2+}\) sensitivity of BK channels is known to be dependent on the presence of β1 accessory subunits, a downregulation of the β1 subunit could explain the changes observed in diabetics. Expression of the BKβ1 subunit in retinal arterioles from nondiabetic and diabetic animals was investigated at the mRNA level. β1 transcripts were approximately 60% less abundant in diabetic than in nondiabetic arterioles (Figure 7A). We also estimated changes in expression of the β1 subunit by immunohistochemistry. A punctuate distribution of BKβ1-associated fluorescence was apparent throughout the VSMC layer of retinal arterioles from nondiabetic animals (Figure 7B). Consistent with the RT-PCR analysis, BKβ1 immunostaining decreased dramatically in retinal arterioles of diabetic rats (Figure 7B) i.e., BKβ1 expression is reduced in retinal VSMC in diabetes.

Besides increasing the Ca\(^{2+}\) sensitivity of the BKα subunit, the BKβ1 subunit also modifies the kinetics and pharmacological properties of BK channels. The β1 subunit increases the stability of BK channel open states. If there is a decrease in the coupling ratio of α/β1 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 mV with 10 μmol/L Ca\(^{2+}\). Histograms were fitted with a single exponential function. BK channels from diabetic myocytes (τ\(_{open}=2.6±1.5\)ms) had shorter open times than those from control VSMCs (τ\(_{open}=6.36±0.54\)ms; P<0.05), supporting the view that β1 subunit function is decreased during diabetes. Recently it has been shown that the xenoestrogen, tamoxifen, markedly increases the peak activity of BK channels from diabetic retinal arterioles; caffeine-evoked BK currents in retinal arterioles from nondiabetic animals increased with increasing Ca\(^{2+}\) concentrations, but the P₀ versus Ca\(^{2+}\) curve was shifted to the right and the Hill slope was reduced for diabetics (Figure 6B). No differences in P₀’s were observed at Ca\(^{2+}\) concentrations of 0.01 and 0.1μmol/L, and this may explain the similarity in voltage-activated, whole cell BK-currents described above. P₀ versus voltage relations were also determined at a single Ca\(^{2+}\) concentration, 10μmol/L (Figure 6C). There was a strong rightward shift along the voltage axis (>100mV) for BK channels from diabetic animals.
It has also previously been reported that BKβ1 channels are markedly less sensitive to blockade by iberiotoxin when compared with BKα channels alone. To determine whether the BKβ1 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 nondiabetic and diabetic retinal VSMCs (Figure 7D). Application of 100 nmol/L Pen A to BK channels from nondiabetic 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 BKβ1 increases the sensitivity of BK channels to Pen A, although further studies using heterologous expression systems are required to confirm this.

**Discussion**

It has been recognized 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. Despite this, surprisingly little is known about the precise mechanisms linking chronic hyperglycaemia to retinal arteriolar vasoconstriction and reduced retinal blood flow before 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 BKβ1 subunit is downregulated in retinal VSMCs, whereas the expression of the pore-forming α-subunit remains unaltered. Consistent with this, we observed a marked reduction in the Ca\(^{2+}\) sensitivity of BK channels and an uncoupling of BK channel activation from Ca\(^{2+}\) release in diabetic retinal VSMCs. Of particular note, the decreased expression of the β1 subunit drastically reduced the ability of spontaneous Ca\(^{2+}\) sparks to activate BK channel-mediated STOCs. Because STOCs act to hyperpolarise and relax VSMCs, 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\(^{2+}\) sparks and STOC activation\(^{30}\) and whole-cell BK current density is reduced in microvascular smooth muscle cells of mesenteric arteries from Zucker diabetic fatty rats\(^{31}\) and fructose-fed, insulin resistant rats.\(^{32}\) 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 BKβ1 expression was not apparent in Zucker diabetic fatty rats.\(^{31}\) 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 because the P\(_o\) of BK channels is increased in thoracic aortic VSMCs of STZ-induced diabetic mice.\(^{33}\)

Hypertension is approximately twice as frequent in patients with diabetes compared with patients without the disease.\(^{34}\) 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.\(^{35}\) 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.\(^{36}\) The present study is the first to raise the possibility that there is a selective downregu-
during diabetes, particularly in small arteries and arterioles that express BK1 mRNA in retinal VSMC cells from diabetic arterioles. BK1 expression in diabetic arterioles is presented relative to nondiabetic vessels. Amplifications were performed in triplicate (same samples as for Figure 5A) and normalized as described for BKα transcripts. B, left, Confocal images of nondiabetic and diabetic retinal arterioles embedded within retinal flatmount preparations and labeled with anti-BKα 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-BKα fluorescence for diabetic samples (n = 6 retinas, 30 vessels) relative to nondiabetics (n = 6 retinas, 25 vessels). C, Sensitivity of single BK channels in inside out patches to 1 μmol/L tamoxifen (holding potential +80 mV; 1 μmol/L free [Ca2+]) from nondiabetic and diabetic retinal VSMCs. Right, summary data showing the differential effects of tamoxifen on the Po of single BK channels from nondiabetic (n = 7) and diabetic (n = 8) vessels. D, Pharmacology of single BK channels from nondiabetic (n = 5) and diabetic (n = 9) retinal VSMCs exposed to Pen A. Mean data are expressed as the % inhibition of Po after 5-minute of exposure to 100 nmol/L Pen A.

Figure 7. BKβ1 subunit expression and function. A, Downregulation of BKβ1 mRNA in retinal VSMCs from diabetic arterioles. BKβ1 expression in diabetic arterioles is presented relative to nondiabetic vessels. Amplifications were performed in triplicate (same samples as for Figure 5A) and normalized as described for BKα transcripts. B, left, Confocal images of nondiabetic and diabetic retinal arterioles embedded within retinal flatmount preparations and labeled with anti-BKα 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-BKα fluorescence for diabetic samples (n = 6 retinas, 30 vessels) relative to nondiabetics (n = 6 retinas, 25 vessels). C, Sensitivity of single BK channels in inside out patches to 1 μmol/L tamoxifen (holding potential +80 mV; 1 μmol/L free [Ca2+]) from nondiabetic and diabetic retinal VSMCs. Right, summary data showing the differential effects of tamoxifen on the Po of single BK channels from nondiabetic (n = 7) and diabetic (n = 8) vessels. D, Pharmacology of single BK channels from nondiabetic (n = 5) and diabetic (n = 9) retinal VSMCs exposed to Pen A. Mean data are expressed as the % inhibition of Po after 5-minute of exposure to 100 nmol/L Pen A.

Another novel observation in the current study was that Ca2+ 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 BKβ1 and loss of Ca2+ spark/BK channel coupling because the spatiotemporal properties of Ca2+ sparks were unaltered in VSMCs of BKβ1 knockout animals.19,20 Ca2+ spark amplitude should depend on the number and activity (open times) of RyR channels in each spark unit and the driving force for Ca2+ efflux from the SR. It seems unlikely that the larger Ca2+ spark amplitudes can be attributed to changes in the SR Ca2+ load, because caffeine-evoked [Ca2+]i transients and caffeine-activated Cl− currents were similar in retinal VSMCs from nondiabetic and diabetic animals. Diabetic retinal vessels are known to accumulate increased levels of advanced glycation end-products41 and these adducts are known to accumulate heavily on RyR channels during diabetes.42 The possible contribution of AGE crosslinking of RyRs to alterations in Ca2+ 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 BKβ1 subunit and consequently decreases Ca2+ 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 devel-
ompment of hypertension in diabetic patients, although more extensive studies are needed to fully evaluate this possibility.

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Disclosures
None.

References
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Animal Preparation

Male Sprague-Dawley rats (200-250 g) were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg) freshly added to isotonic saline buffered by 20 mmol/L citrate, pH 4.6. After 2 weeks the rats were weighed and diabetes was confirmed by measuring blood glucose concentrations. Three months after the STZ injection rats were weighed and blood glucose measured again and percentage levels of glycosylated haemoglobin (GHb; Helena Biosciences, UK) determined. Only animals with a blood glucose concentration >15 mmol/L and GHb values >10% were included in the diabetic group. Non-injected, age-matched rats were used as controls. Rats were anesthetized with CO₂ and killed by cervical dislocation and those that had lost weight during the three month period were excluded. Retinal arterioles devoid of surrounding neuropile were mechanically isolated in low Ca²⁺ Hanks’ solution (in mmol/L: 140, NaCl; 6, KCl; 5, D-glucose; 0.1, CaCl₂; 1.3, MgCl₂; 10, HEPES (pH 7.4 with NaOH)) as previously described¹. For tissues from diabetic rats, all bathing solutions, including the low Ca²⁺ Hanks’ contained 25 mmol/L D-glucose.

Whole Cell and Single Channel Electrophysiology

BK currents were recorded from retinal VSMCs still embedded within their native arterioles at 37°C using the whole-cell perforated patch-clamp technique². Prior to electrophysiological recording, vessels were anchored down in the recording bath and digested for 20 min with an enzyme cocktail of collagenase 1A (0.1 mg/ml) and protease type XIV (0.01 mg/ml) to remove surface basal lamina and to electrically uncouple the
endothelial cells from overlying VSMCs cells. Pipette resistances were 1-2 MΩ. Arterioles were continuously superfused with Hanks’ solution (in mmol/L): 140, NaCl; 6, KCl; 5, D-glucose; 2, CaCl₂; 1.3, MgCl₂; 10, HEPES (pH 7.4 with NaOH). The internal solution was composed of (in mmol/L): 138 KCl; 1 MgCl₂; 0.5 EGTA; 0.2 CaCl₂; 10 HEPES (pH adjusted to 7.2 using KOH); free Ca²⁺: 100 nM, to which 300 µg/ml amphotericin B was added. Enzyme and drug solutions were delivered via an 11-way micro-manifold with an exchange time of 1 s as measured by switching over to dye solution. The flow from the manifold into the bath was through a single tube (350 µm in diameter, 6 mm in length, 0.2 µl volume) long enough to allow the temperature to equilibrate with the solution flowing through the bath. An Axopatch-1D (Axon Instruments) amplifier was used and recordings were low pass filtered at 0.5 kHz and sampled at 2 kHz by a National Instruments PC1200 interface using WINWCP V3.5 (John Dempster, University of Strathclyde). Leakage currents were subtracted off-line from the active currents with the use of the standard leak subtraction protocol contained within the Patch software suite. Series resistance (~30 MΩ) was routinely compensated by >70%. For determination of whole-cell current densities, cell membrane capacitance was determined from the time constant of a capacitance transient elicited by a 20 mV hyperpolarisation from -60 mV with a sampling frequency of 20 kHz. For recording of caffeine-induced BK currents arterioles were bathed in low Cl⁻ Hanks’ solution (in mmol/L: 140, NaGlutamate; 6, KCl; 5, D-glucose; 2, CaCl₂; 1.3, MgCl₂; 10, HEPES adjusted to pH 7.4 with NaOH) containing 1 mmol/L 9-anthracene carboxylic acid (9AC) to minimize contamination from Ca²⁺-activated Cl⁻ currents (Cl⁺⁺ current). For measurement of voltage-dependent BK currents 10 mmol/L 4-aminopyridine (4AP) was
added to this solution to minimize contamination by other voltage dependent K⁺ currents. Spontaneous transient outward currents (STOCs) were often superimposed on the background of the voltage-dependent BK currents. The sustained voltage-activated BK currents were measured from the base of the STOC events in the final 100 ms of the voltage pulse.

Single channel BK currents were recorded from inside-out patches under symmetrical K⁺ (140 mmol/L). Patches were bathed in solution containing (in mmol/L): 140, KCl; 1, MgCl₂; 10, HEPES; 1, HEDTA; 10, 4AP adjusted to pH 7.2 with KOH). Variable amounts of CaCl₂ were added to the solution to give the desired free Ca²⁺ concentrations (determined by using WebMAXC, Chris Patton, Stanford University). The patch pipette contained (in mmol/L): 140, KCl; 1.3, MgCl₂; 5, Glucose; 2, CaCl₂; 10, HEPES; 1, 9AC adjusted to pH 7.4 with KOH. Recordings (low pass filtered at 0.5 kHz and sampled at 5 kHz) were made and analyzed using WINEDR V2.5.9 (John Dempster, University of Strathclyde). Single channel currents were recorded at +80mV and all experiments were performed at 37°C. BK channel number, conductance, and open probability (Pₒ) were determined from all-points amplitude histograms; only recordings with stable Pₒ values for a minimum of 30 s were analyzed. The number of BK channels per patch was estimated while patches were held at +80 mV in the presence of 100µM Ca²⁺ or by the addition of the BK channel opener phloretin (10µmol/L;⁴).

**Conventional Ca²⁺ measurements**

VSMCs within arteriolar segments were loaded with the Ca²⁺ indicator dye fura 2. Arterioles were incubated with 5 µmol/L fura 2-AM for 2 hours. They were then washed
and superfused with Hanks’ solution at 37°C in a perfusion bath mounted on the stage of an inverted microscope (Nikon Eclipse TE2000). They were illuminated by 340/380 nm light from a dual monochromator (5 nm bandwidth) and light chopper (Cairn Research Ltd., Faversham, UK). Emitted fluorescence was measured from the side port of the microscope via an adjustable rectangular window, a filter (510 nm) and a photon counting photomultiplier tube (PMT) in the light path. Fluorescence equipment was controlled by Acquisition Engine (Cairn) software (V1.1.5) which was also used for analysis of the fluorescence data. Fluorescence ratios were converted to Ca\(^{2+}\) concentrations as previously described\(^1\).

**Sub-cellular Ca\(^{2+}\) imaging**

Ca\(^{2+}\) sparks and global Ca\(^{2+}\) oscillations were measured in retinal VSMCs retained within vascular fragments loaded with the fluorescent Ca\(^{2+}\) indicator Fluo-4AM (10 \(\mu\)mol/L) using a Biorad MR-A1 laser scanning confocal microscope coupled to a Nikon Eclipse TE300 inverted microscope fitted with a X60 oil-immersion lens (numerical aperture = 1.4)\(^5\). Changes in [Ca\(^{2+}\)]\(_i\) were recorded in line-scan mode along a line orientated transversely across the smooth muscle cells with a line-scan duration of 0.2 ms and a scan rate of 500 scans s\(^{-1}\). Experiments were performed at 37°C. Ca\(^{2+}\) sparks and global Ca\(^{2+}\) oscillations were detected using custom analysis software written in our laboratory by Dr Norman Scholfield (V1.20, Borland Delphi, UK). Fluorescence images were background corrected using an image frame collected without excitation. Detection of Ca\(^{2+}\) events was based on a gradient-change algorithm to detect rapid increases in fluorescence.
Events for which the maximum increase in F/F₀ was < 2 x peak-peak noise for resting fluorescence were excluded.

**Quantitative RT-PCR**

Total RNA was extracted from retinal arterioles using an RNeasy Micro kit (Qiagen, Crawley, UK), according to the manufacturer’s protocol. The RNA samples were quantified by Ribogreen (Invitrogen Inc.) using a microplate reader. Equal amounts of RNA samples were reverse transcribed into cDNA using a Sensiscript Reverse Transcription Kit from Invitrogen Inc. (Cat. No.205211). Quantitative PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, UK). Primers were designed using Vector NTI (Invitrogen Inc.) to amplify BKα (NM_031828, forward primer 5’CTCGAAGTGAGCTGCCGATGA3’, Reverse primer 5’ACTCCCGCTTGAAGCTTGA3’), BKβ1 (NM_019273, forward primer 5’ACCAATCTTCTTCTGACACGCAGC3’, Reverse primer 5’AGAGCTGTGACGTGCCTCTT3’) and β–actin (NM_031144, forward primer 5’TCCCTGGAGGAAGCTTACGC3’, Reverse primer 5’GTTCATGGATGCCACAGGATT3’). The reaction mix contained 1 µl of cDNA, 0.3 µmol/L of forward and reverse primer each, 5 µl of Quantitect SYBR Green I mix (Qiagen) and 3.4 µl of RNase free water making a total reaction of 10 µl. The PCR conditions were as follows: Denaturation, 95°C for 15 min; Amplification (45 cycles), 94°C for 15 s, 55°C for 30 s, and 72°C for 15 s; Melting curve, from 65°C to 95°C at 0.1°C·s⁻¹. Relative expression was quantified using the LinReg analysis package⁶.
Immunohistochemistry

Immunohistochemistry was carried out on retinal arterioles embedded within retinal flatmount preparations. Freshly enucleated eyes were placed in low Ca²⁺ Hanks’ solution, the cornea and lens dissected off and the posterior eye-cup fixed in 4% paraformaldehyde for 20 minutes. The preparation was then washed repeatedly in phosphate-buffered saline (PBS) for 4 hours. Retinae were detached and placed in permeabilization buffer (0.5% Triton X-100 in PBS), with 5% normal donkey serum (Chemicon International, Temecula, CA, US) to block non-specific antibody binding. Tissue was incubated at 4°C overnight with polyclonal antibodies raised in rabbit against residues 90-103 of the human BKβ1 potassium channel subunit (Affinity Bioreagents, Inc, CO, US) diluted 1:200 in the permeabilisation buffer and then washed for 4 hours at 21°C. Incubation and wash steps were repeated using a 1:200 dilution of 2° antibody, i.e., donkey anti-rabbit IgG, labelled with Alexa-488 (Molecular Probes Europe BV, Leiden, The Netherlands). To facilitate rapid identification of arterioles within the retinal neuropile, propidium iodide (PI) nuclear stain was used. Retinae were incubated in 5 µg/L of PI (Invitrogen, Carlsbad, CA, US) in PBS for 30 minutes at 37°C. Retinae were flattened by placing four radial cuts from the retinal periphery to points within 1 mm from the optic disk and mounted in Vectashield (Vector Laboratories Ltd, Burlingame, CA, US). Images were acquired using an Olympus BX60 fluorescence microscope (Olympus UK Ltd., London, UK) fitted with a MicroRadiance confocal-scanning laser microscope (Bio-Rad). To confirm specific binding of the BKβ1 antibody, the binding was blocked with corresponding excess antigen or absence of primary antibody (data not shown). BKβ1 immunofluorescence was only detectable in retinal blood vessels and not in other cell
types of the retina, consistent with BKβ1 being expressed predominantly in VSMCs (data not shown;\(^7\)). BKβ1 immunofluorescence was quantified using Image J (NIH, US) by measuring the intensity of pixels above a threshold, which was defined as 2SD above the mean fluorescence intensity at the optic disc (background). An average intensity value was generated for each arteriole by sampling areas of ~2000µm\(^2\) at three equidistant points along the vessel length starting at the optic disc and finishing at the edge of the retinal periphery.

**Arteriolar tone**

Vessels, 25-42 µm outside diameter and 400-4000 µm long, were isolated as described above and pipetted into a recording bath mounted on an inverted microscope. A tungsten wire slip (75x2000 µm) was laid on one end of the vessel which provided anchoring and occluded of the distal open end. The vessel was then superfused with Ca\(^{2+}\)-free Hanks’ (in mmol/L: 140, NaCl; 6, KCl; 5, D-glucose; 1.3, MgCl\(_2\); 10, HEPES (pH 7.4 with NaOH)) at 37°C. Cannulations were performed using glass pipettes (tip diameters 3-10µm) filled with Ca\(^{2+}\)-free medium held in a patch electrode holder and connected to a pressure transducer and water manometer. After the pipette was pushed into the open end of the vessel, Hanks’ solution containing 2 mmol/L Ca\(^{2+}\) flowed over the preparation for 15 min while sealing of the pipette to the vessel wall took place. The vessel was then inflated to 70 mmHg which was maintained throughout the experiment. The following criteria were used to show that the vessel lumen was sealed: (i) the vessel distal to the tungsten wire slip showed no indication of inflation, (ii) the pressurized vessel was evenly distended along its length after switching to a Ca\(^{2+}\)-free medium (iii) at the end of
the experiment, the vessel could be inflated to higher pressures (up to 400 mmHg) without the occluded end leaking and without the cannula becoming detached.

A section of vessel at least 150 µm away from the cannula was viewed under a x40 NA 0.6 objective focused midway through its depth. Experiments were started after a stable level of arteriole tone had been obtained for a period ≥10 min. Vessels were then treated for a further 10-min with Hanks solution containing 100 nmol/L penitrem A. The internal diameter was measured manually from saved video images.

**Chemicals and Data Analysis**

All chemicals used in this study were purchased from Sigma-Aldrich (Poole, UK). Values are expressed as means ± SEM. The following labeling convention has been used to indicate the statistical significance of differences between non-diabetic and diabetic data in all figures: no asterisk, NS; *, P<0.05; **, P<0.01; ***, P<0.001. Curve fitting was performed with Graphpad Prism V4 (Graphpad Software, Inc).

Ca\(^{2+}\) dose-response curves were fitted with the Hill equation:

\[
P_o = P_{o,max}/1+(K_d/[Ca^{2+}])^n
\]  

(1)

The K_d (dissociation constant) and n (Hill slope) were determined from this fit.

Po-Voltage relations were fitted with a Boltzmann function:

\[
P_o = P_{o,max}/1+e^{-zF(V-V_{1/2})/RT}
\]  

(2)

The V_{1/2} (half-activation voltage) was determined from this fit.
Results

Online Table I. Spatiotemporal features of Ca$^{2+}$ sparks and global Ca$^{2+}$ oscillations in non-diabetic and diabetic retinal VSMCs. Numbers of Ca$^{2+}$ events (amplitudes and duration) and cells (frequency) analyzed from 8 non-diabetic and 9 diabetic vessels are given in parentheses.

<table>
<thead>
<tr>
<th>Measurement Event</th>
<th>Non-diabetics Mean ± SEM (n)</th>
<th>Diabetics Mean ± SEM (n)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude (F/F0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Spark</td>
<td>0.42 ± 0.03 (282)</td>
<td>0.92 ± 0.06 (427)</td>
<td>***</td>
</tr>
<tr>
<td>Spark on Oscillation</td>
<td>0.36 ± 0.04 (133)</td>
<td>1.56 ± 0.21 (190)</td>
<td>***</td>
</tr>
<tr>
<td>Oscillation</td>
<td>1.24 ± 0.12 (65)</td>
<td>1.37 ± 0.10 (94)</td>
<td>NS</td>
</tr>
<tr>
<td>Duration (ms)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Spark</td>
<td>18.55 ± 1.28 (282)</td>
<td>18.38 ± 0.80 (427)</td>
<td>NS</td>
</tr>
<tr>
<td>Spark on Oscillation</td>
<td>22.00 ± 1.69 (132)</td>
<td>23.49 ± 2.23 (190)</td>
<td>NS</td>
</tr>
<tr>
<td>Oscillation</td>
<td>5386.37 ± 684.95 (65)</td>
<td>5968.06 ± 990.01 (94)</td>
<td>NS</td>
</tr>
<tr>
<td>Frequency (events/s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Spark</td>
<td>0.17 ± 0.02 (14)</td>
<td>0.20 ± 0.06 (18)</td>
<td>NS</td>
</tr>
<tr>
<td>Spark on Oscillation</td>
<td>0.34 ± 0.09 (14)</td>
<td>0.25 ± 0.09 (18)</td>
<td>NS</td>
</tr>
<tr>
<td>Oscillation</td>
<td>0.03 ± 0.00 (14)</td>
<td>0.03 ± 0.01 (18)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Reference List


