Functional Impairment of Renal Afferent Arteriolar Voltage-gated Calcium Channels in Rats with Diabetes Mellitus

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Abstract

Experiments were performed to test the hypothesis that diabetes mellitus is associated with impaired afferent arteriolar responsiveness to opening of voltage-gated calcium channels. Diabetes was induced by injection of streptozocin (65 mg/kg, i.v.) and insulin was administered via an osmotic minipump to achieve moderate hyperglycemia. Sham rats received vehicle treatments. 2 wk later, the in vitro blood-perfused juxtamedullary nephron technique was used to allow videomicroscopic measurement of afferent arteriolar contractile responses to increasing bath concentrations of either Bay K 8644 or K^+. Baseline afferent arteriolar diameter in kidneys from diabetic rats (26.4±1.2 μm) exceeded that of Sham rats (19.7±1.0 μm). Bay K 8644 evoked concentration-dependent reductions in afferent diameter in both groups of kidneys; however, arterioles from Sham rats responded to 1 nM Bay K 8644 while 100 nM Bay K 8644 was required to contract arterioles from diabetic rats. The EC_{50} for K^+-induced reductions in afferent arteriolar diameter was greater in diabetic kidneys (40±4 mM) than in kidneys from Sham rats (28±4 mM; $P < 0.05$). In afferent arterioles isolated by microdissection from Sham rats and loaded with fura 2, increasing bath [K^+] from 5 to 40 mM evoked a 98±12 nM increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ responses to 40 mM K^+ were suppressed in afferent arterioles from diabetic rats ($\Delta = 63 \pm 5$ nM), but were normalized by decreasing bath glucose concentration from 20 to 5 mM. These observations indicate that the early stage of insulin-dependent diabetes mellitus is associated with a functional defect in afferent arteriolar L-type calcium channels, an effect which may contribute to suppressed afferent arteriolar vasoconstrictor responsiveness and promote glomerular hyperfiltration. (*J. Clin. Invest.* 1996. 98:2564–2571.) Key words: Bay K 8644 • diltiazem • KCl • renal vasoconstriction • streptozocin

Introduction

The contractile status of vascular smooth muscle (VSM)[1] is critically dependent upon intracellular calcium concentration ($[Ca^{2+}]_i$). Contraction requires elevations in $[Ca^{2+}]_i$ achieved through Ca^{2+} release from intracellular stores and/or Ca^{2+} influx. Within the renal microvasculature, Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) is critically involved in the regulation of afferent arteriolar resistance, while efferent arteriolar contractile events are relatively independent of this process (1). Accordingly, organic calcium channel antagonists, which block passage of the ion through VGCCs, are potent preglomerular vasodilators (2) and inhibit a broad spectrum of afferent arteriolar vasoconstrictor events, without markedly altering efferent arteriolar resistance or contractile responsiveness (1).

Because of the segmentally specific functional distribution of VGCCs within the renal microvasculature, a defect in expression or regulation of these channels should selectively influence preglomerular function. Indeed, the renal microvascular effects of pharmacological VGCC blockade are reminiscent of the functional changes which engender hyperfiltration during the early stages of insulin-dependent diabetes mellitus (IDDM). Data from micropuncture studies indicate that afferent arteriolar resistance is reduced in IDDM, with efferent arteriolar resistance either normal or decreased to a lesser extent than afferent resistance (3–5). Videometric data have confirmed that juxtamedullary afferent arterioles are dilated and exhibit reduced vasoconstrictor responsiveness to norepinephrine during diabetic hyperfiltration, while these parameters of efferent arteriolar function remain unaltered (6). Afferent arteriolar myogenic and tubuloglomerular feedback responses (7, 8), as well as autoregulation of renal blood flow (9, 10), are also abated in IDDM. These processes are all normally subject to inhibition by VGCC blockers (11–13).

Based on these considerations, it is intriguing to speculate that a defect in VGCC activity might contribute to the altered renal hemodynamic function evident during the early stages of IDDM. Bank et al. (14) first suggested that insulinopenia in IDDM might impair Ca^{2+} movement through VGCCs in the renal vasculature. More recently, cultured rat aortic VSM cells exposed to high extracellular glucose concentrations for periods exceeding 12 h have been shown to exhibit suppressed voltage-sensitive and agonist-induced $^{45}Ca^{2+}$ uptake responses (15), an observation which links hyperglycemia with depressed VGCC activity. The present study was designed to evaluate the hypothesis that diabetic hyperglycemia is associated with impaired functional expression of VGCCs in the rat renal af-

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; fura 2-AM, fura 2-acetoxymethyl ester; IDDM, insulin-dependent diabetes mellitus; ST2, streptozocin; VGCC, voltage-gated Ca^{2+} channel; VSM, vascular smooth muscle.

ferent arteriole. Attention was focused on discerning the impact of streptozocin-induced IDDM on afferent arteriolar responses to stimuli which normally evoke contraction through receptor-independent opening of VGCCs. Initial studies determined the impact of IDDM on afferent arteriolar contractile responses to pharmacological enhancement of the open probability of L-type VGCCs using the dihydropyridine agonist Bay K 8644 (16, 17). As membrane depolarization is the primary stimulus for opening of VGCCs (18), further experiments probed the influence of IDDM on afferent arteriolar contractile and $[Ca^{2+}]_i$ responses to K^+-induced membrane depolarization.

Methods

Induction of diabetes mellitus

The procedures used in this study were approved by the Tulane University School of Medicine Advisory Committee on Animal Resources, and the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Male Sprague-Dawley rats were anesthetized (either 80 mg/kg ketamine plus 12 mg/kg xylazine or 50 mg/kg methohexital sodium, i.p.) to facilitate intravenous injection of streptozocin (STZ; Upjohn Co., Kalamazoo, MI, 65 mg/kg). Sham rats received vehicle treatment. The rats were allowed to recover from anesthesia and housed overnight with ad libitum access to food and water. The following morning, blood glucose levels were measured (Accu-Check III model 766; Boehringer Mannheim Co., Indianapolis, IN) and the rats were anesthetized to allow intraperitoneal implantation of an osmotic minipump (2002; Alzet, Palo Alto, CA). STZ rats received insulin (3.0–3.5 $U \cdot kg^{-1}$ per day, Iletin II; Eli Lilly and Co., Indianapolis, IN) via the minipump, while Sham rats received diluent. Each rat received penicillin G procaine (60,000 U, i.m.) after surgery. Thereafter, blood glucose concentration and body weight were measured twice weekly.

In vitro blood-perfused juxtamedullary nephron studies

Basic techniques. 2 wk after induction of diabetes, acute experiments were performed using the in vitro blood-perfused juxtamedullary nephron technique (6, 19, 20). Most experiments used two rats (blood donor and kidney donor), although a single rat provided both tissues in a few experiments. The kidney donor was anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and the right renal artery was cannulated via the superior mesenteric artery. The kidney was perfused in situ with Tyrode's solution containing 52 grams/liter dialyzed BSA (Sigma Chemical Co., St. Louis, MO), a mixture of L-amino acids (20), and D-glucose at a concentration of either 5 mM (Sham rats) or 20 mM (STZ rats). The rat was exsanguinated into a heparinized syringe and the kidney was harvested for in vitro study. Renal perfusion was maintained throughout the dissection procedure needed to reveal the tubules, glomeruli, and related vasculature of juxtamedullary nephrons. Tight ligatures were placed around the most distal accessible segments of the large arterial branches that supply the exposed microvasculature.

Blood donor rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), bilaterally nephrectomized, and exsanguinated via a carotid arterial cannula into a heparinized syringe. Blood collected in this manner was pooled with blood from the kidney donor and processed to remove leukocytes and platelets (6, 20). The resulting blood perfusate was stirred continuously in a closed reservoir that was pressurized by a 95% O_2–5% CO_2 tank, an arrangement that provided both oxygenation and the driving force for perfusion of the dissected kidney at a constant renal arterial pressure of 110 mmHg. Kidneys from STZ rats were perfused with blood from STZ rats and kidneys from Sham rats were perfused with blood from Sham rats.

The perfusion chamber was warmed and the tissue surface was superfused continuously with Tyrode's solution containing 10 grams/liter BSA at 37°C. A 15-min equilibration period ensued before initiating one of the protocols described below. The renal microvasculature was visualized by videomicroscopy and an afferent arteriole was selected for study based on visibility and acceptable blood flow. All protocols were designed to assess afferent arteriolar diameter at a single measurement site (> 100 μm upstream from the glomerulus) under several experimental conditions. Each protocol consisted of consecutive 5-min treatment periods, during which images of the selected arteriole were recorded on videotape. In rare instances when two vessels could be visualized within the same field of view, responses of both vessels were recorded simultaneously and analyzed separately during videotape playback.

Effect of pharmacologic activation of L-type calcium channels. Bay K 8644 (Research Biochemicals Inc., Natick, MA) was prepared as a 1-mM stock solution in ethanol and stored at −80°C. On the day of the experiment, the stock solution was diluted with Tyrode's solution to the desired final concentration and was protected from light until reaching the kidney surface. Cumulative concentration-response profiles were generated by measuring arteriolar lumen diameter during sequential exposure to 1, 10, 100, and 1,000 nM Bay K 8644. Previous studies in our laboratory found no effect of the ethanol vehicle on afferent arteriolar diameter (12).

Afferent arteriolar contractile responses to K^+-induced depolarization. The effects of membrane depolarization on renal afferent arterioles were assessed through isosmotic substitution of KCl for NaCl in the Tyrode's bathing solution. Cumulative concentration–response profiles were produced by documenting the afferent arteriolar lumen diameter response to bathing solutions containing increasing concentrations of K^+ (2.7, 10, 20, 40, 55, and 80 mM). During continued exposure to 80 mM K^+, most vessels were exposed to 10 μM diltiazem HCl (Marion Merrell Dow, Inc., Kansas City, MO), a VGCC antagonist (18).

Data analysis. Arteriolar lumen diameters were determined from videotaped images using a calibrated digital image-shearing monitor (Instrumentation for Physiology & Medicine, San Diego, CA). Diameter measurements were reproducible to within < 1 μm and were obtained at 12-s intervals from a single site along the vessel length. The average diameter during the final 2 min of each 5-min treatment period was used for statistical analysis.

Isolated arteriole studies

Basic techniques. 2 wk after induction of diabetes, acute experiments were performed on afferent arterioles isolated from Sham or STZ rats. After anesthetization with methohexital sodium (50 mg/kg, i.p.), the kidneys were removed, immersed in cold Ringer's solution, and cut into 1–2 mm thick longitudinal slices. A medial slice was selected and placed in Ringer's solution containing 7 μM fura 2-acetoxymethyl ester (fura 2-AM), 0.09 grams/dl DMSO, and 0.018 grams/dl Pluronic F-127 (Molecular Probes Inc., Eugene, OR). Segments of interlobular artery with single attached afferent arterioles (but devoid of glomeruli or tubular segments) were isolated by microdissection, taking care not to touch or stretch the afferent arterioles. These arterioles were of mixed cortical origin, as no specific emphasis was placed on restricting these studies to juxtamedullary arterioles. After 1-h incubation at room temperature in the fura 2-AM solution, the specimen was transferred to a temperature-controlled chamber mounted on the stage of an inverted microscope (Diaphot 300; Nikon Inc., Melville, NY). The interlobular artery and the cut end of the afferent arteriole were each held in position at the bottom of the chamber by gentle suction from holding pipettes. The chamber was warmed to 35°C and Ringer's solution (without fura 2-AM) was continuously exchanged at a rate of 4 ml/min, with the chamber volume maintained at ~ 300 μl.

$[Ca^{2+}]_i$ measurements. Dual excitation wavelength fluorescence microscopy was used to monitor afferent arteriolar $[Ca^{2+}]_i$ in fura 2–loaded arterioles, using methods similar to those described previously (21). Briefly, the tissue was illuminated alternately with light at 340 and 380 nm wavelengths. An adjustable optical sampling window was positioned over the afferent arteriole and emission fluorescence (510

nm) was collected using a photometer assembly. After background subtraction, arterioles which displayed emitted fluorescence intensities < 500,000 cps at either excitation wavelength were excluded from the study. Data were collected at a rate of 5 points/s, and were stored and processed using the OSCAR software package (Photon Technologies International Inc., Monmouth Jct., NJ). Calibration of the fura 2 signal was performed according to the method of Grynkiewicz et al. (22), as described in detail previously (21).

In addition to the ultraviolet illumination needed for determining $[Ca^{2+}]_i$ using fura 2, the tissue was transilluminated continuously with red light (610 nm). A dichroic cube positioned in the side port of the microscope allowed the red light image (> 600 nm) to be diverted to a monochrome CCD camera (Javelin Electronics, Torrance, CA) and displayed on a video monitor, while transmitting the lower wavelength signals to the photometer assembly. Thus, we were able to monitor simultaneously both the 510-nm fura 2 emissions and the 610-nm video image of the arteriole, the latter allowing continuous verification of its stable position within the optical sampling window. Preliminary experiments verified that the red light illumination did not interfere with detection of fura 2 emissions from afferent arterioles.

Solutions and drugs. A bicarbonate-free Ringer's solution provided the basis for all bathing solutions used in the isolated arteriole experiments (21). Except where noted, tissue from Sham rats was bathed in Ringer's solution containing 5 mM D-glucose, while arterioles from STZ rats were dissected and studied in Ringer's solution containing 20 mM glucose. As a control for the osmotic impact 20 mM glucose in Ringer's solution, some experiments used a Ringer's solution containing 5 mM glucose and 15 mM mannitol. High-K^+ (40 mM) Ringer's solution was prepared by equimolar replacement of NaCl with KCl. Diltiazem HCl (10 μM) was added to some solutions to block VGCCs (18). All solutions were adjusted to pH 7.40 and bubbled continuously with 100% O_2.

Data analysis. Fura 2 calibrations were performed daily, yielding R_{min} and R_{max} values averaging 0.53±0.03 and 44.01±4.06, respectively (n = 16). $[Ca^{2+}]_i$ responses to K^+-induced depolarization are reported as the peak value obtained. All other data are reported as the average $[Ca^{2+}]_i$ observed during the treatment period.

Statistics

Data were analyzed by ANOVA for repeated measures and Newman-Keuls tests, nonlinear regression analysis, or unpaired t tests, as appropriate. P values < 0.05 were considered significant. All data are reported as the mean±SEM.

Results

Animal characteristics. At the time of injection of either STZ or vehicle, the 104 rats used in this study weighed 324±5 grams and exhibited blood glucose concentrations averaging 4.7±0.1 mM. During the 2 wk after injection of STZ or vehicle, blood glucose concentration averaged 20.0±0.4 mM in STZ rats (n = 57) and 4.8±0.1 mM in Sham rats (n = 47; $P < 0.001$ vs STZ). Thus, rats receiving STZ were moderately hyperglycemic relative to Sham (vehicle-treated) rats. Rats treated with STZ gained weight at a slower rate than nondiabetic (Sham) rats. Accordingly, STZ rats weighed significantly less (333±6 grams) than Sham rats (384±8 grams; $P < 0.001$) at the time of the microvascular function studies. Despite the lower body weight of STZ rats, left kidney weight was greater in STZ rats (1.56±0.06 grams; n = 12) than in Sham rats (1.26±0.06 grams; n = 14; $P < 0.005$).

Diameter responses to pharmacologic activation of L-type calcium channels. Fig. 1 illustrates afferent arteriolar diameter responses to s(−)-Bay K 8644. In kidneys from Sham rats, baseline afferent arteriolar lumen diameter averaged 20.0±1.4 μm (n = 13 arterioles) and was decreased by Bay K 8644 in a concentration-dependent fashion. The lowest Bay K 8644 concentration used in this study (1 nM) significantly reduced afferent diameter by 9±2%, and the highest concentration (1,000 nM) decreased afferent diameter by 52±4%. In six efferent arterioles from Sham rats, lumen diameter averaged 21.7±1.7 μm under baseline conditions and was not influenced by Bay K 8644 at any concentration used in the present study (1 nM, 21.8±1.7 μm; 10 nM, 22.0±1.7 μm; 100 nM, 22.2±1.6 μm; 1000 nM, 21.4±1.6 μm).

In kidneys from STZ rats, baseline afferent diameter averaged 27.4±1.4 μm (n = 13 arterioles; P = 0.001 vs Sham) and was unaltered by either 1 or 10 nM Bay K 8644. Accordingly, afferent arteriolar responses to 1 and 10 nM Bay K 8644 in STZ kidneys were significantly less than those observed in Sham kidneys. Both 100 and 1,000 nM Bay K 8644 evoked concentration-dependent reductions in afferent arteriolar diameter in STZ kidneys, and these responses did not differ significantly from those observed in Sham kidneys.

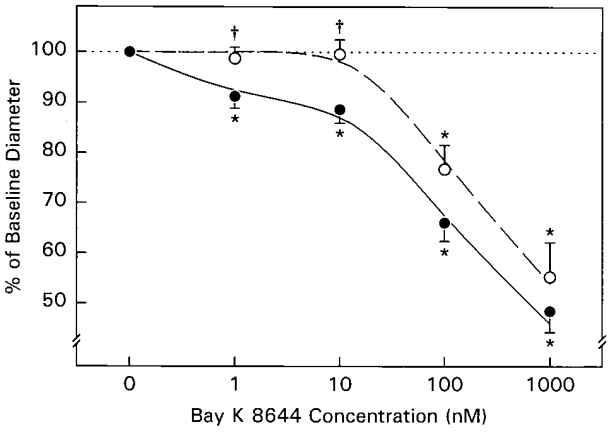

Figure 1. Effect of diabetes mellitus on renal arteriolar contractile responses to Bay K 8644. Data are shown for afferent arterioles from Sham rats (●) and STZ rats (○). See text for baseline values. Data are mean±SEM. *$P < 0.05$ vs baseline (0 nM Bay K 8644); †$P < 0.05$ vs Sham.

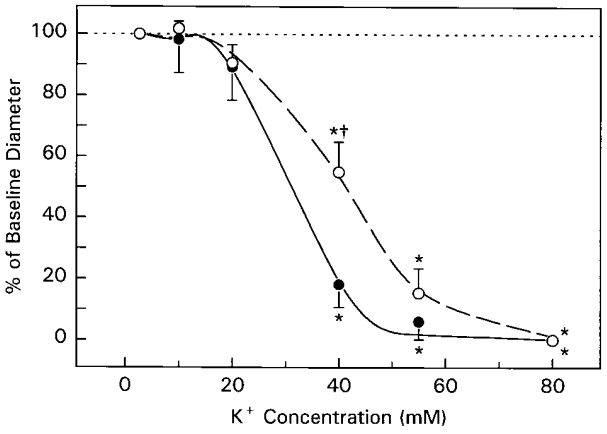

Figure 2. Effect of diabetes mellitus on renal arteriolar contractile responses to increases in bath $[K^+]$. Responses are shown for afferent arterioles from Sham rats (●) and STZ rats (○). See text for baseline values. Data are mean±SEM. *$P < 0.05$ vs baseline (2.7 mM K^+); †$P < 0.05$ vs Sham.

Diameter responses to K^+-induced depolarization. Fig. 2 illustrates the impact of increases in bath $[K^+]$ on juxtamedullary afferent arterioles from Sham and STZ rats. In kidneys from Sham rats, afferent arteriolar lumen diameter averaged 19.1±1.4 μm under baseline conditions (bath $[K^+]$ = 2.7 mM; n = 7 arterioles) and increases in bath $[K^+]$ evoked concentration-dependent diameter reductions. Significant reductions in afferent arteriolar lumen diameter were observed in response to 40 mM K^+ (82±7% decrease in diameter), five of the seven Sham afferent arterioles were maximally vasoconstricted (lumen diameter = 0) by 55 mM K^+, and 80 mM K^+ closed all vessels. Addition of 10 μM diltiazem to the bathing solution reversed the afferent diameter response to 80 mM K^+ (diameter restored to 110±5% of baseline; n = 4 arterioles).

In kidneys from STZ rats, baseline afferent diameter averaged 25.2±2.0 μm (n = 11 arterioles; $P < 0.05$ vs Sham). Afferent arterioles from STZ rats also exhibited concentration-dependent diameter reductions in response to increases in bath $[K^+]$. However, although 40 mM K^+ reduced afferent diameter in STZ kidneys (45±10% decrease), this response was significantly less than that observed in Sham kidneys ($P <$ 0.05). Nevertheless, 55 mM K^+ closed 8 of the 11 vessels in this group, and 80 mM K^+ closed all vessels. Diltiazem (10 μM) reversed the response to 80 mM K^+, restoring lumen diameter to 120±5% of baseline (n = 7 arterioles). Nonlinear regression analysis, using a three parameter logistic function, revealed that the EC_{50} for the diameter response to increases in bath $[K^+]$ was greater in kidneys from STZ rats (40±4 mM) than in kidneys from Sham rats (28±4 mM, $P < 0.05$).

Effect of K^+-induced depolarization on arteriolar $[Ca^{2+}]_i$. Fig. 3 shows representative afferent arteriolar $[Ca^{2+}]_i$ responses to K^+-induced membrane depolarization. Baseline $[Ca^{2+}]_i$ did not differ significantly between afferent arterioles from Sham rats (5 mM glucose) and STZ rats (20 mM glucose), averaging 38±4 (n = 14) and 36±4 nM (n = 24), respectively. Exposure to 40 mM K^+ evoked rapid increases in afferent arteriolar $[Ca^{2+}]_i$; however, the average response in arterioles from STZ rats (Δ = 63±5 nM) was smaller than that observed in arterioles from Sham rats (Δ = 98±12 nM; $P <$ 0.005). In both groups of vessels, return to the normal Ringer's bathing solution restored $[Ca^{2+}]_i$ to values not significantly different from baseline. Fig. 3 also illustrates the reproducibility of $[Ca^{2+}]_i$ responses to K^+-induced depolarization within individual arterioles, which was verified in four Sham and five STZ arterioles. In other experiments, addition of 10 μM diltiazem to the high-K^+ bathing solution restored $[Ca^{2+}]_i$ to values averaging 90±10 and 92±9% of baseline in Sham (n = 3) and STZ (n = 4) arterioles, respectively.

Effect of glucose concentration on $[Ca^{2+}]_i$ responses to depolarization. Fig. 4 illustrates the impact of acute changes in bath glucose concentration on afferent arteriolar $[Ca^{2+}]_i$ responses to K^+-induced depolarization. In arterioles from STZ rats (Fig. 4 *A*), 40 mM K^+ increased $[Ca^{2+}]_i$ by 54±8 nM (n = 8) in the presence of 20 mM glucose. Reducing bath glucose concentration from 20 to 5 mM did not alter baseline $[Ca^{2+}]_i$. However, within ~ 10 min after reducing bath glucose concentration, these same vessels responded to the depolarizing stimulus with a 111±9 nM increase in $[Ca^{2+}]_i$ ($P < 0.001$ vs 20 mM glucose). Thus, in afferent arterioles from STZ rats, acute restoration of normal bath glucose concentration enhanced $[Ca^{2+}]_i$ responsiveness to membrane depolarization, achieving values which did not differ significantly from those observed in arterioles from Sham rats. Moreover, the $[Ca^{2+}]_i$ response to depolarization was also enhanced when six arterioles from STZ rats were exposed to Ringer's solution containing both 5 mM glucose and 15 mM mannitol (Fig. 4 *B*). In arterioles from Sham rats (Fig. 4 *C*), 40 mM K^+ evoked $[Ca^{2+}]_i$ transients averaging 86±11 nM in the presence of 5 mM glucose and 107±18 nM after ~ 10-min exposure to 20 mM glucose (n = 9; NS vs 5 mM). Moreover, afferent arterioles from Sham rats displayed normal $[Ca^{2+}]_i$ responses to depolarization after 2–3 h exposure to 20 mM glucose (Δ = 101±17 nM, n = 5). Thus, afferent arterioles from Sham rats did not exhibit sensitivity to bath glucose concentration within the time frame of these experiments.

Discussion

Glomerular hyperfiltration is typically evident during the early phase of IDDM and is thought to engender eventual development of diabetic nephropathy. Although preglomerular vasodilation and reduced vasoconstrictor responsiveness appear to elicit the hyperfiltration, the underlying mechanism remains speculative. The present studies were performed to evaluate the hypothesis that the afferent arteriole exhibits impaired functional expression of VGCCs in a moderately hyperglycemic rat model of STZ-induced IDDM. These rats display elevated renal plasma flow and GFR (6). Moreover, in vitro blood perfused juxtamedullary nephrons from these rats exhibit increased single nephron GFR, afferent arteriolar vasodilation, and decreased afferent vasoconstrictor responses to ex-

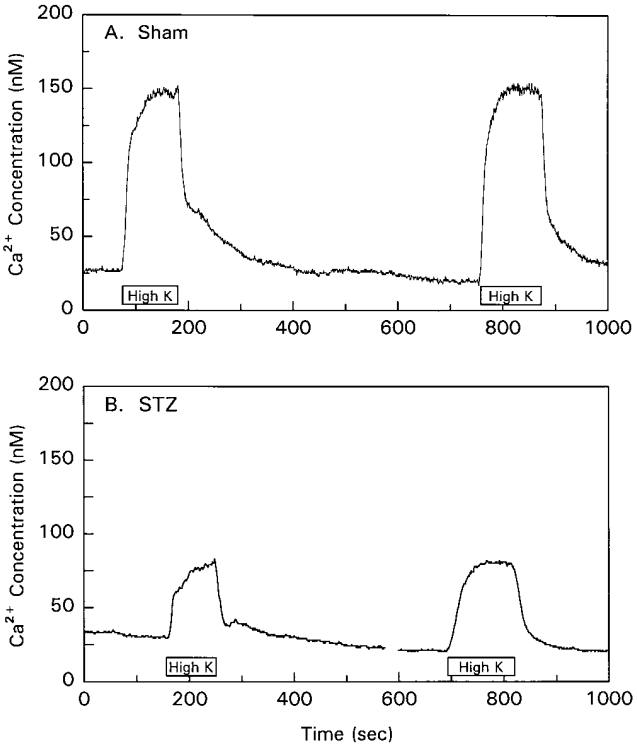

Figure 3. Afferent arteriolar $[Ca^{2+}]_i$ responses to increases in extracellular $[K^+]$. Vessels were exposed to Ringer's solution containing either normal (5 mM) or high (40 mM) concentrations of K^+. (*A*) Responses of an afferent arteriole isolated from a Sham rat, during continuous exposure to 5 mM glucose. (*B*) Responses of an afferent arteriole isolated from an STZ rat, during continuous exposure to 20 mM glucose. See text for mean data.

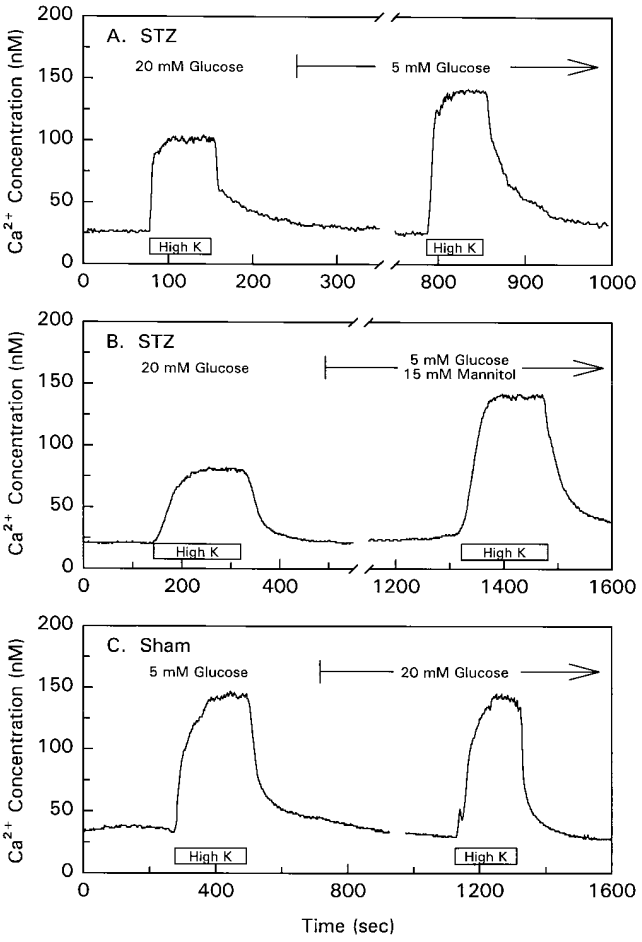

Figure 4. Effect of bath glucose concentration on afferent arteriolar $[Ca^{2+}]_i$ responses to elevated extracellular $[K^+]$. (*A*) $[Ca^{2+}]_i$ response to High K (40 mM) in an STZ arteriole before and after decreasing bath glucose concentration from 20 to 5 mM. (*B*) $[Ca^{2+}]_i$ response to 40 mM K^+ in an STZ arteriole before (*20 mM glucose*) and after isosmotic restoration of normal bath glucose concentration (*5 mM glucose plus 15 mM mannitol*). (*C*) $[Ca^{2+}]_i$ response to 40 mM K^+ in a Sham arteriole before and after increasing bath glucose concentration from 5 to 20 mM. See text for mean data.

ogenous norepinephrine (6). Results of the present study extend these observations by revealing that afferent arteriolar contractile and $[Ca^{2+}]_i$ responses to opening of VGCCs are diminished in this model of IDDM. These results are consistent with the postulate that a functional defect in afferent arteriolar VGCCs contributes to the renal microvascular dysfunction evident during diabetic hyperfiltration.

Two mechanistically different approaches were used to evoke opening of VGCCs: exposure to Bay K 8644 and K^+-induced membrane depolarization. Bay K 8644 acts directly and specifically on L-type VGCCs to prolong their open state (16–18). Studies using the in vivo hydronephrotic kidney first unveiled the ability of this compound to evoke preglomerular vasoconstriction without significantly altering efferent arteriolar diameter (23). The preferential impact of Bay K 8644 on the preglomerular microvasculature of the normal rat kidney is confirmed by our observations. Moreover, the concentration of Bay K 8644 required to constrict afferent arterioles was $\sim$ 100-fold greater in kidneys from STZ rats than in Sham kidneys. While this observation is consistent with the postulate that functional expression of afferent arteriolar VGCCs is suppressed in IDDM, other explanations may underlie our results. For example, there may be a defect in dihydropyridine binding to functional VGCCs in IDDM. A second possibility arises from that fact that Bay K 8644 prolongs the open state of VGCCs after physiological activation, rather than opening the channels directly (18). Since basal tone of the afferent arteriole is highly dependent upon the status of VGCCs (2), one would predict that the open probability of VGCCs in a dilated afferent arteriole would be lower than in an afferent arteriole with normal tone. This phenomenon might underlie a reduced contractile sensitivity to Bay K 8644 in the typically vasodilated afferent arterioles from STZ rats. Thus, the results of the Bay K 8644 experiments alone are not sufficient to conclude that a defect in afferent arteriolar VGCCs accompanies IDDM, although the data are consistent with this possibility.

As membrane depolarization is the primary physiological stimulus for opening VGCCs, we compared depolarization-induced afferent arteriolar vasoconstrictor responses in kidneys from Sham and STZ-treated rats. In VSM, the membrane depolarization evoked by elevated extracellular $[K^+]$ increases the open-state probability of VGCCs, allowing Ca^{2+} influx down its concentration gradient, a rise in $[Ca^{2+}]_i$, and vasoconstriction. We have shown previously that afferent arteriolar $[Ca^{2+}]_i$ responses to K^+-induced membrane depolarization require extracellular Ca^{2+} and are blocked by nifedipine (21). Accordingly, increases in extracellular $[K^+]$ evoke dihydropyridine-sensitive afferent arteriolar vasoconstriction (24), evidently via Ca^{2+} influx through L-type VGCCs. It is possible that some component of the response to K^+-induced depolarization encompasses neurotransmitter release from residual sympathetic nerve terminals in our preparations. However, both afferent and efferent arterioles of juxtamedullary nephrons constrict in response to renal nerve stimulation in the rat hydronephrotic kidney (25), while only afferent arterioles constrict in response to K^+-induced membrane depolarization. Moreover, phentolamine does not alter the afferent vasoconstrictor response to 30 mM K^+ in isolated perfused hydronephrotic kidneys (24). Thus, it is likely that Ca^{2+} influx through L-type VGCCs in VSM cells is primarily responsible for the afferent arteriolar vasoconstrictor response to K^+-induced membrane depolarization.

Previous studies of VSM function in rat models of IDDM have revealed suppressed (26–28), unaltered (29) or accentuated (30–32) maximal contractile responses to increases in bath $[K^+]$, together with no change (26–28, 30, 32) or an increase in the EC_{50} (33). These discordant observations are generally attributed to variations in the duration and/or severity of IDDM, as well as tissue-specific heterogeneities in the vascular alterations accompanying IDDM. The results of the present study reveal that, during the hyperfiltration stage of IDDM in the rat, renal afferent arterioles display altered vasoconstrictor responses to K^+-induced depolarization which include reduced responsiveness to 40 mM K^+ and an increase in the EC_{50}. The ability of diltiazem to reverse the afferent arteriolar vasoconstrictor response to 80 mM K^+ in kidneys from both Sham and STZ rats supports the contention that these events involve gating of VGCCs. While these observations indicate that electromechanical coupling is disrupted in afferent arteriolar VSM during IDDM, some potential caveats deserve consideration. First, an unavoidable complication of studies of afferent arteri-

olar function during diabetic hyperfiltration involves the substantial difference in baseline lumen diameter typically observed between STZ and Sham rats. To normalize for this phenomenon, contractile responses are reported here as percentage of baseline diameter. It is possible that the apparent suppression of contractile responsiveness to opening of VGCCs in STZ rats is a mathematical consequence of this normalization procedure. In addition, reduced baseline tone in arterioles from STZ rats may underlie the suppressed responsiveness as a result of an altered activation status of the intracellular contractile machinery. To address these issues, the STZ vessels used in evaluating vasoconstrictor responses to K^+-induced depolarization were arbitrarily divided into two groups according to baseline diameter. When grouped in this manner, the largest STZ arterioles exhibited baseline diameters averaging 31.4±1.6 μm (n = 5) and the smallest STZ arterioles had diameters averaging 20.0±1.0 μm (n = 6; NS vs Sham). There was no significant difference between the contractile responses evoked by 40 mM K^+ in these subsets of STZ arterioles. Moreover, responses to 40 mM K^+ in the smallest STZ arterioles were significantly less than those observed in Sham arterioles, despite similar baseline diameters. Thus, the defect in contractile responsiveness to increases in extracellular [K^+] in afferent arterioles from STZ rats cannot be attributed to increased baseline diameter per se or reduced tonic activation of the contractile machinery.

Based on the contractile studies alone, it remained possible that VGCC gating was unaltered in IDDM (evoking normal $[Ca^{2+}]_i$ responses to Bay K 8644 or K^+-induced membrane depolarization), while the contractile apparatus was less sensitive to the resulting change in $[Ca^{2+}]_i$. This type of phenomenon has been reported to underlie reduced contractility of mesangial cells from STZ rats (34). To address this possibility, fura 2 was used to monitor $[Ca^{2+}]_i$ responses to K^+-induced membrane depolarization in afferent arterioles isolated from Sham and STZ rats. This technique has been used in a number of laboratories to study arteriolar $[Ca^{2+}]_i$ regulation (35–37), and its utility relies on the validity of several assumptions that we have considered in detail previously (21). Of principal importance is the assumption that VSM cells provide the major source of fura 2 fluorescence emissions in this experimental setting. In support of this contention are reports that introduction of fura 2-AM from the abluminal aspect of isolated arterioles yields selective loading of VSM cells (37, 38). However, if endothelial loading of fura 2 does occur, emissions originating from these cells (which lack VGCCs [39]) would blunt the apparent VSM $[Ca^{2+}]_i$ response to membrane depolarization. This effect should be constant throughout our acute experimental manipulations and, thus, could not underlie the rapidly reversible, glucose-sensitive phenomenon unveiled by our experiments. Moreover, it seems likely that fluorescence emissions from endothelial cells would be eclipsed by those originating from the larger mass of VSM cells, and would thus represent a minor component of the overall signal. In light of these considerations, our observation of a diminished diltiazem-sensitive $[Ca^{2+}]_i$ response to 40 mM K^+ in afferent arterioles from STZ rats implicates reduced Ca^{2+} influx (through VGCCs) into VSM cells. This contention is in accord with previous reports that rat aortic VSM cells cultured in a high glucose environment display depressed $^{45}Ca^{2+}$ uptake responses to Bay K 8644 and 65 mM K^+ (15). However, we cannot rule out the lingering possibility that intracellular [K^+] or membrane K^+ conductance is altered in afferent arterioles from STZ rats, thus shifting the relationship between extracellular [K^+] and membrane potential and altering the $[Ca^{2+}]_i$ and contractile responses to increases in bath [K^+]. Direct electrophysiological studies will be necessary to confirm our assumption that afferent arteriolar VSM cells from Sham and STZ rats exhibit comparable membrane potential responses to alterations in bath [K^+]. Nevertheless, since the STZ arterioles exhibited quantitatively similar defects in the $[Ca^{2+}]_i$ and contractile responses to 40 mM K^+, the most likely explanation of our data is that a decreased Ca^{2+} influx through L-type VGCCs lessens the $[Ca^{2+}]_i$ response to membrane depolarization which, in turn, curtails the vasoconstrictor response to this stimulus.

Since baseline afferent arteriolar tone is normally dependent on Ca^{2+} influx through L-type VGCCs (1, 2, 20), it is easy to envision how a defect in these channels could contribute to the afferent vasodilation observed in moderately hyperglycemic states and associated with glomerular hyperfiltration. Ca^{2+} influx through VGCCs is also an important component of the afferent arteriolar response to numerous vasoconstrictor agonists (1, 20), such that impaired Ca^{2+} entry through this pathway could contribute to diminished agonist-induced vasoconstriction in IDDM (40–42). For example, the selective preglomerular defect in norepinephrine responsiveness in our model of IDDM (6) correlates well with the functional localization of VGCCs within the renal microvasculature (1, 20), and is consistent with the postulate that a defect in VGCC activity might underlie altered norepinephrine responsiveness in IDDM. Reduced myogenic and tubuloglomerular feedback responsiveness have also been reported in IDDM (7, 8). Since these afferent arteriolar responses are highly dependent upon Ca^{2+} influx through dihydropyridine-sensitive channels, a VGCC defect could underlie impaired renal autoregulation in IDDM (10) and thus contribute to the accelerated glomerular damage elicited by the development of systemic hypertension in diabetic patients.

The hallmark of diabetes is hyperglycemia, and control of blood glucose levels slows the development and progression of diabetic nephropathy. It has long been recognized that reduction of blood glucose levels to normal in newly diagnosed IDDM patients readily returns GFR to normal or near-normal values (43, 44). Therefore, we postulated that restoration of normal extracellular glucose concentration would restore responsiveness to membrane depolarization. Indeed, the $[Ca^{2+}]_i$ response to 40 mM K^+ reverted to typically normal behavior in arterioles isolated from STZ-treated rats within 10 min of exposure to normal extracellular glucose levels. In contrast, 2–3 h exposure to hyperglycemic media failed to attenuate the $[Ca^{2+}]_i$ response to depolarization in arterioles from Sham rats. The normalization of $[Ca^{2+}]_i$ responsiveness to depolarization in STZ arterioles cannot be attributed to an osmotic effect of reducing bath glucose concentration from 20 to 5 mM, since this phenomenon was also evident when osmolality was maintained by including 15 mM mannitol in the 5 mM glucose Ringer's solution. This observation suggests that the beneficial effect of decreasing bath glucose concentration is dependent on the metabolic impact of glucose on cellular function. It is intriguing to note that the suppressed VGCC-dependent response to a hyperglycemic environment is relatively slow in onset, but rapidly reversible.

Few studies have examined by direct methods the impact of IDDM or hyperglycemia on VGCC activity. Ventricular myocytes from diabetic rats have been reported to exhibit nor-

mal (45) or reduced (46) L-type VGCC current densities. A recent preliminary report indicates that coronary artery VSM cells from diabetic pigs display reduced L-type VGCC current density, which implies that fewer channels are available (47). Indeed, 48-h glucose infusion reduces L-type VGCC mRNA and blunts the response to Bay K 8644 in rat pancreatic β-cells, suggesting that hyperglycemia may directly reduce channel expression (48). However, the literature contains conflicting evidence regarding the number of dihydropyridine binding sites in diabetic rat heart and skeletal muscle (49–51). The results of the present study do not likely reflect a decreased expression of VGCCs (i.e., reduced number of channels), since the phenomenon is rapidly reversible. Moreover, glycosylation of the VGCC or one of its regulatory proteins does not likely underlie these changes, since protein glycosylation processes develop over a period of weeks and are irreversible (52). The ability to rapidly restore normal responsiveness through acute alterations in extracellular glucose concentration suggests that the defect reflects an altered regulation of channel activity. Changes in basal dephosphorylation represent a major determinant of L-type VGCC kinetics in VSM cells (53, 54) and likely underlie the reported effect of protein kinase C to reduce the ability of VGCCs to open upon depolarization (55). Hence, glucose-induced activation of protein kinase C in VSM (56) could suppress VGCC activity in IDDM through this type of phosphorylation-dependent process. Alternatively, the development of hyperglycemia-induced redox imbalance as a result of increased flux through the sorbitol pathway (57) may favor a reduction in voltage-dependent Ca^{2+} entry by altering the oxidative state of cysteine residues within the pore region of VGCCs (58). Further studies at the single cell and/or single channel level will be necessary to determine the validity of these postulates.

In summary, the results of the present study reveal that renal juxtamedullary afferent arteriolar contractile responses to Bay K 8644 and K^+-induced membrane depolarization are impaired during the hyperfiltration stage of IDDM. These changes are accompanied by suppressed afferent arteriolar $[Ca^{2+}]_i$ responses to membrane depolarization, a phenomenon which is readily reversible upon normalization of extracellular glucose concentration. These observations suggest that hyperglycemia in IDDM engenders a functional defect in the L-type VGCCs that normally play a prominent role in the regulation of afferent arteriolar baseline tone and vasoconstrictor responsiveness. Because of the unique functional distribution of VGCCs within the renal microvasculature, the selective preglomerular impact of this defect can be expected to promote glomerular hyperfiltration in the early stages of IDDM.

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