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Noradrenaline-induced changes in intracellular Ca^{2+} and tension in mesenteric arteries from diabetic rats

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1 The purpose of this investigation was to determine whether enhanced contractile responses to noradrenaline (NA) of mesenteric arteries from rats with chronic streptozotocin-induced diabetes are associated with increases in mean cytosolic $[Ca^{2+}]_i$.

2 $[Ca^{2+}]_i$ was measured with fura 2-AM, and was monitored simultaneously with tension in perfused endothelium-denuded mesenteric arterial rings from 12–14 week diabetic rats and age- and gender-matched control rats.

3 Basal $[Ca^{2+}]_i$ (expressed as R_n, the normalized fura 2 ratio) was not significantly different in arteries from control and diabetic rats. Similarly, no differences between control and diabetic arteries in the tension or $[Ca^{2+}]_i$ responses to 80 mM KCl in the presence of phentolamine were detected.

4 The rate of tension development, peak tension and integrated tension in response to 30 μM NA were all significantly greater in diabetic than control arteries. However, this was not associated with enhancement of the corresponding $[Ca^{2+}]_i$ responses in the diabetic arteries.

5 Peak contractile responses to perfusion with both 0.3 and 3 μM NA, but peak $[Ca^{2+}]_i$ only in response to 0.3 μM NA, were significantly greater in diabetic than control arteries.

6 NA (30 μM) produced a greater increase in both peak tension and $[Ca^{2+}]_i$ in diabetic than control arteries perfused with Ca^{2+}-free solution containing 1 mM EGTA. Neither the rate nor the magnitude of NA-induced Ca^{2+} influx appeared to be altered in the diabetic arteries.

7 The enhanced sustained contractile response of diabetic arteries to NA appears to be dissociated from increases in $[Ca^{2+}]_i$, and may be due to other factors, such as an increase in the Ca^{2+} sensitivity of the contractile proteins.

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Abbreviations: Ach, acetylcholine; ANOVA, analysis of variance; IP_3, inositol 1,4,5 trisphosphate; NA, noradrenaline; PE, phenylephrine; STZ, streptozotocin

Introduction

Efforts to investigate the effects of diabetes on the responsiveness of arterial blood vessels have been furthered by studies conducted in genetically and chemically induced diabetic animal models. Although the results of these studies have not all been consistent, in this laboratory we have found that maximal contractile responses of arteries from rats with streptozotocin (STZ)-induced diabetes of 12–14 weeks duration to noradrenaline (NA) acting at α_1-adrenoceptors are enhanced (MacLeod, 1985; Abebe *et al*., 1990; 1994; Weber *et al*., 1996). The increased reactivity to NA cannot be attributed to a generalized increase in the responsiveness of the arteries because vessels (aortas, mesenteric and tail arteries) from both diabetic and control rats exhibit responses of similar magnitude to depolarizing KCl concentrations, in the presence of phentolamine to block the effects of endogenously released noradrenaline (NA) (MacLeod, 1985; Abebe *et al*., 1994; Weber & MacLeod, 1994; Weber *et al*., 1996). However, since contractile responses of diabetic arteries to direct stimulation of G-proteins with NaF are also enhanced (Weber *et al*., 1996), the increased responsiveness to NA appears to result from a change in the signal transduction process downstream from the α_1-adrenoceptor.

The increased reactivity of diabetic arteries to α_1-adrenoceptor stimulation was found to be largely dependent on the presence of extracellular Ca^{2+} but there was also an apparent increased release of intracellular Ca^{2+} in response to high concentrations of agonist (Abebe *et al*., 1990; 1994). The initial rapid component of the contractile response to NA is partially dependent on Ca^{2+} released from inositol 1,4,5 trisphosphate (IP_3)-sensitive intracellular stores (Watras *et al*., 1989; Ferris & Snyder, 1992; Weber *et al*., 1995), and we have found that the breakdown of [^{32}P]-phosphatidylinositol 4,5-bisphosphate, and the production of [^{3}H]-inositol phosphates and IP_3 were enhanced in response to maximal stimulation by NA in diabetic arteries (Abebe & MacLeod, 1991; 1992). We therefore proposed that the increased levels of IP_3 promote greater release of Ca^{2+} from intracellular stores, thereby contributing to higher cytosolic Ca^{2+} levels and the enhanced contractile responses of diabetic arteries to NA.

The results of a number of studies have suggested, indirectly, that Ca^{2+} influx through dihydropyridine sensitive channels is also enhanced in arteries from diabetic rats

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(Scarborough & Carrier, 1984; Agrawal & McNeill, 1987; White & Carrier, 1988; Abebe *et al*., 1990; 1994). This is based mainly on the greater magnitude of inhibitory effect of dihydropyridine Ca^{2+} channel blockers or removal of extracellular Ca^{2+} on maximum contractile responses of diabetic arteries to vasoconstrictors. Nifedipine-sensitive $^{45}Ca^{2+}$ influx and contraction induced by 5-HT was reported to be greater in diabetic rat aortas than in controls (Hattori *et al*., 1995), also suggesting that the enhanced contractile responses of diabetic arteries to receptor stimulation is associated with increased Ca^{2+} influx.

These studies suggest that increased $[Ca^{2+}]_i$ contributes to the enhanced maximum contractile responsiveness of arteries from diabetic rats to NA, but to our knowledge this has not been directly demonstrated. Only a few studies have measured $[Ca^{2+}]_i$ in diabetic rat arteries (Ohara *et al*., 1991; Tam *et al*., 1997), and none have correlated agonist-induced changes in $[Ca^{2+}]_i$ with contraction in the same preparation. Therefore, in the present experiments, changes in $[Ca^{2+}]_i$ and contractile tension in response to stimulation with NA were measured simultaneously, and compared with changes in tension and $[Ca^{2+}]_i$ produced by depolarization with high K^+, in mesenteric arteries from diabetic rats and age- and gender-matched control rats. In addition, NA-induced release of intracellular Ca^{2+} and influx of extracellular Ca^{2+} and their associated changes in tension were compared in mesenteric arteries from control and diabetic rats.

Methods

Male Wistar rats weighing between 150–175 grams were obtained from the U.B.C. Animal Care Unit (Vancouver, B.C.) and were housed and treated in accordance with the guidelines of the Canadian Council on Animal Care. Diabetes was induced by injection of STZ (60 mg kg^{-1}) into the lateral tail vein of rats lightly anaesthetized with halothane. Control rats were treated in the same manner, but received the citrate buffer vehicle. STZ-treated rats with blood glucose levels of 13 mmol l^{-1} or greater (measured with an Ames glucometer) 1 week after the injection were considered diabetic and were kept for experiments. Control and diabetic rats were housed separately but maintained in similar environments and had food and water readily available. At the end of 12–14 weeks, animals were weighed and given a 65 mg kg^{-1} injection (or more as necessary) of sodium pentobarbital. When the animals were deeply anaesthetized, the chest was opened and blood collected by cardiac puncture for later assay of plasma insulin and glucose levels. The superior mesenteric artery was excised, placed in Krebs solution (composition in mM: NaCl 124, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2 and dextrose 11.5) at room temperature and carefully cleaned of fat and connective tissue. The endothelium was removed by rubbing a thin wire against the lumenal wall of the vessel. Arteries were then cut into 4 mm rings for loading with fura 2-AM.

The method used to load the mesenteric artery with fura 2-AM was a modification of that of Himpens & Somlyo (1988). Arteries were placed in a loading solution consisting of 5 ml of Krebs solution containing 10 μM fura 2-AM, 0.1% (v v^{-1}) cremophor El and 0.02% (wt v^{-1}) pluronic F-12 and left to incubate at 37°C for 2 h in the dark while under constant aeration with 95% O_2/ 5% CO_2. At the end of the loading period, each arterial ring was mounted between two triangular hooks in a 3 ml jacketed organ bath. One of the hooks was attached to a Grass FT.03 isometric force displacement transducer while the other was fastened to an immobile post. The arterial ring was then lowered over the top of a bifurcated randomized light guide that was attached to the bottom of the organ bath. The artery was illuminated alternately with light of 340 and 380 nm wavelength *via* a Nikon HMX2 100 watt mercury arc lamp equipped with a filter wheel containing 340 and 380 nm interference filters that rotated at 50 Hz. The emitted fluorescence was selected by a 500 nm filter, amplified by a photomultiplier and demodulated, using equipment obtained from the Biomedical Instrumentation Group at the University of Pennsylvania. The demodulated signals corresponding to the two excitation wavelengths (F_{340} and F_{380}), and a signal from the force transducer were sampled at a rate of 1000 s^{-1}, averaged, and recorded and displayed at a rate of 1 s^{-1}, using custom-written software.

Arteries were placed under 1 gram of tension, and perfused with 95% O_2/ 5% CO_2-aerated Krebs solution at a rate of 4–6 ml per min for 25–30 min to allow washout of excess fura 2-AM and tissue equilibration before experiments were begun. The artery rings were then perfused with Krebs solution containing 3 μM NA and allowed to contract maximally, following which Ach (3 μM) was added to the perfusate. The absence of a relaxant response to Ach confirmed successful removal of the endothelium. Arteries were then perfused with Krebs solution for 45–60 min, until both tension and fluorescence had returned to baseline values, before being perfused with 0.3, 3 or 30 μM NA. In experiments where responses to more than one concentration of NA were obtained, arteries were perfused with Krebs solution between each concentration of NA until tension and fluorescence values had returned to baseline levels. In a separate set of experiments, we used a Ca^{2+}-free- Ca^{2+} re-introduction protocol (Samain *et al*., 1999) to assess NA-induced intracellular Ca^{2+} release and extracellular Ca^{2+} influx. First, responses to 3 and 30 μM NA were obtained in normal Krebs solution, and arteries washed until tension and fluorescence had returned to baseline levels. Arteries were then perfused with Ca^{2+}-free Krebs solution containing 1 mM EGTA for 3 min before being perfused with 30 μM NA in the same solution. When the fluorescence had returned to baseline levels, tissues were then perfused with Krebs buffer containing 2.5 mM Ca^{2+} and 30 μM NA.

At the completion of each experiment, arteries were perfused with 0.5 μM phentolamine for 15 min, then with Krebs solution containing 80 mM KCl and 0.5 μM phentolamine. When the maximum response to KCl was attained the arteries were perfused with various buffers containing 10 μM ionomycin to determine the minimum and maximum fluorescence ratios (Grynkiewycz *et al*., 1985; Himpens & Somlyo, 1988). The minimum fluorescence ratio (R_{min}) was obtained by perfusing the tissues with a Ca^{2+}-free physiological solution containing 140 mM KCl and 2 mM EGTA. The maximum fluorescence ratio (R_{max}) was then obtained by perfusing the tissues with physiological solution containing 80 mM KCl and 2.5 mM $CaCl_2$. Lastly, the tissue autofluorescence (A_f) was determined by perfusing the tissues with physiological solution containing 20 mM $MnCl_2$. A_f values at

340 and 380 nm, which were not significantly different between control and diabetic arteries, were subtracted from all other F_{340} and F_{380} values prior to calculation of fluorescence ratios ($R_{340/380}$). No significant differences between control and diabetic arteries were detected in R_{min}, R_{max} or β, which is the ratio of F_{380} at R_{min} to F_{380} at R_{max} (Table 1). Increasing the ionomycin concentration to 50 μM did not alter R_{min}, R_{max} or β. Because of uncertainties in the determination of the K_d for fura 2 in intact arteries, absolute values of $[Ca^{2+}]_i$ were not calculated in the present investigation. To allow comparison between control and diabetic arteries, normalized fluorescence ratios (R_n) were calculated according to the following formula (VanBavel *et al*., 1998), and used as an index of $[Ca^{2+}]_i$:

$$R_n = (R_{340/380} - R_{min})/(R_{max} - R_{min}) \quad (1)$$

In addition, the effects of NA on both fluorescence and on tension were calculated as a per cent of the maximum response of the same preparation to 80 mM KCl in the presence of phentolamine.

The effects of 30 μM NA and 80 mM KCl on tension and $[Ca^{2+}]_i$ were characterized by calculating the following parameters: the maximum rates of tension and R_n development, the peak tension and R_n, and the integrated tension and R_n. The rates were calculated from the maximum slope of the initial rapid phase of each response. Maximum slope was determined by calculating the slope for each data point in the selected time interval (usually between 10 and 50 s after the response threshold), using a 15 point linear regression analysis (7 points on either side of each data point). The integrated tension and R_n were calculated by determining the area under the curve of each response measured between time zero (the time of the response threshold) and 300 s for NA, or 200 s for KCl (times corresponding to the maximum contractile response to each agonist.

Statistical analysis

Results are expressed as the mean ± s.e.mean, and *n* refers to the number of animals. Results were compared for significant differences using one-way or two-way analysis of variance (ANOVA) followed by the Neuman-Keuls test for multiple comparisons. In all cases, the level of significance was set at 0.05.

Drugs and chemicals

Fura-2 AM was obtained from Molecular Probes (Eugene, OR, U.S.A.) Ionomycin was purchased from Calbiochem (La Jolla, CA, U.S.A.). Pluronic F-17, cremophor EL, Ach, and NA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other drugs and chemicals were obtained from BDH Inc. (Toronto, Canada).

Table 1 Calibration parameters for fura 2 in mesenteric arteries from control and diabetic rats

	Control (n = 11)	*Diabetic* (n = 12)
R_{min} (minimum fluorescence ratio)	0.59 ± 0.01	0.59 ± 0.01
R_{max} (maximum fluorescence ratio)	2.54 ± 0.11	2.83 ± 0.25
A_f (autofluorescence)	0.49 ± 0.01	0.49 ± 0.01
β (F_{380min}/F_{380max})	1.45 ± 0.03	1.58 ± 0.08

All parameters determined as described in methods. There were no significant differences between control and diabetic arteries in any parameter.

Results

STZ-diabetic rats exhibited many of the signs and symptoms of a poorly controlled diabetic state, including polyuria, polydipsia and cataracts. The diabetic rats had significantly elevated plasma glucose levels (25.7 ± 0.6 vs 9.5 ± 0.2 mmol l^{-1}, $n = 15$ in each group), reduced plasma insulin levels (0.4 ± 0.05 vs 4.3 ± 0.3 ng ml^{-1}) and weighed less at the time of sacrifice (383 ± 8 vs 555 ± 11 g) than did control rats.

Changes in R_n and tension in response to perfusion with 30 μM NA followed by 80 mM KCl in mesenteric artery rings from a control and a diabetic rat are illustrated in Figure 1. Perfusion with this concentration of NA, which is maximal for contraction, resulted in an initial rapid rise in tension for the first 50 – 60 s, followed by a more slowly developing phase which reached a maximum after approximately 5 min. This was associated with an initial rapid rise in $[Ca^{2+}]_i$ which preceded the initial rise in tension. In both control and diabetic arteries, $[Ca^{2+}]_i$ levels reached their peak prior to the contractile response, and then tended to fall off while contraction was sustained. Following washout of NA, perfusion with 80 mM KCl in the presence of 0.5 μM phentolamine resulted in a rapid increase in both tension and $[Ca^{2+}]_i$. Phentolamine alone had no significant effect on either the tension or $[Ca^{2+}]_i$.

No statistically significant difference was evident between the mean basal R_n calculated for control (0.19 ± 0.03, $n = 11$) compared to diabetic (0.24 ± 0.03, $n = 12$) arteries. Similarly, no differences between control and diabetic arteries in either

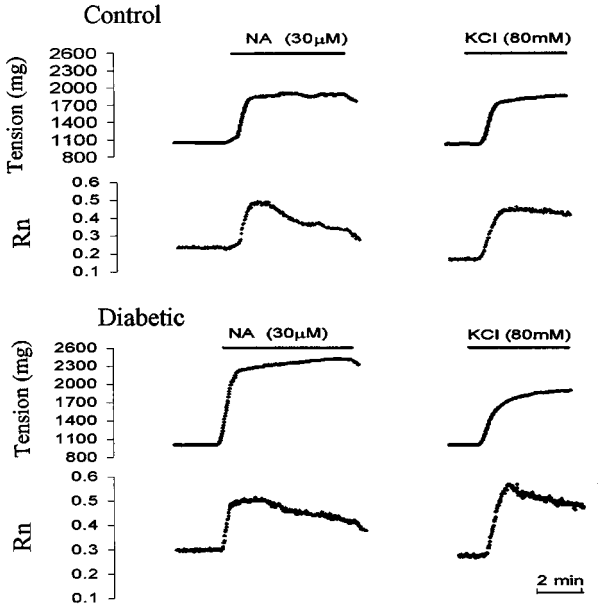

Figure 1 Representative changes in the normalized fura 2 ratio (R_n) and tension of mesenteric arteries from control (top) and diabetic (bottom) rats in response to 30 μM NA and to 80 mM KCl.

the tension or the $[Ca^{2+}]_i$ responses to KCl were detected. Neither the maximum rate of increase in tension or R_n (Figure 2A), the peak increase in these parameters (Figure 2B), nor the integrated tension and R_n (Figure 2C) in response to KCl were significantly different between control and diabetic arteries. Subsequently, in order to allow comparison of responses of arteries from control and diabetic rats to NA, these were expressed as a percentage of the corresponding response of the same vessel to 80 mM KCl.

In contrast to the KCl response, 30 μM NA produced a greater increase in tension in diabetic than control mesenteric arteries. The rate of tension development, peak tension and the area under the tension curve in response to NA were all significantly greater in diabetic than in control arteries (Figure 3A,B,C). However, this was not associated with a significant difference in the effects of NA on $[Ca^{2+}]_i$ in diabetic compared to control arteries (Figure 3). Comparison of the rate of increase and the peak increase in $[Ca^{2+}]_i$ in diabetic and control arteries revealed no significant differences (Figure 3A,B). Similarly, although the $[Ca^{2+}]_i$ response appeared to be sustained to a greater extent in the diabetic arteries (Figure 1), there was no difference in the area under the curve for $[Ca^{2+}]_i$ in control compared to diabetic arteries (Figure 3C). Although the large standard error in the diabetic arteries could have obscured a significant difference in the latter response, re-calculation of the integrated $[Ca^{2+}]_i$ in diabetic arteries without one outlying value resulted in a reduction in the mean$\pm$s.e. mean to 132$\pm$9%, a value still not significantly different from control (105$\pm$9%).

Peak contractile responses of diabetic mesenteric arteries to NA were also significantly greater than control at lower concentrations of NA (0.3 and 3 μM) (Figure 4B). However, while the peak increase in $[Ca^{2+}]_i$ in response to 0.3 μM NA was significantly greater in diabetic than control arteries, the increase in $[Ca^{2+}]_i$ in response to 3 μM NA was not significantly different (Figure 4A). The data from Figures 3B and 4 was used to construct peak tension-$[Ca^{2+}]_i$ curves for NA in control and diabetic arteries (Figure 5). These indicate that the tension-$[Ca^{2+}]_i$ relationship is much steeper for diabetic than control arteries, in that for a given increase in $[Ca^{2+}]_i$ there is a much greater increase in tension in the diabetic arteries.

In order to investigate whether there is any evidence for altered Ca^{2+} influx or release from intracellular stores in diabetic arteries, responses to 30 μM NA of control and diabetic arteries perfused with Ca^{2+}-free Krebs solution containing 1 mM EGTA, followed by Krebs solution containing 2.5 mM Ca^{2+} were compared. A typical tracing of the effects of this treatment on $[Ca^{2+}]_i$ is shown in Figure 6. Perfusion of arteries with Ca^{2+}-free Krebs solution containing 1 mM EGTA resulted in a small decrease in basal $[Ca^{2+}]_i$. Addition of NA produced a rapid but transient increase in $[Ca^{2+}]_i$, which returned to basal levels within 3–4 min. Preliminary results (data not shown) indicated that under these experimental conditions, exposure of arteries to caffeine also resulted in a rapid, transient increase in $[Ca^{2+}]_i$ and abolished the response to subsequent perfusion with NA, suggesting that the increase in $[Ca^{2+}]_i$ was due to release from

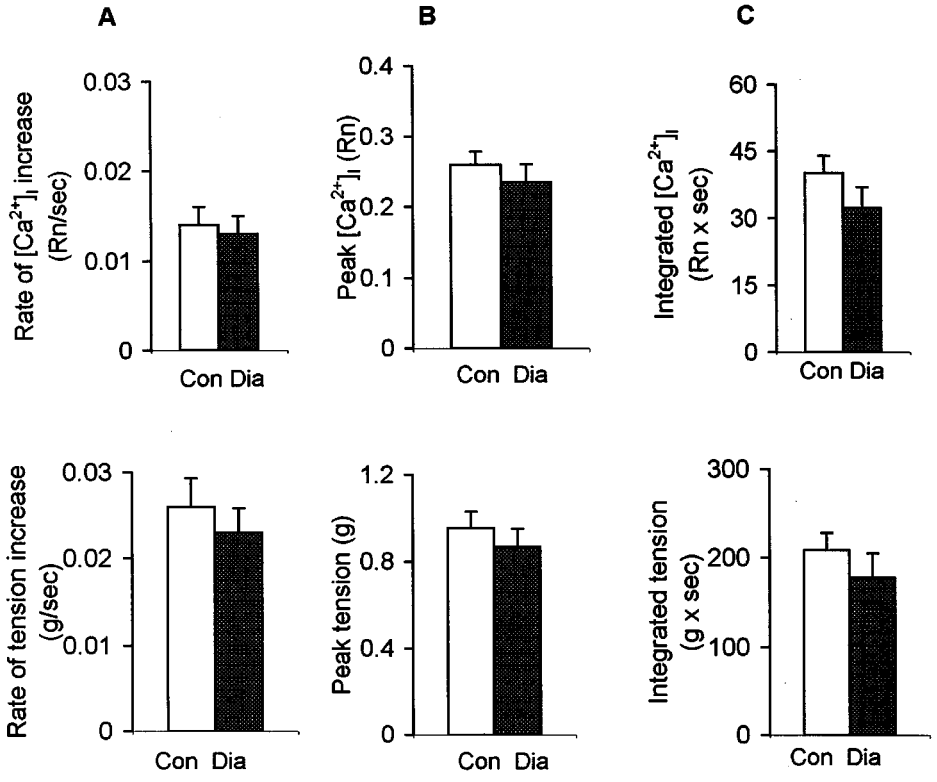

Figure 2 Effect of 80 mM KCl in the presence of 0.5 μM phentolamine on (A) the rate of increase in $[Ca^{2+}]_i$ and tension development, (B) peak $[Ca^{2+}]_i$ and tension, and (C) integrated $[Ca^{2+}]_i$ and tension in mesenteric arterial rings from control (Con, $n=11$) and diabetic (Dia, $n=12$) rats.

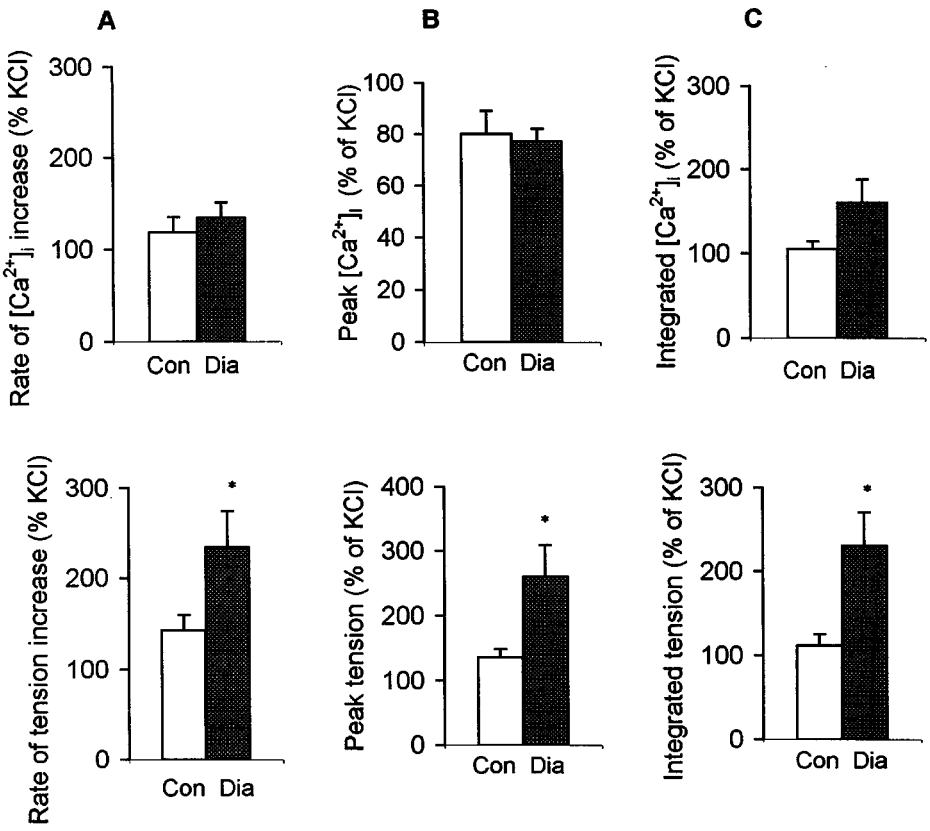

Figure 3 Effect of 30 μM NA on (A) the rate of increase in $[Ca^{2+}]_i$ and tension, (B) peak $[Ca^{2+}]_i$ and tension, and (C) integrated $[Ca^{2+}]_i$ and tension in mesenteric arterial rings from control ($n=11$) and diabetic ($n=12$) rats. Responses are expressed as a percentage of the corresponding response to 80 mM KCl in the presence of 0.5 μM phentolamine obtained in the same preparation. *$P<0.05$ compared to corresponding control responses (one-way ANOVA).

intracellular stores. When arteries were then perfused with Krebs solution containing 2.5 mM Ca^{2+} and NA, there was a further increase in $[Ca^{2+}]_i$ which was sustained. Both peak tension and integrated tension in response to 30 μM NA were significantly greater in diabetic than in control arteries perfused with Ca^{2+}-free solution containing 1 mM EGTA (Figure 7B). However, under these conditions the peak increase in $[Ca^{2+}]_i$ was also increased in diabetic compared to control arteries (Figure 7A). While the average integrated $[Ca^{2+}]_i$ was also greater in diabetic than control arteries, there was a high degree of variability in the area under the curve for $[Ca^{2+}]_i$ in diabetic arteries in Ca^{2+}-free solution and the difference was not significant. On subsequent perfusion of arteries with Krebs solution containing 2.5 mM Ca^{2+} in the presence of NA, the rates of increase in both tension and $[Ca^{2+}]_i$ were much slower than was observed in regular Krebs solution (compare Figure 8 with Figure 3), and were not significantly different between diabetic and control arteries. In addition, although the peak tension was significantly greater in the diabetic arteries, there was no significant difference in peak $[Ca^{2+}]_i$ between control and diabetic arteries (Figure 8).

Discussion

The results of the present investigation demonstrate that the enhanced maximum contractile response of mesenteric arteries from diabetic rats to NA is not associated with a corresponding increase in $[Ca^{2+}]_i$ levels. Furthermore, the data suggest that while the release of Ca^{2+} from intracellular stores may be enhanced in response to NA stimulation, the NA-induced increase in influx of extracellular Ca^{2+} is not altered in diabetic compared to control arteries. The dissociation between tension and $[Ca^{2+}]_i$ in response to NA in diabetic arteries suggests that a mechanism other an increase in mean cytosolic $[Ca^{2+}]_i$ levels is responsible for the enhanced contractile responses of diabetic arteries to NA.

In the present investigation, normalized fluorescence, R_n, was used as an index of basal and KCl-induced increases in $[Ca^{2+}]_i$, since the K_d for fura 2 *in situ* in mesenteric arteries is unknown. However, for R_n to be a valid index of $[Ca^{2+}]_i$ for purposes of comparison between control and diabetic arteries, the K_d for fura 2 in these arteries must be the same. Although differences in the intracellular environment between control and diabetic arteries could alter the binding of Ca^{2+} to fura 2, and thus the K_d, the similarity in the calibration parameters, R_{min}, R_{max} and β between control and diabetic arteries, suggests that the intracellular behaviour of fura 2 is similar in these arteries. Furthermore, a difference in K_d between control and diabetic arteries would not affect the comparison of changes in $[Ca^{2+}]_i$ produced by NA relative to KCl, or of the rate of increase in R_n.

Using R_n as an index of intracellular Ca^{2+}, the basal $[Ca^{2+}]_i$ in control and diabetic rats did not appear to be

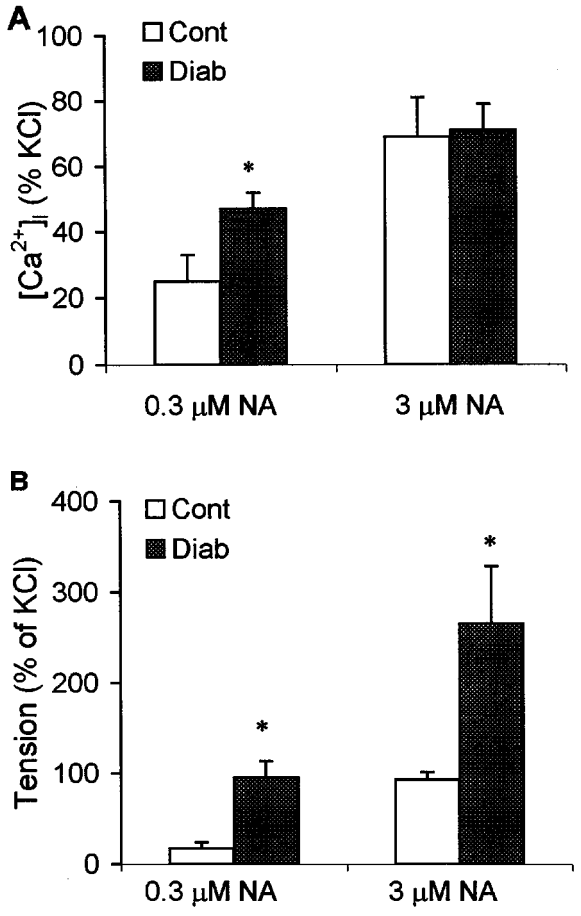

Figure 4 Peak $[Ca^{2+}]_i$ (A) and tension (B) in mesenteric arteries from control ($n=6$) and diabetic ($n=6$) rats in response to 0.3 and 3 μM NA. Responses are expressed as a percentage of the response to 80 mM KCl in the presence of 0.5 μM phentolamine obtained in the same preparation. *$P<0.05$ compared to corresponding control responses (2-way ANOVA followed by Neuman-Keul's test).

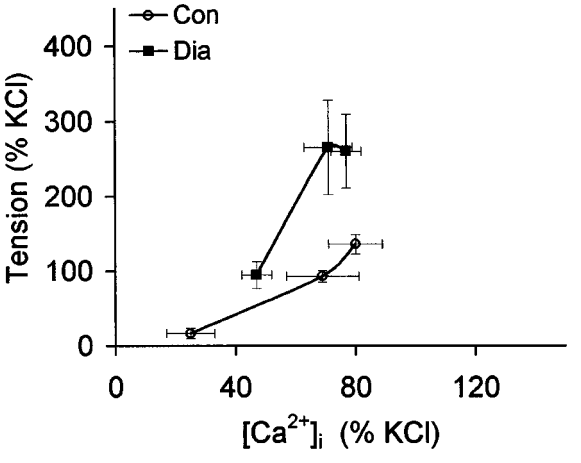

Figure 5 Peak tension-$[Ca^{2+}]_i$ relationship for NA in mesenteric arteries from control and diabetic rats. Data re-plotted from Figures 3B and 4.

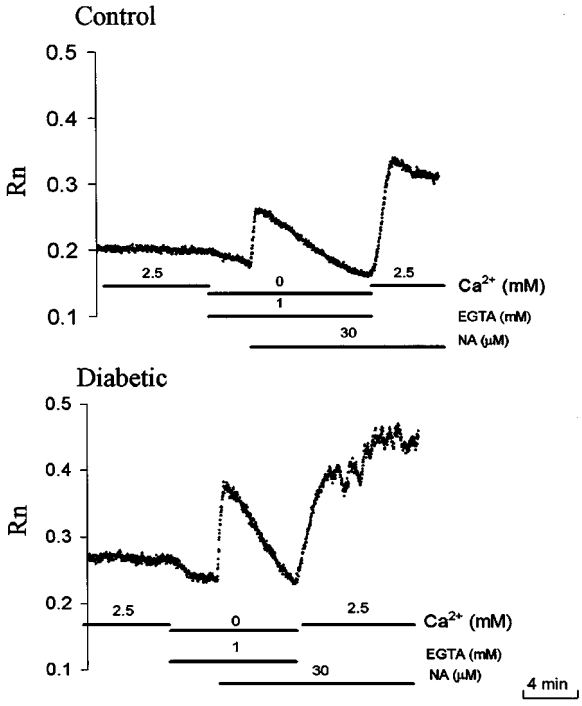

Figure 6 Representative changes in R_n obtained in mesenteric arteries from control (A) and diabetic (B) rats to NA, on exposure to the Ca^{2+}-free-Ca^{2+}-re-introduction protocol.

significantly different, although there was a tendency for levels to be higher in the diabetic mesenteric arteries. Similar results were obtained in vascular smooth muscle cells isolated from the tail artery of rats with STZ-induced diabetes of 12–13 weeks duration (Tam *et al.*, 1997). In contrast, resting $[Ca^{2+}]_i$ levels were reported to be significantly higher in aortic vascular smooth muscle cells isolated from rats with STZ-induced diabetes of 7 and 14 days duration (Ohara *et al.*, 1991). This difference could be due either to the shorter duration of diabetes or the different artery used in the latter investigation.

We have previously reported that contractile responses of mesenteric arteries from 12–14 week STZ-diabetic rats to KCl are not significantly different from those of arteries from age-matched control animals (MacLeod, 1985; Abebe *et al.*, 1994; Weber *et al.*, 1996). The observation in the present investigation that the increase in R_n in response to KCl was not different in control and diabetic arteries is consistent with the lack of difference in contractile responses of diabetic arteries to this agent, and supports our previous suggestion that there is no generalized increase in either $[Ca^{2+}]_i$ or tension in diabetic mesenteric arteries. However, in contrast to our working hypothesis, the results of the present investigation suggest that the enhanced contractile responses of arteries from diabetic rats to NA are largely independent of changes in $[Ca^{2+}]_i$. In response to the lowest concentration of NA tested (0.3 μM, a concentration near the EC_{50} for NA), $[Ca^{2+}]_i$ in arteries from diabetic rats was increased to approximately double the level in arteries from control rats, while tension in the diabetic arteries was increased about 5 fold over that in control arteries. Furthermore, the enhanced

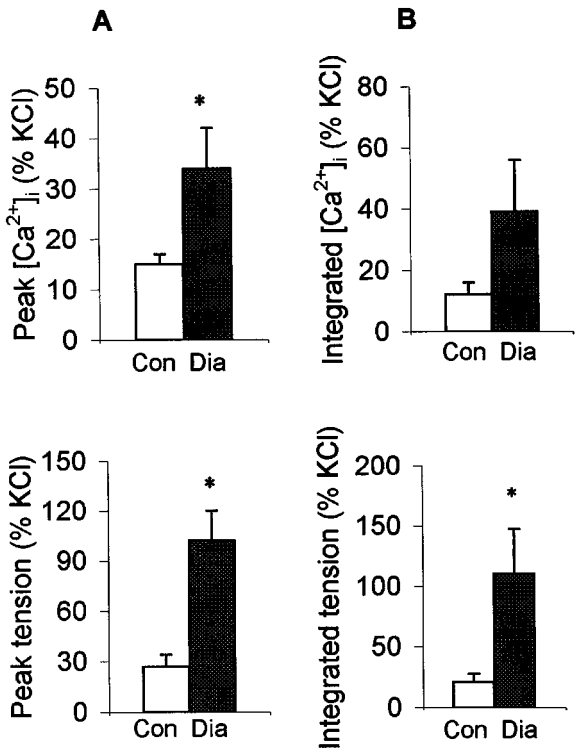

Figure 7 Peak $[Ca^{2+}]_i$ and tension (A) and integrated $[Ca^{2+}]_i$ and tension (B) in response to 30 μM NA in mesenteric arteries from control ($n=6$) and diabetic ($n=6$) rats perfused with Ca^{2+}-free Krebs solution containing 1 mM EGTA. Responses are expressed as a percentage of the response to 80 mM KCl obtained in the same preparation. $^*P<0.05$ compared to corresponding control responses (one-way ANOVA).

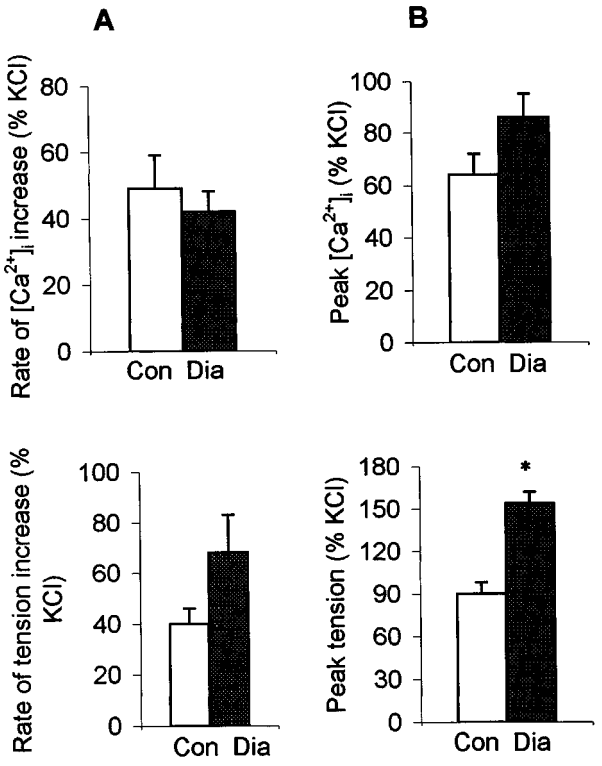

Figure 8 Effect of re-perfusion of arteries with Krebs solution containing 2.5 mM Ca^{2+} on the maximum rate of increase in $[Ca^{2+}]_i$ and tension (A) and on peak $[Ca^{2+}]_i$ and tension (B) in response to 30 μM NA in mesenteric arteries from control and diabetic rats. Responses are expressed as a percentage of the response to 80 mM KCl obtained in the same preparation. $^*P<0.05$ compared to corresponding control responses (one-way ANOVA).

peak tension response of the diabetic compared to control arteries to the two higher concentrations of NA tested was not associated with an increase in peak $[Ca^{2+}]_i$. Therefore, as demonstrated by the peak tension-$[Ca^{2+}]_i$ relationship, the increase in tension for a given increase in $[Ca^{2+}]_i$ in response to NA was greater in diabetic than control arteries, and was greater at higher than lower concentrations of NA. The reason for the greater increase in $[Ca^{2+}]_i$ in response to 0.3 μM NA in arteries from diabetic than control rats was not investigated. However, it appears that whatever the source, it is not sufficient to explain the enhanced contractile responses of diabetic arteries to NA.

Ca^{2+} is generally accepted to be an essential second messenger in the contractile response of smooth muscle to agonists and K^+-depolarization. The major mechanism of smooth muscle contraction is believed to be the Ca^{2+}-dependent activation of myosin light chain kinase (MLCK) leading to phosphorylation of myosin light chain (MLC), allowing it to interact with actin. Since MLC is dephosphorylated by myosin light chain phosphatase (MLPP), the extent of myosin phosphorylation is dependent on the MLCK/MLPP activity ratio (reviewed in Karaki *et al.*, 1997). It is now clear that contractile force can be modulated by agonists independently of $[Ca^{2+}]_i$, a process known as Ca^{2+} sensitization (e.g. Bradley & Morgan, 1987; Himpens *et al.*, 1990). While a number of different mechanisms for Ca^{2+} sensitization have been proposed, the major mechanism appears to be an increase in the MLCK/MLPP activity ratio due to inhibition of MLPP activity (reviewed in Somlyo *et al.*, 1999). Recent studies have suggested that phosphorylation of MLPP by one of a number of possible kinases, including Rho kinase (Uehata *et al.*, 1997) and protein kinase C (PKC) (Masuo *et al.*, 1994) activated in response to agonist stimulation of G-protein-coupled receptors, results in inhibition of its activity. The results of the present investigation suggest that there may be a relatively greater increase in Ca^{2+} sensitization in response to NA in diabetic than control arteries. Recently, NA-induced Ca^{2+} sensitization in rat mesenteric small arteries was attributed to a PKC-mediated mechanism (Buus *et al.*, 1998). Previous studies from this laboratory have suggested that diacylglycerol production in response to NA is increased in arteries from diabetic rats (Abebe & MacLeod, 1991; 1992), and have demonstrated that the PKC inhibitor staurosporine blocks the enhanced sustained contractile responses of diabetic mesenteric arteries to stimulation with a maximal concentration of NA (Abebe & MacLeod, 1990). Therefore, increased Ca^{2+} sensitization of the contractile proteins in response to increased activation of PKC could be largely responsible for the enhanced contractile responses of diabetic mesenteric arteries to NA.

An alternate explanation for the increased Ca^{2+} sensitivity of mesenteric arteries from diabetic rats in response to NA is a decrease in activation of β-adrenoceptors by this non-selective agonist. Stimulation of β-adrenoceptors leading to

elevation of intracellular cyclic AMP levels has been demonstrated in some arteries to decrease Ca^{2+} sensitivity, possibly by activating MLPP (reviewed in Karaki *et al*., 1997). In order to investigate this possibility, in preliminary studies we measured contractile responses and $[Ca^{2+}]_i$ in response to the α_1-adrenoceptor selective agonist, phenylephrine (PE). As we found with NA, treatment with a maximal concentration of PE (10 μM) produced a significantly greater increase in tension in mesenteric arteries from diabetic ($233 \pm 38\%$, $n=3$) than from control ($125 \pm 10\%$) rats, although there was no significant difference in $[Ca^{2+}]_i$ between diabetic and control arteries (84 ± 11 and $77 \pm 4\%$, respectively). This suggests that any increase in Ca^{2+} sensitivity of the diabetic arteries is not due to a diminished contribution of β-adrenoceptors to the NA response.

We have previously shown that the production of IP_3 in response to stimulation with a maximal concentration of NA is also enhanced in mesenteric arteries from diabetic rats (Abebe & MacLeod, 1992). This led us to hypothesize that there would be greater release of intracellular Ca^{2+} response to this concentration of NA in diabetic compared to control arteries. The results of the present investigation are in agreement with this hypothesis, since peak increases in $[Ca^{2+}]_i$ and tension of diabetic mesenteric arteries incubated in Ca^{2+}-free solution to NA were significantly greater in diabetic than in control arteries. These data are not consistent with the results obtained in another study in which NA-induced changes in $[Ca^{2+}]_i$ were measured, in vascular smooth muscle cells isolated from 12–13 week diabetic rat tail arteries (Tam *et al*., 1997). In that investigation, there appeared to be increased release of $[Ca^{2+}]_i$ in response to low concentrations of NA but decreased release of $[Ca^{2+}]_i$ in response to maximal NA, in cells from diabetic compared to control rats. However, since neither contractile tension nor IP_3 production in response to NA stimulation were measured in the latter investigation, it is not clear whether the other changes that we have detected in mesenteric arteries from diabetic rats also occur in the tail artery. It is also interesting that the relatively greater release in $[Ca^{2+}]_i$ in response to NA in diabetic arteries does not contribute to an overall increase in cytosolic $[Ca^{2+}]_i$ levels when extracellular Ca^{2+} is present. It is possible that the increased Ca^{2+} release is offset by a relatively smaller increase in the influx of extracellular Ca^{2+} in the diabetic arteries. Although the present investigation did not directly address this question, the results do suggest that when Ca^{2+} stores are depleted, both the rate and magnitude of NA-stimulated Ca^{2+} influx (as assessed by the Ca^{2+}-free-Ca^{2+} re-introduction protocol) are similar in control and diabetic arteries. Therefore, if decreased Ca^{2+} influx does occur when Ca^{2+} stores are intact in diabetic arteries, this may occur due to the activation of other regulatory processes, such Ca^{2+}-dependent K^+ channels, in the diabetic arteries.

As noted in the Introduction, the results of studies of vascular reactivity in diabetes have not all been consistent. While we and others have found that responsiveness of arterial preparations to NA and PE is increased, in other studies responsiveness to these agonists was found to be unchanged or even decreased (see Subramanian & MacLeod, 1999 for review). To a certain extent, these differences appear to be attributable to methodological differences between studies. For instance, we have found that the increased reactivity of mesenteric arteries to NA does not occur until at least 6–8 weeks after diabetes induction (Bardell & MacLeod, unpublished observations), while contractile responses of rat aorta and tail artery to α-adrenoceptor agonists were reported to be unchanged 2 weeks (Fulton *et al*., 1991), and 4 weeks (Wang *et al*., 1998) after the induction of diabetes, respectively. However, the discrepancy between our results and reports of a generalized increase in contractile responses of mesenteric arteries from 3 month diabetic rats to all vasoconstrictors, including KCl, (Agrawal & McNeill, 1987; White & Carrier, 1988) is more difficult to explain. However, Rodriguez-Manas *et al*. (1998) found that impairment of endothelium-dependent relaxation of 8 week diabetic rat aorta to acetylcholine increased as glycaemic control worsened, suggesting that the severity of the diabetic state is also an important determinant of vascular reactivity and could explain some of the differences between studies. It should also be noted that recently, Wang *et al*. (2000) reported that the current density of L-type Ca^{2+} channels, measured by the whole-cell patch clamp technique, was significantly lower in vascular smooth muscle cells isolated from 1–3 month diabetic rat tail arteries, suggesting the Ca^{2+} influx through these channels is impaired. While this observation does not seem consistent with the reports of unchanged or even increased contractile responses to KCl in diabetic mesenteric arteries, this may be attributable to differences between preparations or in the experimental conditions.

The physiological significance of the enhanced contractile responsiveness of the diabetic mesenteric arteries to NA that we have detected is not known at present. We have previously reported that systolic blood pressure is not altered in rats with STZ-induced diabetes of 12–14 weeks duration compared to their age-matched controls (MacLeod, 1985), indicating that the increased reactivity to NA that we have detected occurs prior to any change in blood pressure in these animals. At the same time, the increased reactivity of tail arteries from diabetic rats to NA does not appear to be secondary to the development of autonomic neuropathy (Weber & MacLeod, 1994). It is possible that the enhanced responsiveness contributes to an eventual increase in blood pressure in long-term diabetes, or that it contributes to the maintenance of cardiovascular homeostasis in the face of diabetes-induced changes in other systems involved in cardiovascular regulation (reviewed in Hebden, 1999). Further studies are required to resolve this.

In summary, the results of indirect studies in the past have suggested that enhanced maximum contractile responses of diabetic arteries to receptor stimulation are associated with both increased Ca^{2+} influx and increased release of $[Ca^{2+}]_i$, leading to an increased cytosolic Ca^{2+}. However, direct measurement of $[Ca^{2+}]_i$ in diabetic arteries indicates that while release of $[Ca^{2+}]_i$ in response to a maximal concentration of NA is enhanced in arteries from diabetic rats, there is no apparent increase in Ca^{2+} influx or in mean cytosolic Ca^{2+}. The relatively greater increase in the rate of tension development and in peak tension in response to NA may be due to an increase in the Ca^{2+} sensitivity of the contractile proteins. Further investigation will be required to establish whether this is due to increased activation of PKC in the diabetic arteries.

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