



Enhanced contractile responses of arteries from streptozotocin diabetic rats to sodium fluoride

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1 Previous studies from this laboratory have demonstrated that α_1 -adrenoceptor-mediated increases in tension and phosphoinositide metabolism are enhanced in the aorta and mesenteric arteries from diabetic rats. The purpose of the present investigation was to determine whether contractile responses to sodium fluoride (NaF), which directly stimulates GTP-binding proteins (G-proteins), are also enhanced in diabetic arteries.

2 NaF (1–20 mM) in the presence of 10 μ M aluminium chloride produced slowly developing, concentration-dependent contractions in mesenteric arteries from three month streptozotocin-diabetic (60 mg kg⁻¹, i.v.) male Wistar rats and age-matched control rats. The maximum contractile response but not the sensitivity to NaF was significantly greater in mesenteric arteries from diabetic than from control rats, as was the response to noradrenaline (NA). Maximum contractile responses of aorta and caudal artery from diabetic rats to NaF were also significantly enhanced.

3 Removal of the endothelium and denervation with 6-hydroxydopamine did not significantly alter the maximum contractile response of mesenteric arteries from either control or diabetic rats to NaF. Similarly, NaF had no effect on cyclic AMP levels in aorta, and no difference in cyclic AMP levels, either basally or in the presence of NaF, was detected between control and diabetic rat aorta.

4 Contractile responses of mesenteric arteries from both control and diabetic rats to NaF were diminished in calcium-free Krebs solution, but the NaF response remained significantly elevated in mesenteric arteries from diabetic rats compared to control.

5 Ryanodine (30 μ M) which depletes intracellular calcium stores, nifedipine (3 μ M) which blocks dihydropyridine-sensitive calcium channels and calphostin C (0.5 μ M) which selectively inhibits protein kinase C, all significantly inhibited maximum contractile responses of mesenteric arteries from control and diabetic rats to NaF. There were no significant differences between control and diabetic arteries in the relative magnitude of the inhibition produced by the three antagonists.

6 These data suggest that there may be increased activation of the same signalling processes that mediate NA-stimulated vasoconstriction, perhaps contraction-associated G-proteins or the effectors coupled to these G-proteins, in response to NaF in mesenteric arteries from diabetic rats. This may also be responsible for the enhanced contractile responses of these arteries to α_1 -adrenoceptor stimulation.

Keywords: Diabetes mellitus; mesenteric artery; G-proteins; vasoconstriction; sodium fluoride

Introduction

Diabetes mellitus is associated with an increased incidence of cardiovascular disease, which has been suggested to be due at least in part to a change in reactivity of blood vessels to noradrenaline (NA) and other circulating hormones (Christlieb *et al.*, 1976). Studies in animal models of diabetes have been conflicting, with reports of increased maximum contractile responses (Ramanadham *et al.*, 1984; MacLeod, 1985; Agrawal & McNeill, 1986; Harris & MacLeod, 1988; White & Carrier, 1988; Abebe *et al.*, 1990; Weber & MacLeod, 1994), increased sensitivity with no change in the maximum responses (Cohen *et al.*, 1990), and decreases in the maximum responses (Pfaffman *et al.*, 1982; Hart *et al.*, 1988; Takiguchi *et al.*, 1989) of arteries from animals with chronic, chemically induced diabetes to α -adrenergic stimulation. The reason for these differences is not entirely clear, but contributing factors may be differences in the species, duration of diabetes, and vascular preparation studied. This laboratory has consistently shown that maximum contractile responses to NA of aorta, mesenteric and caudal arteries from rats with streptozotocin-induced diabetes of 12 to 14 weeks duration are increased, with little or no increase in the sensitivity (pD₂ or -log EC₅₀) compared to responses of arteries from age-matched control rats. The enhanced responsiveness to NA is not due to a generalized in-

crease in contractility of the arteries from diabetic rats since we have previously observed that the contractile responses to depolarizing concentrations of KCl in the presence of phenolamine are not different between arteries from control and diabetic rats (MacLeod, 1985; Abebe *et al.*, 1994; Weber & MacLeod, 1994). Previous studies in this laboratory have also determined that the increase in the contractile response of the arteries from diabetic rats to NA is not due to a decrease in the release of endothelium-derived relaxing factors (Harris & MacLeod, 1988), nor to the development of autonomic neuropathy (Weber & MacLeod, 1994), but instead is mediated by an enhanced response to stimulation of α_1 -adrenoceptors (Abebe *et al.*, 1990).

The vascular α_1 -adrenoceptor is thought to be coupled to two signal transduction pathways. It has been proposed that one of these is a guanine nucleotide binding protein (G-protein) that is sensitive to pertussis toxin (PTX) and activates a calcium channel (Nichols *et al.*, 1989). The second of these is a PTX-insensitive G-protein that activates phospholipase C (PLC). PLC catalyzes hydrolysis of phosphatidyl inositol-4,5-bisphosphate (PIP₂) to form the two second messengers: inositol-1,4,5-trisphosphate (IP₃) which causes release of intracellular calcium, and diacylglycerol (DAG) which activates protein kinase C (PKC) (Abdel-Latif, 1986). These events then result in increases in intracellular calcium, phosphorylation of various proteins, and contraction of vascular smooth muscle. Studies from this laboratory have demonstrated that the en-

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hanced contractile response to NA is associated with increased breakdown of PIP₂, formation of total inositol phosphates, and production of IP₃ in aorta and mesenteric arteries from diabetic as compared to control rats (Abebe & MacLeod, 1991a, b; 1992). Increased activation of PKC and increased influx of extracellular calcium also appear to contribute to the enhanced contractile response of aorta and mesenteric arteries from diabetic rats to NA (Abebe & MacLeod, 1990; Abebe *et al.*, 1994). These results suggest that there is an enhancement at one or more points in the signalling pathway coupled to the α_1 -adrenoceptor in arteries from diabetic rats.

The purpose of the present investigation was to compare contractile responses of aorta, mesenteric, and caudal artery from 12–14 week diabetic rats and their age-matched controls to sodium fluoride (NaF) in the presence of aluminium chloride (AlCl₃), which has been shown to stimulate G-proteins (Ratz & Blackmore, 1990). If NaF-induced contractions are mediated by stimulation of the same G-proteins as those activated by the α_1 -adrenoceptor, then removal of extracellular calcium or incubation of tissues with drugs that are known to interfere with α_1 -adrenoceptor signalling by inhibiting the actions of IP₃, PKC, and calcium channels should diminish the NaF-induced contraction. Therefore, the effects of removal of extracellular calcium, and of pretreatment of tissues with ryanodine (an intracellular calcium depletor), and/or nifedipine (a calcium channel blocker), and calphostin C (a PKC inhibitor) on contractile responses to NaF were compared in mesenteric arteries from both control and diabetic rats. Since NaF would be expected to activate all G-proteins non-selectively, we also investigated possible contributions of activation of G-proteins located in other cell types (neural and endothelial), and of G-proteins coupled to other signal transduction systems (adenylyl cyclase) to the contractile response to NaF in arteries from control and diabetic rats.

Methods

Male Wistar rats (170–230 g) were housed and treated according to the guidelines of the Canadian Council on Animal Care. Rats were given a single i.v. injection of streptozotocin (60 mg kg⁻¹) or the citrate buffer vehicle. Streptozotocin-treated rats with a blood glucose of greater than 180 mg dl⁻¹ (measured with an Ames glucometer) one week after injection were considered to be diabetic and were retained for the experiment. Control and diabetic rats were housed separately and given free access to food and water. Three months later, the blood glucose of the diabetic rats was checked again with an Ames glucometer and only those animals that were still diabetic (>180 mg dl⁻¹) were used for experiments. The animals were weighed, killed with an overdose of pentobarbitone (65 mg kg⁻¹), and the mesenteric artery removed.

The mesenteric arteries were cleaned of connective tissue and fat, then cut into 4 mm rings. Each ring was suspended between the bases of two triangular-shaped wires in an isolated tissue bath containing Krebs solution (composition in mM: NaCl 113, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25 and dextrose 11.5), maintained at 37°C, and oxygenated with 95% O₂/5% CO₂. One wire was attached to a fixed tissue support while the other wire was attached by a cotton thread to a Grass FT.03 force displacement transducer connected to a Grass 7E polygraph. The rings were placed under 1.0 g resting tension, which was determined in preliminary experiments to be optimal for mesenteric artery. The mesenteric arteries were handled in a manner that has been shown in previous experiments in this laboratory to leave the endothelium functionally intact (Harris & MacLeod, 1988), except where indicated.

Tissues were allowed to equilibrate for a period of 90 min and the bathing solution was changed every 20 min before the addition of any drug. Contractile responses to cumulative addition of NA were obtained in untreated control and diabetic mesenteric arteries, the arteries were washed for 1 h and

then a concentration-response curve to NaF in the presence of 10 μ M AlCl₃ was obtained. In preliminary experiments, the contractile response to NaF was found to be optimal in the presence of this concentration of AlCl₃. In a second series of experiments, contractile responses of mesenteric arteries to 80 mM KCl (preincubated with 0.1 μ M phentolamine for 20 min) were obtained, the arteries washed for 1 h and then responses to NaF (20 mM) in the presence of 10 μ M AlCl₃ were obtained in the same tissues. The contribution of the endothelium to the contractile responses to NaF was determined in a third series of experiments. Endothelium was removed from half of the arterial rings by inserting a wire into the lumen and gently rotating the ring around the wire several times. The integrity of the endothelium was tested by determining the ability of arteries pre-contracted with 0.5 μ M NA to relax in response to 10 μ M acetylcholine (ACh). The tissues were then washed for 1 h and a maximum contractile response to NaF was measured in the presence of 10 μ M AlCl₃. A fourth series of experiments was performed to determine if there was a neural contribution to NaF responses. An initial contractile response to 30 μ M NA was obtained in mesenteric arteries, which were then washed for 1 h, treated with either 1 mM 6-hydroxydopamine (6-OHDA) or its vehicle for 20 min, and washed for a further 2 h. This procedure has been shown previously in this lab to abolish neurally-mediated responses in rat caudal artery (Weber & MacLeod, 1994). Then responses to 30 μ M NA were obtained again, the tissues washed for 1 h, and concentration-response curves to NaF were obtained in the presence of 10 μ M AlCl₃. To determine the effects of removal of extracellular calcium, contractile responses of mesenteric arteries incubated for 20 min in either normal Krebs buffer or calcium-free Krebs plus 1 mM EGTA to a maximum concentration of NaF (20 mM) in the presence of 10 μ M AlCl₃ were compared. In the final series of experiments in mesenteric artery, contractile responses to 20 mM NaF in the presence of 10 μ M AlCl₃ were determined in untreated arteries, and the arteries washed for 1 h. Then arteries were incubated with vehicle (ethanol or dimethyl sulphoxide (DMSO)), ryanodine (30 μ M for 30 min) and/or nifedipine (3 μ M for 30 min), or with calphostin C (0.5 μ M for 1 h) and a second response was obtained. Contractile responses to NaF in the presence of ethanol and DMSO were not significantly different from each other, so NaF responses in vehicle-treated tissues were pooled.

To extend the investigation to other arteries, aorta or caudal arteries were removed from control and diabetic rats, cleaned of connective tissue and fat, then cut into 4 mm and 2 mm rings, respectively. Each ring of aorta or caudal artery was suspended as described above, under their optimum resting tensions of 2.0 g (aorta) or 0.5 g (caudal artery). While the aorta retains functional endothelium through this process, preliminary experiments in this laboratory have shown that in handling the caudal artery, endothelial function is consistently lost (data not shown). After equilibration of the aorta and caudal arteries, contractile responses to a maximal concentration of NaF in aorta (30 mM) and caudal artery (20 mM) from control and diabetic rats was obtained in the presence of 10 μ M AlCl₃.

The effect of 30 mM NaF in the presence of 10 μ M AlCl₃ on cyclic AMP levels was determined in rings of aorta that were pre-equilibrated in oxygenated Krebs buffer, warmed to 37°C for 90 min. All rings had the endothelium removed in the manner described above for mesenteric artery. Twelve minutes after addition of NaF and AlCl₃ tissues were frozen with clamps cooled in liquid nitrogen and stored at -70°C until assay. In preliminary time course experiments, 12 min was the average time to the maximum contractile response to NaF in aorta. The tissues were quickly weighed while frozen and soluble cyclic AMP was extracted from the tissues by grinding the frozen arteries in a dental amalgamator with ice-cold 6% trichloroacetic acid (TCA) solution. The TCA was removed from the aqueous homogenate by extraction 4 times with 4 ml of water-saturated diethyl ether, and the samples frozen at -70°C. Cyclic AMP levels were measured by radio-

immunoassay, using the acetylation method of a Biotrak dual range scintillation proximity assay kit (Amersham, Oakville, Ontario, Canada).

Data analysis

In most cases, contractile responses are expressed as stress, in grams of tension per cross-sectional area of the artery. Cross-sectional area was calculated according to the following equation:

cross-sectional area (mm²) =

$$\frac{\text{mass (mg)}}{\text{length (mm)} \times \text{density (mg mm}^{-3}\text{)}}$$

where the density was assumed to be 1.05 mg mm⁻³. In the series of experiments with 6-OHDA, the response to NaF was expressed as a percentage of the maximum NA response after treatment with 6-OHDA or its vehicle obtained in the same tissue. In other cases, responses to the second NaF exposure in the absence or presence of antagonist were expressed as a percentage of the maximum contractile response to the same NaF concentration obtained previously in the same tissue. All values were expressed as mean \pm s.e.mean. Numbers (*n*) represent the number of animals used in each experiment. Results were compared for significant differences using a one-way or two-way analysis of variance (ANOVA), followed by a Bonferroni post-hoc test as appropriate. Results were considered to be significantly different if $P < 0.05$.

Drugs

Noradrenaline hydrochloride, nifedipine, EGTA, 6-OHDA hydrobromide, acetylcholine chloride and TCA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ryanodine was purchased from Calbiochem Corporation (La Jolla, CA, U.S.A.). Calphostin C was obtained from Kanimaya Biomed (1000 Oaks, CA, U.S.A.). Sodium pentobarbitone (Somnotol) was obtained from MTC Pharmaceuticals (Cambridge, ON, Canada). All other chemicals were obtained from BDH Inc. (Toronto, ON, Canada).

NA was dissolved in deionized water containing 1 mg ml⁻¹ ascorbic acid to reduce oxidation of the drug. ACh, 6-OHDA, and ryanodine were dissolved in distilled water. Nifedipine solutions were prepared with ethanol, while calphostin C was dissolved in DMSO. Experiments with nifedipine were performed in tissue baths protected from light, while experiments with calphostin C were performed in tissue baths exposed to daylight and fluorescent light.

Results

Twelve to fourteen weeks after STZ injection, rats displayed typical complications of diabetes, such as emaciation, polydipsia, polyuria and cataracts. The diabetic rats had significantly ($P < 0.05$; 1-way ANOVA) decreased body weights (377 ± 10 g; mean \pm s.e.mean; $n = 44$) compared to age-matched control rats (533 ± 9 g; $n = 44$). The cross-sectional areas of the mesenteric arteries from diabetic rats (0.26 ± 0.01 mm²) were also significantly ($P < 0.05$) smaller than those from control rats (0.32 ± 0.01 mm²).

In the presence of 10 μ M AlCl₃, NaF (1–20 mM) induced slowly-developing and concentration-dependent contractions in mesenteric arteries from both control and diabetic rats (Figure 1a and b). The maximum contractile response of mesenteric arteries from diabetic rats was 7.59 ± 0.41 g mm⁻² which was significantly ($P < 0.05$) greater than the maximum response of 5.78 ± 0.47 g mm⁻² ($n = 8$) observed in arteries from control rats (Figure 1a). There was no difference in the sensitivity to NaF between mesenteric arteries from control

and diabetic rats (Figure 1b). Both control and diabetic mesenteric arteries consistently relaxed when the NaF concentration was increased above 20 mM (up to 100 mM NaF in preliminary experiments), indicating that 20 mM produced the maximal contractile response to NaF in this preparation. The NA maximum was also obtained in the same tissues that were used to generate NaF concentration-response curves. The NA maximum was 4.97 ± 0.53 g mm⁻² ($n = 7$) in the mesenteric arteries from diabetic rats, which was similarly significantly ($P < 0.05$) enhanced compared to the maximum of 3.57 ± 0.41 g mm⁻² ($n = 7$) in arteries from control rats. The maximum NaF response was $161 \pm 11\%$ of the NA response in mesenteric arteries from control rats and $180 \pm 15\%$ of the NA response in those from diabetic rats ($P > 0.05$), indicating that the contractile response to NaF was enhanced to a similar extent as the NA response in mesenteric arteries from diabetic rats.

In order to investigate the selectivity of the enhancement of contractile responses of diabetic mesenteric arteries to NaF, responses to 80 mM KCl (in the presence of 0.1 μ M phentolamine) and to 20 mM NaF (plus 10 μ M AlCl₃) were obtained in the same preparation. Contractile responses to NaF, but not to KCl, were significantly increased in mesenteric arteries from diabetic rats compared to those of control rats (Figure 2a). Contractile responses of mesenteric arteries from diabetic rats to NaF remained significantly elevated compared to the responses of the control arteries when the NaF responses were

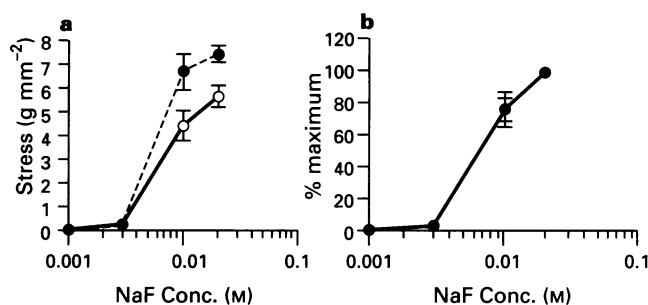


Figure 1 Cumulative concentration-response curve to NaF in mesenteric arteries from control (○, $n = 8$) and diabetic (●, $n = 8$) rats. (a) Results are expressed as stress (g mm⁻²). (b) Results are expressed as a percentage of the maximum response. Each point represents the mean \pm s.e.mean.

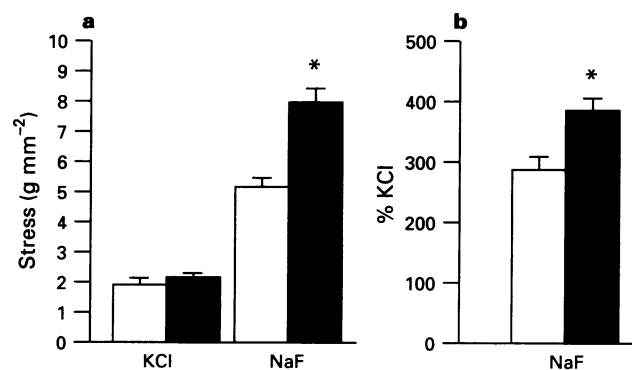


Figure 2 (a) Maximum contractile responses of mesenteric arteries from control (open columns, $n = 7$) and diabetic rats (solid columns, $n = 7$) to 80 mM KCl (plus 0.1 μ M phentolamine) and to 20 mM NaF expressed as stress (g mm⁻²). (b) Contractile responses to NaF expressed as a percentage of the KCl response in the same tissue. Each column represents the mean and each error bar the s.e.mean. *Significantly ($P < 0.05$; 1-way ANOVA) different from the corresponding control.

expressed as a percentage of the KCl response obtained previously in the same tissue (Figure 2b). The NaF response was $288 \pm 22\%$ ($n=7$) of the KCl response in mesenteric arteries from control rats and $386 \pm 21\%$ ($n=7$) of the KCl response in those from diabetic rats (Figure 2b).

Other arteries were examined for their response to NaF in the presence of $10 \mu\text{M}$ AlCl_3 . The contractile response to a maximal concentration of NaF was significantly greater in aorta from diabetic rats than that of aortae from control rats (Figure 3). Similarly, the maximum contractile response to NaF in caudal arteries from diabetic rats was significantly higher than the response obtained in caudal arteries from control rats (Figure 3).

Although vasoconstriction is the observed effect of NaF, activation of vasorelaxation signalling cascades could potentially modulate the response to this agent. This was investigated by measuring levels of the G_s -associated second messenger, cyclic AMP, in endothelium-denuded aorta from control and diabetic rats. The aorta was used for cyclic AMP determinations, rather than the relatively smaller mesenteric artery, to ensure that cyclic AMP levels could be detected. NaF (30 mM plus $10 \mu\text{M}$ AlCl_3) had no effect on cyclic AMP levels in aortae from control or diabetic rats (Table 1). Furthermore, no differences in either the basal cyclic AMP levels or cyclic AMP levels in the presence of NaF were detected between arteries from control and diabetic rats (Table 1). The effect of removal of the endothelium on responses to NaF was also investigated in mesenteric arteries from control and diabetic rats. Removal of the functional endothelium did not significantly change the maximum response to NaF in either control or diabetic arteries (Figure 4). Following endothelium removal, the maximum response remained significantly ($P < 0.05$) enhanced in mesenteric arteries from diabetic rats

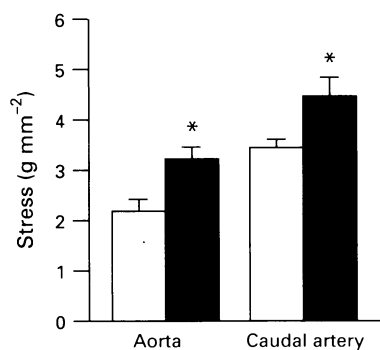


Figure 3 Maximum contractile responses to 30 mM NaF in aorta ($n=5$) and to 20 mM NaF in caudal artery ($n=6$) from control (open columns) and diabetic (solid columns) rats. The cross-sectional area of aortic rings from diabetic rats was $0.81 \pm 0.07 \text{ mm}^2$ and that of control aorta was $1.16 \pm 0.04 \text{ mm}^2$ ($P < 0.05$), while that of the diabetic caudal artery was $0.19 \pm 0.01 \text{ mm}^2$ and the control caudal artery was $0.24 \pm 0.02 \text{ mm}^2$ ($P < 0.05$). Each column represents the mean and each error bar the s.e.mean. *Significantly ($P < 0.05$; 1-way ANOVA) different from the corresponding control.

Table 1 Levels of cyclic AMP in aortae from control and diabetic rats exposed to 30 mM NaF or vehicle for 12 min, corrected for the frozen weight of the arteries

	Basal (fmol mg ⁻¹)	NaF (fmol mg ⁻¹)
Control	103 ± 16 ($n=5$)	136 ± 45 ($n=5$)
Diabetic	174 ± 75 ($n=4$)	148 ± 28 ($n=5$)

($6.80 \pm 0.46 \text{ g mm}^{-2}$; $n=12$) compared to the response of arteries from control rats ($5.30 \pm 0.20 \text{ g mm}^{-2}$; $n=12$) (Figure 4).

The contribution of neurally released factors to the NaF response was investigated by pretreating mesenteric arteries from control and diabetic rats with either 1 mM 6-OHDA or its vehicle. The response to $30 \mu\text{M}$ NA obtained after treatment with 6-OHDA was diminished slightly compared to the response to the same concentration of NA obtained prior to treatment in both control and diabetic arteries, indicating some direct effects of 6-OHDA on smooth muscle function. Therefore the NaF contractile response was expressed as a percentage of the response to $30 \mu\text{M}$ NA obtained after 6-OHDA or its vehicle in the same tissue. As noted above, expressing the data in this way eliminates the difference between control and diabetic tissues since the responses to NaF and NA are enhanced to the same extent in diabetic arteries. Treatment with 6-OHDA did not significantly affect the maximum responses of mesenteric arteries from either control or diabetic rats to NaF (Figure 5).

Removal of extracellular calcium significantly decreased the responses to 20 mM NaF (in the presence of $10 \mu\text{M}$ AlCl_3) of mesenteric arteries from both control and diabetic rats, to $1.54 \pm 0.09 \text{ g mm}^{-2}$ and $2.66 \pm 0.15 \text{ g mm}^{-2}$ ($n=7$ for both), respectively (Figure 6). However, the response of the mesenteric arteries from diabetic rats was still significantly larger than that of the control rats. If on the other hand, this remaining portion of the contractile response was expressed as a percentage of the NaF response obtained in the same tissue in

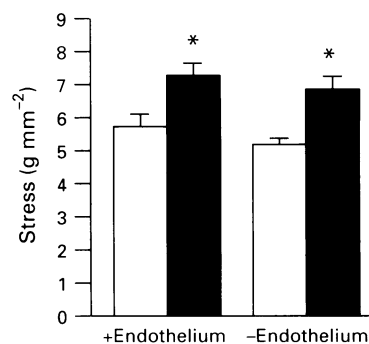


Figure 4 Maximum contractile response to NaF in mesenteric arteries from control (open columns) and diabetic (solid columns) rats, in the presence and absence of endothelium. Results are expressed as stress (g mm^{-2}) and each point represents the mean \pm s.e.mean ($n=12$ in all groups). *Significantly different (2-way ANOVA followed by a Bonferroni post-hoc test; $P < 0.05$) from the corresponding control.

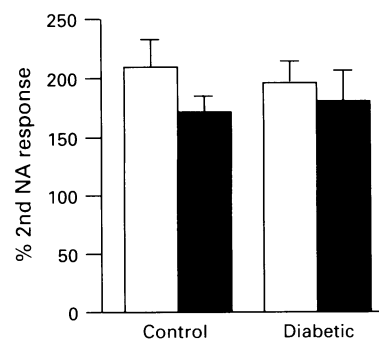


Figure 5 Maximum contractile response to NaF in mesenteric arteries from control ($n=5$) and diabetic ($n=4-5$) rats, pretreated with 1 mM 6-OHDA (solid columns) or its vehicle (open columns). Results are expressed as the mean \pm s.e.mean of the percentage of the response to $30 \mu\text{M}$ NA obtained after treatment with 6-OHDA or its vehicle in each tissue.

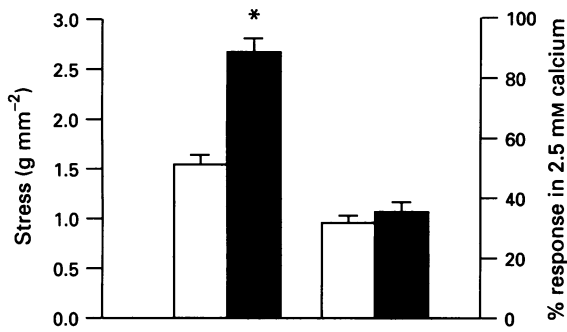


Figure 6 Contractile responses of mesenteric arteries from control (open columns; $n=7$) and diabetic (solid columns; $n=7$) rats to 20 mM NaF in calcium-free Krebs solution containing 1 mM EGTA, expressed (left axis) as stress (g mm^{-2}) and (right axis) as a percentage of the response to the same concentration of NaF obtained in 2.5 mM calcium-containing buffer obtained in the same tissue. Each column represents the mean and each error bar the s.e.mean. *Significantly ($P<0.05$; 1-way ANOVA) different from control.

the presence of extracellular calcium, no significant difference was detected between the responses of the arteries from control and diabetic rats (Figure 6).

The contractile response of vehicle-treated mesenteric arteries to a second exposure to NaF (20 mM plus 10 μM AlCl_3) was diminished to $93.1 \pm 4.6\%$ ($n=11$) in arteries from control rats and $82.0 \pm 4.3\%$ ($n=10$) in arteries from diabetic rats of the first response to NaF, indicating some loss of responsiveness to NaF upon repeated exposure. Accordingly, contractile responses to NaF in the presence of antagonists were compared with the corresponding responses to NaF obtained at the same time in vehicle-treated arteries. Pretreating mesenteric arteries from both control and diabetic rats with ryanodine alone, nifedipine alone, the combination of ryanodine plus nifedipine, or calphostin C alone all resulted in significant inhibition of the NaF-induced contractile response compared to their corresponding vehicle-treated arteries (Figure 7). The combination of ryanodine plus nifedipine reduced the NaF response to a significantly greater extent than any of the antagonists alone in mesenteric arteries from both control and diabetic rats (Figure 7). However, the relative inhibition of the NaF response produced by each of the three antagonists alone or by ryanodine and nifedipine in combination was not significantly different between mesenteric arteries from control and diabetic rats (Figure 7).

Discussion

The results of the present investigation demonstrate that maximum contractile responses to NaF (in the presence of AlCl_3) in the mesenteric artery, as well as in the aorta and caudal artery, from rats with chronic streptozotocin-diabetes are enhanced compared with responses of the corresponding arteries from age-matched control rats. The enhanced maximum contractile response to NaF occurred in mesenteric arteries that also displayed an enhanced response to NA and the magnitude of the increase appeared to be similar with both agents. The presence of extracellular calcium, calcium influx via dihydropyridine-sensitive calcium channels, intracellular calcium release, and activation of PKC all significantly contributed to the contractile response to NaF in mesenteric arteries from control and diabetic rats, with the relative contributions of each being similar between arteries from both types of animals. This suggests that NaF is eliciting the observed enhanced contractile response in arteries from diabetic rats via increased activation of signalling mechanisms similar to those stimulated by NA in smooth muscle, perhaps by stimulation of contraction-associated G-proteins or of the effectors coupled to these G-proteins.

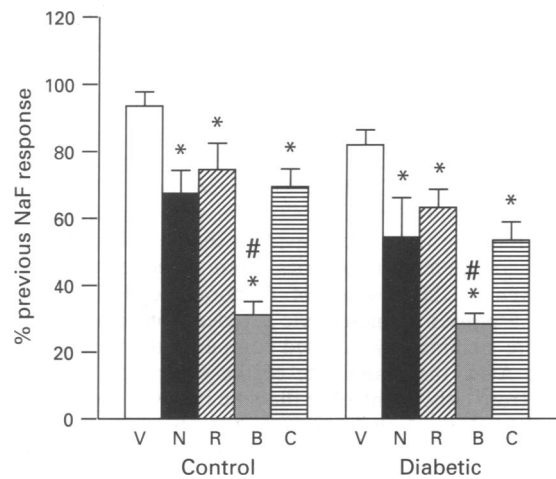


Figure 7 Maximum contractile responses of mesenteric arteries from control and diabetic rats to 20 mM NaF after pretreatment with vehicle (V, $n=10-11$), 30 μM ryanodine (R, $n=7$), 3 μM nifedipine (N, $n=6-7$), the combination of ryanodine plus nifedipine (B, $n=6-7$), or 0.5 μM calphostin C (C, $n=9$). Results are expressed as a percentage of the contractile response to 20 mM NaF obtained previously in the same tissue before treatment. Each column represents the mean and each error bar the s.e.mean. Results were analyzed by 2-way ANOVA, followed by Bonferroni post-hoc tests as needed. *Significantly different ($P<0.05$) from the corresponding vehicle-treated arteries. #Significantly different ($P<0.05$) from all other treatment groups.

The selectivity of the enhancement of the contractile response to NaF in mesenteric arteries from diabetic rats was investigated in the present study. Consistent with previous investigations from this lab (MacLeod, 1985; Abebe *et al.*, 1994; Weber & MacLeod, 1994) contractile responses to a depolarizing concentration of KCl were not different between control and diabetic mesenteric arteries. Furthermore, NaF-induced responses in mesenteric arteries from diabetic rats were still significantly enhanced compared to those from control rats when normalized relative to KCl responses obtained in the same preparation. This indicates that the enhanced contractile response to NaF in the diabetic arteries was selective and is not an artifact of normalizing the data for cross-sectional area.

NaF in the presence of AlCl_3 is thought to directly stimulate G-proteins via the AlF_4^- species (Ratz & Blackmore, 1990) which substitutes for the terminal phosphate in the presence of GDP to mimic GTP, although F^- by itself in the presence of magnesium may also activate G-proteins (Antony *et al.*, 1993). Potentially all G-proteins could be stimulated by the AlF_4^- species, although evidence has suggested that G_i but not G_s can be activated by fluoroaluminates (Inoue *et al.*, 1990). However, the results of the present investigation suggest that at the concentration and exposure time producing the maximum contractile response to NaF in aorta, neither G_i nor G_s were stimulated, since levels of cyclic AMP were not significantly changed by NaF in aortae from either control or diabetic rats. These results agree with another investigation where NaF had no significant effect on cyclic AMP levels in guinea-pig myometrium (Marc *et al.*, 1988), but contrast with a report that NaF decreased cyclic AMP levels in rat caudal artery (Zeng *et al.*, 1989). On the other hand, NaF has also been reported to increase cyclic AMP levels in bovine trachea (Hall *et al.*, 1990) and adenyl cyclase activity in plasmalemma from frog erythrocytes and S49 cells (Stadel & Crooke, 1988). These discrepancies concerning NaF effects on cyclic AMP levels may possibly be explained by differences in time of exposure to NaF and/or by tissue differences. The observation that cyclic AMP levels, measured basally or in the presence of NaF, were not significantly different between arteries from control and dia-

betic rats suggests that an altered production of cyclic AMP does not contribute to the enhanced contractile responses to NaF in arteries from diabetic rats.

Endothelium dysfunction has been suggested as at least a partial explanation for some of the altered contractile responses observed in arteries from diabetic animals (Oyama *et al.*, 1986; Taylor *et al.*, 1994). However, for a number of reasons it seems unlikely that defective release of the endothelium-derived relaxing factors can explain the enhanced contractile response of mesenteric arteries from diabetic rats to NaF. First, even in the absence of functional endothelium, contractile responses of mesenteric arteries from diabetic rats to NaF were significantly elevated compared to those of control arteries. Also, the magnitude of the contractile response of arteries from either control or diabetic rats was not changed by removal of the endothelium. This last observation in control mesenteric arteries is in agreement with other investigations where removal of the endothelium from aorta or coronary arteries did not significantly affect the contractile response to NaF (Cushing *et al.*, 1990; Adeagbo & Triggle, 1991). Furthermore, an enhanced contractile response to NaF was observed in the present investigation in arteries from diabetic rats that had both functional (aorta and mesenteric artery) and non-functional (caudal artery) endothelium. These observations are consistent with previous investigations from this laboratory which have shown that the enhanced contractile response to NA in mesenteric and caudal arteries from diabetic rats is not due to a decrease in the release of endothelium-derived relaxing factors (Harris & MacLeod, 1988; Weber & MacLeod, 1994).

The mechanism by which NaF elicits a contractile response in arteries could include stimulation of adrenergic nerves, and subsequent release of NA and other contractile neurotransmitters. If so, an altered neural input could be responsible for the enhanced contractile response to NaF observed in mesenteric arteries from diabetic rats. However, responses in aorta from diabetic rats were enhanced compared to those of control, despite the fact that this tissue is known to have little or no innervation. In addition, destruction of sympathetic nerves with 6-OHDA had no significant effect on contractile responses of mesenteric arteries from either control or diabetic rats to NaF. This observation indicates that if neurally released factors contribute to the NaF-mediated contractile responses, the contribution is so small as to be undetectable.

Neither an impaired vasorelaxation response nor increased neural input appear to be responsible for the enhanced response to NaF in arteries from diabetic rats, suggesting that the enhanced response is a result of changes at the level of the smooth muscle. In vascular smooth muscle, NaF in the presence of AlCl_3 is thought to stimulate the G-protein coupled to PLC because of the consistent observation that NaF stimulates increases in accumulation of inositol phosphates and/or increases in production of IP_3 in tissues such as rat aortic myocytes (Berta *et al.*, 1988), rat caudal artery (Zeng *et al.*, 1989; Cheung *et al.*, 1990), rabbit femoral artery (Ratz & Blackmore, 1990), and rat and rabbit aorta (Adeagbo & Triggle, 1991). Various lines of evidence suggest that NaF also activates G-proteins coupled to calcium channels. For instance, NaF has been demonstrated to increase ^{45}Ca uptake into rat caudal artery, and this was partially blocked by dihydropyridines (Zeng *et al.*, 1989). In addition, contractile responses to NaF in caudal, mesenteric and femoral arteries were inhibited by both dihydropyridines (Zeng *et al.*, 1989; Ratz & Blackmore, 1990; Boonen & De Mey, 1990) and by PTX (Boonen & De Mey, 1990; Cushing *et al.*, 1990; Abebe *et al.*, 1995). On the other hand, Adeagbo & Triggle (1991) were unable to detect an inhibitory effect of nifedipine on contractile responses of rat and rabbit aorta to NaF, suggesting that the contribution of dihydropyridine-sensitive calcium channels to the NaF response may vary in different arteries. Other known effects of NaF (probably mediated by F^-), such as inhibition of inositol-(1,4)-biphosphate phosphatase (Tiger *et al.*, 1990), prevention of inactivation of type-1 protein phosphatases (Bollen & Stal-

mans, 1988), inhibition of sarcoplasmic reticular Ca-ATPase (Murphy & Coll, 1992), and inhibition of Na,K-ATPase (Murphy & Hoover, 1992) may all potentially contribute to vasoconstriction. However, direct evidence for G-protein activation to the contractile response contributing to NaF was recently obtained in transiently permeabilized, dispersed intestinal smooth muscle cells, in which the contractile response to NaF was partially inhibited by $\text{GDP}\beta\text{S}$ (Murthy & Makhoul, 1994).

The results of the present investigation demonstrate that the contractile response of rat mesenteric artery to NaF is dependent to a large extent on the presence of extracellular calcium, since the NaF response in calcium-free solution was less than 40% of that in the presence of calcium. Nifedipine, in a concentration that has been shown to produce maximum inhibition of ^{45}Ca influx stimulated by KCl and by NA (Godfraind, 1983; Chiu *et al.*, 1986), also significantly inhibited the contractile response of mesenteric arteries to NaF, suggesting that NaF stimulates calcium influx via dihydropyridine-sensitive calcium channels in this preparation. Ryanodine significantly diminished the contractile response of mesenteric arteries to NaF at a concentration that we have shown to abolish almost completely the NA response in calcium-free medium (Weber *et al.*, 1995) suggesting that release of intracellular calcium also contributes to the NaF response. Lastly, the reduction of the NaF response by calphostin C, a selective PKC inhibitor in the concentration used in this investigation (Bruns *et al.*, 1991; Shimamoto *et al.*, 1993; Henrion & Laher, 1993), provides evidence that PKC also contributes to the contractile response to NaF in mesenteric artery. Each of the antagonists alone produced a similar magnitude of inhibition of the contractile to NaF, suggesting that calcium from intracellular and extracellular sources and PKC may each contribute to the same extent to the NaF response. Nifedipine and ryanodine in combination resulted in further significant reduction of the NaF response, although their combined effect appeared to be simply additive. The pattern and magnitude of inhibition of the NaF response in the presence of nifedipine and/or ryanodine and calphostin C in the present investigation is very similar to the pattern and magnitude of inhibition by these antagonists of the tonic response to a maximal concentration of NA in mesenteric artery (Weber *et al.*, 1995).

Since no differences were detected between mesenteric arteries from control and diabetic rats in the relative magnitude of inhibition of the contractile response to NaF by the three antagonists or by the combination of ryanodine and nifedipine, the enhanced contractile response observed in the arteries from the diabetic rats may not be due to a selective enhancement of calcium influx or PLC activation, but rather to a proportionate increase in both of these signalling mechanisms. This is further supported by the observation that the relative reliance of the NaF response on extracellular calcium was not different in mesenteric arteries from control and diabetic rats. This would suggest that the enhancement of the response to NaF in mesenteric arteries from diabetic rats occurs at a point both proximal and common to calcium influx, intracellular calcium release, and PKC activation, such as increased stimulation of the G-proteins associated with vasoconstriction. Furthermore, since the contractile response of mesenteric arteries from diabetic rats to NaF was enhanced to a similar degree as the NA response, the same mechanisms may be mediating the enhancement of both responses, possibly increased stimulation of contraction-associated G-proteins.

In summary, the results of the present study demonstrate that maximum contractile responses of mesenteric arteries from diabetic rats to NaF are enhanced. The enhancement is not mediated by increased release of contractile neurotransmitters, nor is it due to impaired endothelial function or cyclic AMP production. The results also suggest that the NaF-stimulated mechanisms in smooth muscle, perhaps contraction-associated G-proteins or their effectors, are enhanced in mesenteric arteries from diabetic rats, although other non-G-

protein associated mechanisms contributing to the enhancement cannot be ruled out. If NaF produces vasoconstriction in rat arteries predominantly by stimulating G-proteins, the enhanced contractile responses of the arteries from diabetic rats may be linked to the previously observed enhanced α_1 -adrenoceptor stimulation. The maximum contractile response to NaF is increased in aorta and caudal arteries from diabetic rats as well, which may be mediated by similar processes.

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