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Factors underlying the increased sensitivity to field stimulation of urinary bladder strips from streptozotocin-induced diabetic rats

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1 The responses of bladder strips from control, streptozotocin-diabetic, and sucrose-drinking rats to electrical field stimulation were investigated. Sucrose-drinking rats were included as additional controls because they have enlarged bladders as a result of non-diabetic diuresis.

2 Bladder strips from diabetic rats developed more spontaneous activity than those from the two control groups. Indomethacin reduced the amplitude and frequency of spontaneous contractions suggesting that they resulted from endogenous prostaglandin formation. Tetrodotoxin (TTX) had little effect, while α,β-methylene ATP caused increases in spontaneous activity.

3 Bladder strips from diabetic rats responded to field stimulation with greater contractions than controls in the absence of antagonists as well as in the presence of atropine and α,β-methylene ATP. Increasing TTX concentrations caused a step-wise depression of the contractile response to electrical stimulation which was not affected by preincubation with either atropine or α,β-methylene ATP.

4 Atropine and indomethacin had no effect on stength-duration curves constructed to measure threshold contractile responses to five pulses stimulation. The curves were shifted to the right by both TTX and α,β-methylene ATP, indicating that the responses were neurogenic in nature and at least partially, the result of stimulation of P_2-purinoceptors. In the absence of drugs, bladder strips from diabetics responded at lower voltages and pulse widths than those of control and sucrose-drinking rats, suggesting that they were more excitable.

5 The response curve of bladder strips from diabetics to field stimulation at increasing voltage was shifted upwards and to the left compared to strips from control or sucrose-drinking rats.

6 Bladder strips from diabetics responded to stimulation at increasing pulse width with greater responses than those from control or sucrose-drinking rats. At 1.0 ms pulse width, the TTX-resistant response of strips from diabetic rats was still greater than that of the other groups, indicating that a myogenic component was also involved.

7 The data suggest that bladder strips from diabetic rats are more excitable than those of control or sucrose-drinking rats. This may result from diabetes-induced decreases in bladder lipid or other membrane changes, and/or be a result of partial depolarization, perhaps related to diabetic neuropathy.

Keywords: Diabetes mellitus; sucrose; rat bladder; muscle contraction; electric stimulation

Introduction

The autonomic neuropathy associated with diabetes mellitus results in bladder dysfunction, characterized by a large capacity, atonic bladder (Frimodt-Møller, 1976). Although asymptomatic urodynamic changes have been found in diabetic children with or without neuropathy (Faerman *et al.*, 1971; Barkai & Szabo, 1993), the symptoms of diabetic cystopathy are more likely to present problems in older patients.

The urodynamic changes associated with experimental diabetes mellitus are similar to those found in diabetic patients. Micturition frequency and volume are increased; intravesical pressure upon filling remains low, and the amplitude of filling-induced intravesical contractions is reduced (Santicioli *et al.*, 1987; Andersson *et al.*, 1988; Longhurst *et al.*, 1991). However, there are some differences between the characteristics of human and experimental diabetes. Neurophysiological studies on insulin-treated spontaneously diabetic BB rats at 4 and 6 months, and 2 month streptozotocin-diabetic rats show changes in sensory innervation, but only modest changes in motor innervation (Paro *et al.*, 1990; Steers *et al.*, 1990; Nadelhaft & Vera, 1992). Furthermore, electrical field stimulation of strips from diabetic rat bladders fails to show any evidence of neuropathy; decreases in responsiveness have not been observed (Carpenter, 1983; Lincoln *et al.*, 1984; Luheshi & Zar, 1990; 1991; Paro *et al.*, 1990; Longhurst *et al.*, 1991). Some markers of innervation include acetylcholinesterase, choline acetyltransferase, and nerve growth factor (NGF). Decreased acetylcholinesterase and choline acetyltransferase staining has been noted in bladders from human and experimental diabetics when expressed as concentration, suggesting that cholinergic innervation is altered by diabetes (Faerman *et al.*, 1973; Lincoln *et al.*, 1984). Buttyan and co-workers found increased bladder NGF mRNA 4 weeks after streptozotocin (STZ) treatment, but a steady decrease in NGF protein up to 8 weeks, suggesting a decreased efficiency of NGF mRNA translation (Te *et al.*, 1992). More recently, the same group found decreases in NGF expression 6 weeks after induction of diabetes (Koo *et al.*, 1993).

The response of urinary bladder strips to electrical field stimulation is thought to be at least partially the result of stimulation of cholinergic and purinergic nerves. The relative contributions of cholinergic and non-adrenergic, non-cholinergic (NANC) innervation to the contractile response appear to be species and stimulus-dependent (Sibley, 1984; Brading & Williams, 1990). It is generally agreed that the rapid phasic portion of the response to field stimulation, which is relatively unchanged after atropine treatment but lost after

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desensitization of P_2-purinoceptors with α,β-methylene ATP, results from stimulation of purinergic nerves. Conversely, the tonic portion, which is reduced after atropine treatment but unaltered by P_2-purinoceptor desensitization, is thought to result from stimulation of cholinergic nerves (Brading & Williams, 1990). A series of studies by Luheshi & Zar suggested that bladder strips from diabetic rats had a reduced NANC response and increased cholinergic response to field stimulation, as a result of increased acetylcholine release and reduced NANC transmitter release (Luheshi & Zar, 1990; 1991). Previous studies from this laboratory showed that bladder strips from diabetic rats responded to electrical field stimulation with significantly greater responses than those from control or sucrose-drinking rats (Longhurst *et al.*, 1991).

In the present paper we have investigated the possibility that differences in excitability of diabetic rat bladders were responsible for the changes in response to electrical stimulation, by altering stimulation parameters, and using compounds which altered resting membrane potential or nerve conduction. We also examined further the effects of diuresis induced by sucrose consumption on the contractile responses to electrical stimulation to evaluate whether the observed changes in bladders from diabetics resulted from the diabetic state or increases in bladder mass *per se*.

Methods

Animals

Male Sprague-Dawley rats (300–325 g) obtained from Ace Animals Inc. (Boyertown, PA, U.S.A.) were used throughout the study. All animals received food and water *ad libitum*, except when indicated.

Induction of diabetes

Rats were fasted for 18–24 h. Diabetes was induced in approximately one-third of the rats with a single injection of STZ (60 mg kg^{-1}, i.p.) in ice-cold 0.02 M citrate saline. The remainder of the rats were injected with vehicle. Rats were used 9–12 weeks after the induction of diabetes.

Sucrose treatment

After injection of the vehicle, one-half of the control rats was given 5% sucrose in tap water to drink instead of water. This was continued until the day of experimentation. Sucrose consumption causes polyuria and increases in bladder mass (Longhurst *et al.*, 1990b). For this reason this group was included as non-diabetic controls. Rats were used 9–12 weeks after onset of sucrose treatment. The remaining group of control rats was given tap water to drink.

Tissue preparation

Before anaesthesia, blood samples were collected from the tail artery and the serum separated and analyzed for serum glucose by use of the ABTS method of Bergmeyer & Bernt (1974). The rats were then anaesthetized with pentobarbitone (50 mg kg^{-1}, i.p.). The urinary bladder was removed from each rat and placed in ice-cold Krebs-Henseleit buffer of the following composition (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25 and dextrose 5.6 The bladder was separated into bladder body and base at the level of the ureters. Three or four equally sized longitudinal strips of approximately 2 mm × 10 mm were cut from the bladder body, suspended on 000 sutures between a pair of platinum ring electrodes 8 mm apart, and placed in 30 ml organ baths containing Krebs-Henseleit solution equilibrated with 95% O_2, 5% CO_2, and maintained at 32°C (to reduce spontaneous activity). The tissues were connected to Grass force displacement transducers (FT03) and adjusted to 2 g resting tension. Previously it was determined in our laboratory that maximal active tension was generated at 2 g resting tension in all groups (Longhurst *et al.*, 1990a). Responses were recorded on a Grass Model 7E polygraph. All tissues were then given a 30 min equilibration period during which they were washed and the resting tension was adjusted every 10 min. Electrical stimuli were delivered using a Grass S88 stimulator. Rate of tension development was measured using a LS-14 logging analyzer (Buxco Electronics, Inc., Troy, NY, U.S.A.).

Influence of antagonists on response to field stimulation

Frequency-response curves were elicited by stimulating the tissues for 15 s with pulses of 0.05 ms width at 100 V every 2 min. Subsequently after resting periods of 15 min, frequency-response curves were repeated in the presence of different antagonists as described in the results sections.

Construction of strength-duration curves

Using separate strips, the voltage required at each pulse width to produce a just noticeable contractile response to five stimuli at 1 Hz was recorded. After construction of a control (no drug) curve, antagonists were added, and a second curve recorded after an appropriate period of time (see drugs). One strip from each bladder was exposed to each antagonist. Preliminary experiments showed that repetitive contractile responses to stimulation were not altered by time.

Measurement of voltage and pulse width dependence

Using separate strips, the contractile response to 10 pulses at 32 Hz stimulation was measured at increasing voltage using 0.05 ms width, or at increasing pulse width using 100 V stimulus.

Drugs

The following drugs were obtained from Sigma Chemical Company (time of exposure and dose in parentheses): atropine (15 min, 1 μM), α,β-methyleneadenosine 5'-triphosphate (α,β-methylene ATP) (15 min, 100 μM), indomethacin (60 min, 10 μM), and tetrodotoxin (TTX) (15 min, 1 μM). Indomethacin was dissolved in 0.5 ml dimethylsulphoxide (DMSO) and made up to 100 ml with Krebs. This was then added to the organ bath in a 1:10 dilution.

Statistical analysis

Data are presented as means ± s.e.mean or as a percentage of maximal control (no drug) response. To avoid confusion between references to control (no drug) contractile responses and those to control (not diabetic) animals or tissues, control contractile responses will be referred to in the results section as 'no drug'. Differences between the response of a single strip to field stimulation before and after drug treatment during construction of strength-duration curves were compared by the paired t test. Comparisons between groups were done using the Bonferroni test. A probability of $P < 0.05$ was taken as the criterion of significance. In all instances N = number of animals and n = number of strips.

Results

Rat weight, bladder weight, and serum glucose concentration

Diabetes caused the usual decreases in body weight, and increases in serum glucose concentration and bladder weight compared to control and sucrose-drinking rats (Table 1).

Table 1 Effect of streptozotocin (STZ)-induced diabetes and sucrose-consumption on rat weight, bladder and strip weights, and serum glucose concentration

	Control	*STZ*	*Sucrose*
Rat weight (g)	549 ± 14	276 ± 12†	564 ± 19
Bladder weight (mg)	143.5 ± 4.5	243.2 ± 9.2†	187.0 ± 8.3*
Serum glucose (mmol/l)	5.9 ± 0.2	24.5 ± 1.2†	6.0 ± 0.3

Values indicate the mean ± s.e.mean ($N = n = 32-48$). *Significant difference compared to controls; †significant difference compared to both controls and the sucrose group ($P < 0.05$).

Bladder weights were significantly increased in sucrose-drinking rats compared to controls, but there were no differences in rat weight or serum glucose concentration between the control and sucrose group (Table 1).

General observations of the contractile responses of bladder strips

There was a great deal of spontaneous activity generated by the bladder strips, particularly those from diabetic rats. To reduce this as much as possible, the studies were done at 32°C. Addition of indomethacin decreased the amplitude of spontaneous activity. TTX had little or no effect on spontaneous activity, while α,β-methylene ATP increased the amplitude, particularly in strips from diabetics (Figure 1). On several occasions after α,β-methylene ATP treatment it was difficult to distinguish the response to low frequencies of field stimulation from the spontaneous activity, which in some instances was 1–3 g in amplitude.

The mean contractile response of bladder strips from diabetic rats to α,β-methylene ATP was significantly greater than that of controls (Figure 2). Bladder strips appeared to be fully desensitized with one dose of α,β-methylene ATP, because administration of a second dose was without effect. After treatment with indomethacin the contractile response to α,β-methylene ATP was slightly reduced in the control group (Figure 2). In both the STZ and sucrose-drinking group, the response to α,β-methylene ATP was significantly reduced by indomethacin pretreatment to control levels, indicating that the increased tension observed in response to α,β-methylene ATP was probably mediated by prostaglandin release.

Non-cholinergic, non-adrenergic component of the response to field stimulation

There was a difference in the general shape of the response to field stimulation in the absence of drugs between strips from diabetic rats and those from control and sucrose-drinking rats (Figure 3). In particular, strips from diabetic rats had less distinction between the phasic (within 5 s) and tonic

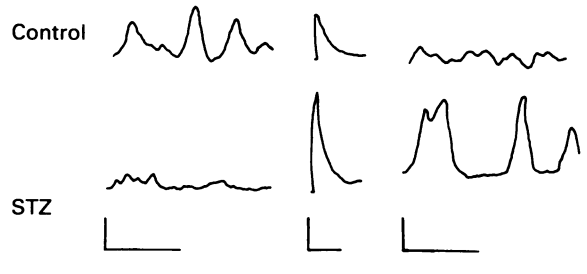

Figure 1 Influence of α,β-methylene ATP (100 μM) on spontaneous activity in bladder strips from control (top) and diabetic (STZ) (bottom) rats. Left panel, spontaneous activity before α,β-methylene ATP; centre panel, response to α,β-methylene ATP; right panel, spontaneous activity after α,β-methylene ATP. Left and right panels, vertical line represents 0.5 g, horizontal line represents 1 min. Centre panel, vertical line represents 2 g, horizontal line represents 4 min.

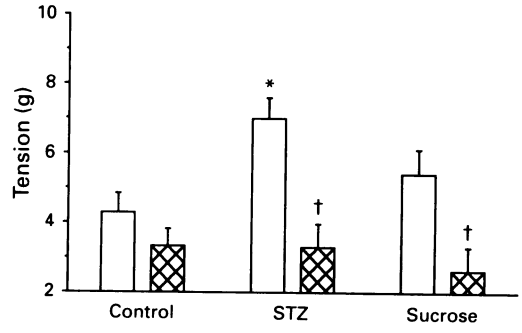

Figure 2 Contractile response of bladder body strips from control, diabetic (STZ), and sucrose-drinking rats to α,β-methylene ATP (100 μM) in the absence (open columns) and presence (cross-hatched columns) of indomethacin (10 μM, 60 min). Each column represents the mean ± s.e.mean ($N = n = 6-9$). *Significantly different from response of control bladder strips; †significantly different from α,β-methylene ATP alone ($P < 0.05$).

Figure 3 Representative responses of bladder strips from control (Con), diabetic (STZ), and sucrose-drinking (Suc) rats to 4 Hz stimulation. Parameters were 100 V, 0.05 ms pulse width, 15 s stimulation.

(after 15 s of stimulation) portion of the response, while in control and sucrose-drinking rats the phasic portion was usually easily distinguishable, and larger than the tonic portion. At all frequencies, the phasic:tonic component of the contractile response of strips from diabetic rats was significantly less than that of control and sucrose-drinking rats. Values at 2 and 32 Hz are shown in Table 2. In general, after treatment with α,β-methylene ATP the phasic component of the response to field stimulation was lost and the contraction appeared more like that shown for the bladder from the diabetic rat in Figure 3. In separate experiments, we calculated the rate of tension development in response to stimulation at low and high rates of frequency. Tension and maximal rate of tension development in response to electrical stimulation at 2 and 32 Hz were significantly greater in strips from sucrose-drinking rats than controls, and greater than controls at 32 Hz for strips from diabetic rats (Table 2).

Frequency-response curves for bladder strips in the absence and presence of atropine, α,β-methylene ATP, and TTX are shown in Figure 4. Because of the difficulty in distinguishing phasic and tonic responses, the data are presented as maximal responses. Strips from diabetics responded to stimulation in the absence of antagonists with significantly

Table 2 Effect of streptozotocin (STZ)-induced diabetes and sucrose-consumption on phasic and tonic components and maximal rate of tension development after 2 and 32 Hz field stimulation

	Control	*STZ*	*Sucrose*
Relative proportion of phasic:tonic component (phasic response (g)/tonic response (g))			
2 Hz	2.53 ± 0.32	1.15 ± 0.10*	2.03 ± 0.23
32 Hz	2.27 ± 0.27	1.35 ± 0.12*	1.59 ± 0.11
Maximal rate of tension development ($g\ s^{-1}$)			
2 Hz	1.49 ± 0.17	2.35 ± 0.42	2.39 ± 0.23*
32 Hz	4.30 ± 0.38	6.58 ± 0.80*	6.87 ± 0.58*

Parameters used were 0.05 ms width, 0.01 ms delay, 100 V, stimulation for 15 s. Values indicate the mean ± s.e.mean (for phasic:tonic experiments, $N = n = 17-20$; for rate experiments, $N = 6-9$, $n = 12-18$). *Significant difference compared to controls ($P < 0.05$).

greater contractions than those of control or sucrose-drinking rats. Incubation with atropine caused a 46% decrease in the response of control bladder strips to 32 Hz field stimulation. The responses of strips from diabetic and sucrose-drinking rats were decreased to a slightly greater extent, by 54% and 50% respectively (Figure 4). The effects of atropine were most noticeable at higher frequencies. Addition of α,β-methylene ATP caused a further decrease in the response to 32 Hz to 27% of the no drug maximum in controls and 16% and 27% in diabetic and sucrose-drinking rats. TTX reduced the contractile response to 32 Hz to 10% of the no drug maximum in controls and 5 and 6% in strips from diabetic and sucrose-drinking rats. In the presence of atropine the absolute responses of strips from diabetic rats were significantly greater than those of the control groups, but there were no significant differences in the degree of antagonist-induced suppression between groups.

Incubation with indomethacin had greater effects on the responses of bladder strips to low frequency stimulation than high frequency (Figure 5). Responses of strips from controls to low frequencies of stimulation were reduced by pretreatment with indomethacin ($P = 0.040$); the decrease was less at higher frequencies. In contrast, strips from diabetic and sucrose-drinking rats were less affected by indomethacin pretreatment. Cumulative addition of atropine and α,β-methylene ATP with indomethacin, or TTX alone caused significant decreases in contractile response of a similar magnitude to those shown in Figure 4.

To try to identify the relative importance of cholinergic vs. purinergic transmitter release in the response to field stimulation, the effects of increasing TTX concentrations were monitored in the presence or absence of atropine or α,β-methylene ATP. Three separate strips from each rat were used. All strips were stimulated first in the absence of drugs (no drug curves). Subsequently one strip was incubated in normal Krebs to establish a time-effect, one with atropine, and one with α,β-methylene ATP before repeating the frequency-response curve (time; atropine; α,β-methylene ATP curves). Then each strip was incubated with increasing concentrations of TTX, 15 min before stimulation. Bladder strips from diabetic rats responded to field stimulation with significantly greater responses than the two control groups, both in the absence and presence of atropine and α,β-methylene ATP. In all groups, TTX caused a sequential decrease in contractile response, which was not altered by the presence of atropine or α,β-methylene ATP. Inhibition was first seen at 20–40 nM TTX. Maximal inhibition was seen with 320 nM TTX. Increasing the concentration of TTX to 640 nM did not reduce the response further (data not shown). There were no differences between the responses of strips from control (Figure 6), diabetic (not shown), or sucrose-drinking rats (not shown) in the absence and presence of atropine and α,β-

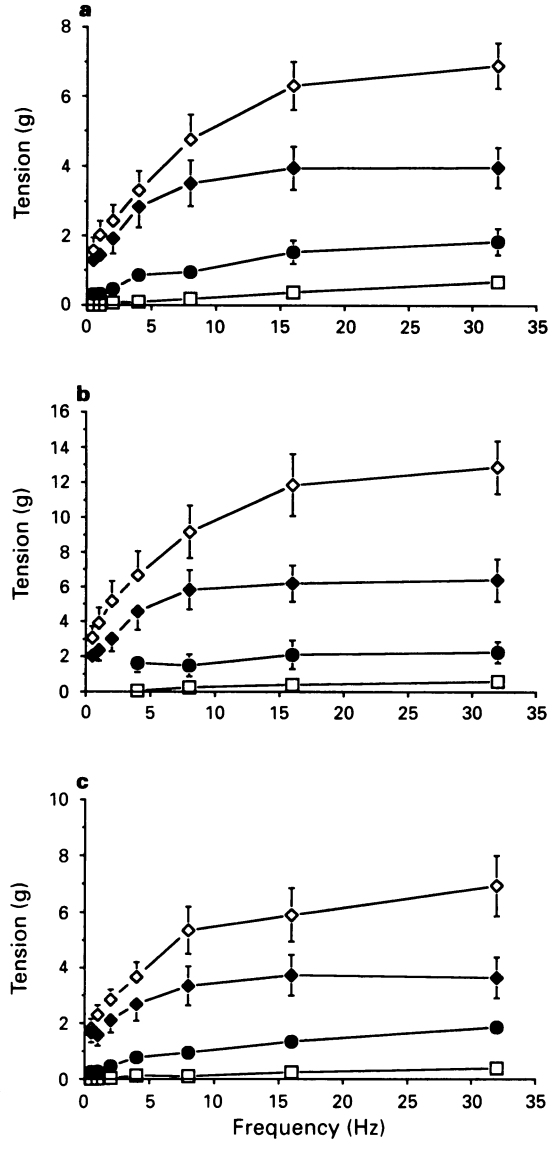

Figure 4 Influence of diabetes mellitus (b) and sucrose-consumption (c) on frequency-response curves of rat bladder body strips after cumulative addition of different antagonists; (a) control: (◇) no drug; (◆) atropine 1 μM, 15 min; (●) atropine + α,β-methylene ATP 100 μM, 15 min; (□) tetrodotoxin 1 μM 15 min. Each point represents the mean ± s.e.mean ($N = n = 7$ or 8).

methylene ATP, and there were no differences in the IC_{50} values for TTX, whether used in the absence or presence of antagonists. Nor were there any differences in TTX IC_{50} values between strips from control, diabetic, or sucrose-drinking rats.

Strength-duration curves

Bladder strips from diabetic rats responded to five pulses of field stimulation at significantly lower voltages and pulse widths than did those of control or sucrose-drinking rats (Figure 7). At 0.1 ms width, 43.2 ± 2.6 V were required to elicit a contraction in strips from control rats ($N = 12$, $n = 36$), 31.5 ± 2.8 V for diabetics ($N = 13$, $n = 39$; $P = 0.003$ vs. controls, $P = 0.058$ vs. sucrose), and 39.4 ± 3.1 V for sucrose-drinking rats ($N = 12$, $n = 36$; $P = 0.35$ vs. controls). Addition of α,β-methylene ATP and TTX caused significant shifts of rat bladder strength-duration curves to the right, while atropine and indomethacin had no effects on the strength-duration curves (Figure 7). No differences were

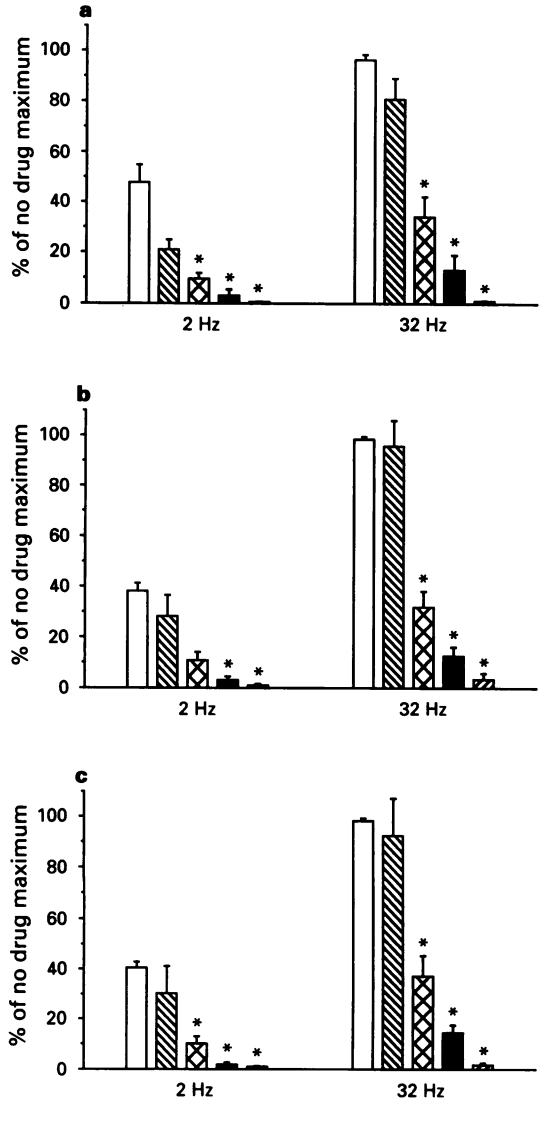

Figure 5 Influence of diabetes mellitus (b) and sucrose-consumption (c) on responses of rat urinary bladder strips to electrical stimulation after cumulative addition of different antagonists; (a) control: no drug (open columns); indomethacin 10 μM, 60 min (left hatched-columns); indomethacin + atropine 1 μM, 15 min (cross-hatched columns); indomethacin + atropine + α,β-methylene ATP 100 μM, 15 min (solid columns); TTX 1 μM, 15 min (right hatched columns). Data are expressed as percentage of no drug maximum. Each bar represents the mean ± s.e.mean ($N = n = 6$–9). *Significantly different from the no drug response ($P < 0.05$).

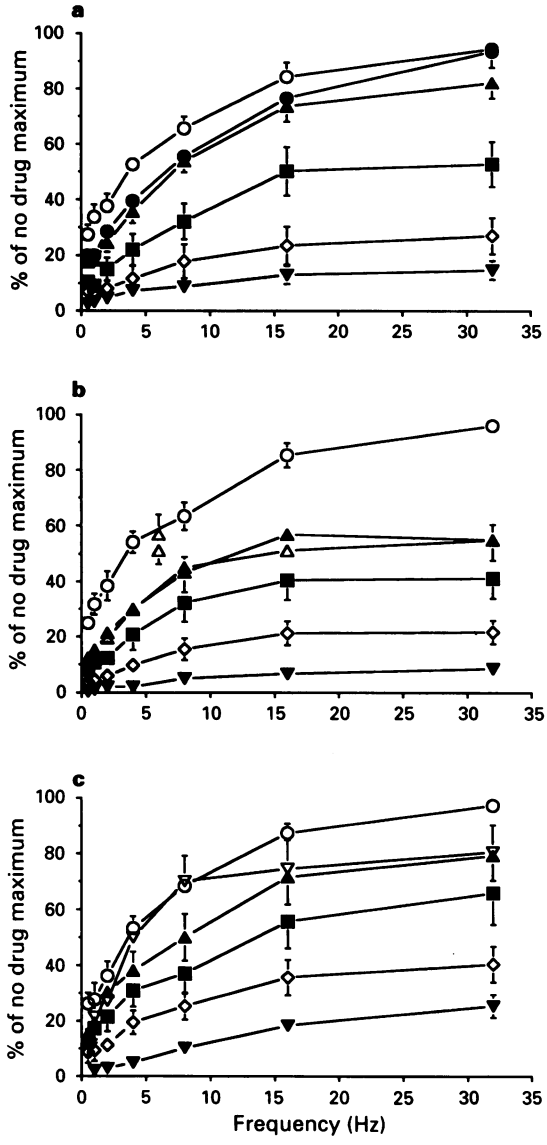

Figure 6 Effects of increasing tetrodotoxin (TTX) concentrations on the frequency-response curves of control rat bladder body strips. Data are expressed as percentage of the maximum no drug response. Each point represents the mean ± s.e.mean ($N = n = 5$–9). (a) (○) No drug; (●) time curve; (▲) TTX 20 nM; (■) TTX 80 nM; (◇) TTX 160 nM; (▼) TTX 320 nM. (b) (○) No drug; (△) atropine 1 μM, 15 min; (▲) atropine + TTX 20 nM; (■) atropine + TTX 80 nM; (◇) atropine + TTX 160 nM; (▼) atropine + TTX 320 nM. (c) (○) No drug; (▽) α,β-methylene ATP 100 μM, 15 min; (▲) α,β-methylene ATP + TTX 20 nM; (■) α,β-methylene ATP + TTX 80 nM; (◇) α,β-methylene ATP + TTX 160 nM; (▼) α,β-methylene ATP + TTX 320 nM. Curves generated in the presence of 10, 40 and 640 nM TTX have been omitted for clarity.

noted in the responses of bladders strip from control, diabetic, or sucrose-drinking rats in the sensitivity of the strength-duration curves to antagonists.

Responses to increasing voltage or pulse width

The increased spontaneous activity and increased sensitivity to electrical stimulation of the strips from diabetic rats during construction of the voltage-duration curves led us to investigate the responses to increasing voltage and pulse width more closely. Diabetes caused a significant shift of the voltage-response curve upwards and to the left compared to the two control groups, indicating that the strips were more excitable (Figure 8). The responses of the strips from diabetics were significantly greater than those of controls at all voltages studied. There were no differences between the responses of strips from sucrose-drinking and control rats.

The effects of increasing pulse width while stimulating at 32 Hz and 100 V were also determined. At 0.05 and 0.1 ms pulse width the contractile responses of strips from diabetics were significantly greater than those from control or sucrose-drinking rats (Figure 9). At all pulse widths the contractile responses of strips from sucrose-drinking rats were the same as those of controls. Incubation with TTX almost completely suppressed the response to stimulation at widths less than 0.2 ms, and at these widths there were no differences in response between the three groups. Increasing the pulse width to 1.0 ms duration in the presence of TTX caused significantly greater responses of strips from diabetics (4.18 ± 0.60 g, $N = n = 8$) than those from control (2.40 ±

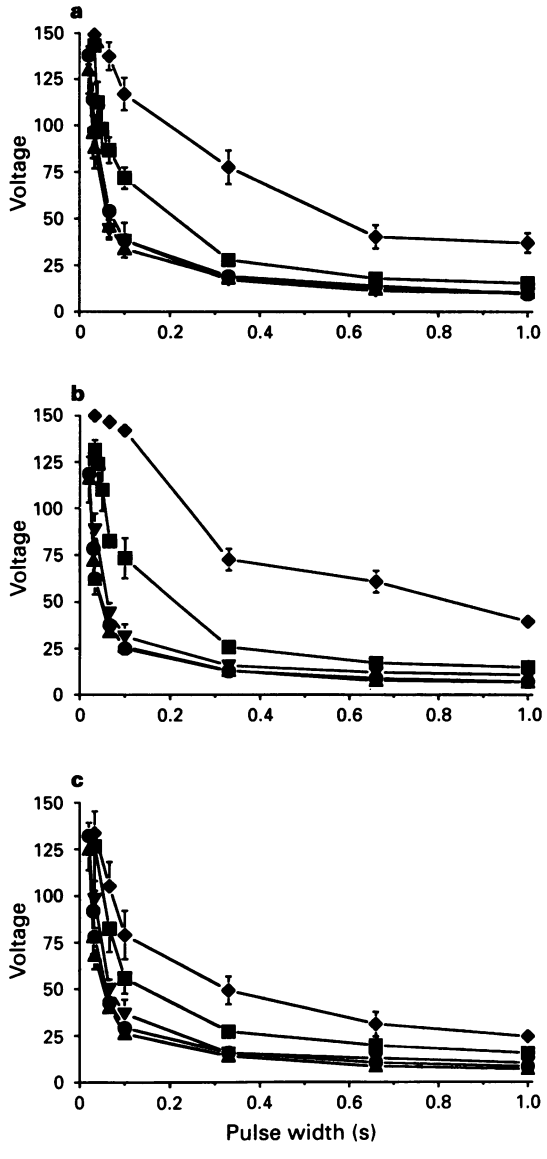

Figure 7 Influence of diabetes mellitus (b) and sucrose-consumption (c) on strength-duration curves of rat urinary bladder body strips; (a) control: (●) no drug; (■) α,β-methylene ATP 100 μM, 15 min; (▲) indomethacin 10 μM, 60 min; (◆) tetrodotoxin 1 μM, 15 min; (▼) atropine 1 μM, 15 min. Each point represents the mean ± s.e.mean of response to five stimuli at 1 Hz ($N = n = 5-15$).

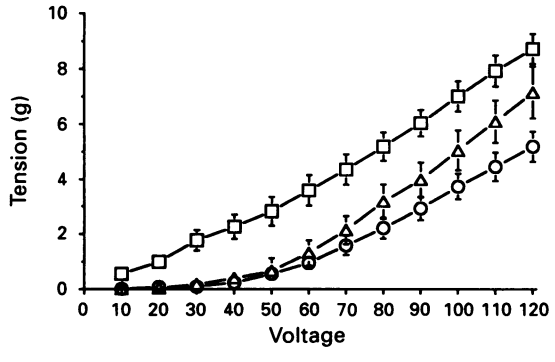

Figure 8 Influence of diabetes mellitus and sucrose-consumption on the contractile response of rat bladder body strips to ten pulses at increasing voltage during stimulation at 32 Hz and 0.05 ms width: (○) control; (□) diabetic; (△) sucrose. Each point represents the mean ± s.e.mean ($N = n = 5-12$). Responses of strips from diabetic rats were significantly greater than those of controls at all voltages studied ($P < 0.05$).

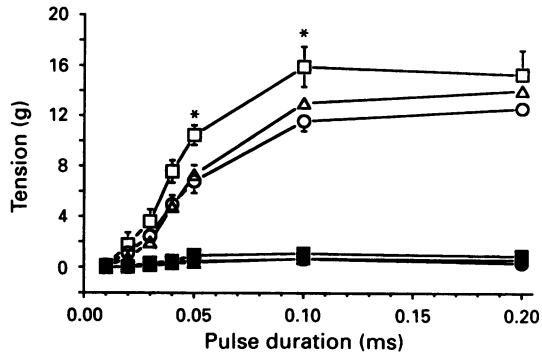

Figure 9 Influence of diabetes mellitus and sucrose-consumption on the contractile response of rat bladder body strips to ten pulses with increasing width at 32 Hz and 100 V. Open symbols are in the absence, and closed symbols in the presence of tetrodotoxin TTX (1 μM, 15 min): (○, ●) control; (□, ■) diabetic; (△, ▲) sucrose. Each point represents the mean ± s.e.mean ($N = n = 8$). *Significantly different from response of control bladder strips ($P < 0.05$).

0.32 g, $N = n = 8$) or sucrose-drinking rats (2.88 ± 0.19 g, $N = n = 8$).

Discussion

Several laboratories have shown that bladders from diabetic rats respond to field stimulation and contractile agents with greater responses than those of controls (Latifpour *et al.*, 1989; Longhurst *et al.*, 1991). The non-specificity of this increased responsiveness implies that the mechanism is unlikely to be the result of changes in specific receptors, but rather, could result from increases in bladder excitability or post-receptor events. The findings of the current study suggest that bladders from diabetic rats are more excitable and therefore more sensitive to membrane depolarization than are bladders from control or sucrose-drinking rats.

Previous studies from this laboratory found that bladder strips from diabetic rats responded to electrical field stimulation with significantly greater responses than those of control or sucrose-drinking rats, and this finding was confirmed in the present study. The shape of the response to field stimulation was different between groups: the phasic component, which is thought to result from ATP release, was smaller in relation to the tonic portion in bladder strips from diabetic compared to control and sucrose-drinking rats, and resembled the response obtained with control strips in the presence of the acetylcholinesterase inhibitor, physostigmine (Longhurst & Tammela, unpublished observation), suggestive of an increase in the cholinergic component. This indirect quantitative evidence supports the finding of Luheshi & Zar who found that in the presence of atropine, the non-cholinergic response was significantly smaller in diabetics than controls (Luheshi & Zar, 1990), while in the presence of 1 μM nifedipine, the cholinergic response was significantly larger (Luheshi & Zar, 1991). They postulated that this might result from early degenerative cholinergic nerve changes resulting in loss of normal control over quantal transmitter release, or alternatively a compensatory overactivity of the cholinergic component, resulting in increases in release of the cholinergic transmitter and decreases in release of the NANC transmitter(s).

Bladder strips from diabetic rats exhibited an increased amplitude of spontaneous activity and had an increased sensitivity to increasing voltage and pulse width compared to those from control and sucrose-drinking rats. The spontaneous activity was probably partially the result of prostaglandin release, as previously suggested by Maggi *et al.* (1984), since the amplitude was substantially reduced by

indomethacin. Anderson & Kohn (1978) found a non-competitive antagonism of the calcium dose-response curve in 80 mM K^+ depolarized rabbit bladder strips, and suggested that indomethacin had effects on calcium channels as well as on prostaglandin synthesis. However, other studies have shown only minor suppressant effects of 10 μM indomethacin on bladder strip responses to acetylcholine (Maggi *et al.*, 1984), carbachol (Anderson, 1982), or palytoxin (Posangi *et al.*, 1992). Additionally, indomethacin had no effects on carbachol-induced calcium uptake by rabbit bladder strips (Anderson, 1982). Bladders from diabetic rats have an increased basal prostacyclin release (Jeremy *et al.*, 1986) and bradykinin-stimulated prostaglandin $F_{2\alpha}$ release (Pinna *et al.*, 1992) compared to controls. Increased prostaglandin release could therefore account, at least in part, for the greater spontaneous activity observed in bladders from diabetic rats. However, the lack of effect of indomethacin on strength-duration and frequency-response curves indicates that increases in prostaglandin release cannot explain the increased responsiveness to electrical stimulation.

In our experiments, incubation with α,β-methylene ATP caused contraction followed by a considerable degree of spontaneous activity. This was particularly noticeable with the strips from diabetic rats, was long-lasting, and presumably the result of generation of spontaneous action potentials. Previous studies have shown that the contractile response of the urinary bladder to ATP is partially dependent on prostaglandin synthesis (Dean & Downie, 1978; Andersson *et al.*, 1980; Choo & Mitchelson, 1980). Although incubation with indomethacin had little effect on the contractile response of control bladder strips to α,β-methylene ATP, the responses of strips from diabetic and sucrose-drinking rats were significantly reduced, suggesting that the response was at least partially the result of prostaglandin synthesis. Increased prostaglandin synthesis in bladders from diabetic rats could therefore explain the changes in activity after α,β-methylene ATP treatment, as well as basal spontaneous activity.

Increases in spontaneous activity are frequently associated with changes in membrane excitability. In the vas deferens and other smooth muscles, spontaneous activity increases after denervation, and this is associated with significant decreases in the contractile response to field stimulation and non-specific increases in responses to agonists (Fleming *et al.*, 1973). This postjunctional supersensitivity is thought to be due to a reduction in the resting membrane potential (Fleming & Westfall, 1975). One explanation for the apparent increase in excitability in bladders from diabetic rats is that it could result from depolarization, causing the Em to be closer to the threshold voltage required to generate action potentials or excitatory junction potentials, similar to the mechanism proposed in the vas deferens to explain postjunctional supersensitivity (Fleming & Westfall, 1975). Electrophysiological studies have not been done on bladders from diabetics rats, therefore the presence of depolarization has not been established.

Removal of the pelvic ganglion or chemical sympathectomy causes increases in spontaneous activity of rat bladder strips, which are insensitive to TTX, but no difference in the response to field stimulation between denervated and control bladder strips (Ekström & Uvelius, 1981), and supersensitivity to autonomic agonists (Ekström, 1981; Ekström & Malmberg, 1984), which may be related to bladder distension and hypertropy. Studies of the influence of denervation or decentralization on choline acetyltransferase, which is used as a marker for cholinergic innervation, found that although initially decreased, levels increased rapidly after surgery and returned to control levels 10 to 25 days after surgery (Ekström, 1981). This suggests that measurement of choline acetyltransferase and other enzymes modulating acetylcholine synthesis, as well as evaluation of the contractile response to field stimulation, may not be very specific tests for denervation in the bladder. The possibility that diabetic neuropathy-induced changes could cause a postjunctional supersensitivity in bladders from two month-diabetic rats has seemed unlikely because of the findings of increases (Longhurst *et al.*, 1991) or no changes (Carpenter, 1983; Lincoln *et al.*, 1984; Luheshi & Zar, 1990; 1991; Paro *et al.*, 1990) in responses to electrical stimulation, and increases in the total activities of acetylchlinesterase and choline acetyltransferase per bladder (Lincoln *et al.*, 1984). However, acetylcholinestserase activity was unchanged in a study by Kudlacz *et al.* (1989). Luheshi & Zar concluded that possible mechanisms contributing to the relative increase in the cholinergic component of the response of diabetic bladder strips to electrical field stimulation were increased transmitter release, decreased inactivation, or degenerative nerve changes. They could find no difference in responses to acetylcholine in the presence of 1 μM nifedipine, and this in conjunction with the findings of increased cholinesterase activity of Lincoln *et al.* (1984), led them to conclude that decreased inactivation was unlikely to be a factor. However, if we consider that removal of the pelvic ganglia fails to affect the contractile responses of bladder strips to nerve stimulation (Ekström & Uvelius, 1981), and produces only temporary changes in choline acetyltransferase activity (Ekström, 1981), the findings of increases or no change in contractile response of bladder strips from diabetic rats to electrical stimulation, associated with variable levels of acetylcholinesterase and choline acetyltransferase, cannot be used as circumstantial evidence that two months after induction of diabetes with streptozotocin, there is no evidence of neuropathy in the bladder. The possibility remains that the increased responsiveness of bladder strips from diabetic rats to agonists could therefore result from some form of denervation supersensitivity, maybe related to diabetic neuropathy.

Strength-duration curves provide information about the general excitability of tissues. Similar to the findings of Brading & Williams (1990), atropine had little effect on the strength-duration curves of bladder strips from any group, implying that acetylcholine is not the predominant transmitter involved in the response to field stimulation. Similarly, indomethacin had little effect on the strength-duration curves. However, both atropine and indomethacin had quite significant depressant effects on the frequency-response curve. The explanation for this apparent discrepancy is probably related to the difference between transmitter release stimulated by five pulses (strength-duration) vs. 15 s stimulation in the frequency-response curves (ranging from seven pulses to 0.5 Hz to 960 pulses at 64 Hz). The amount of acetylcholine released by a small number of pulses (as seen in the strength-duration curves at at low frequencies of stimulation) is probably too low to stimulate influx of extracellular calcium or initiate pharmacomechanical coupling and cause a contraction. Apparently ATP can be released by low numbers of pulses, because the strength-duration curve is shifted to the right by α,β-methylene ATP, and the response to low frequencies of stimulation in frequency-response curves is more sensitive to α,β-methylene ATP than to atropine.

The experiments using increasing concentrations of TTX were done to determine whether acetylcholine and ATP, the presumed major transmitters responsible for field stimulation-induced contraction in the rat bladder, were released from the same nerve terminals, or whether separate cholinergic and purinergic nerves were present. Our rationale was that if the transmitters were released from the same nerve, the sensitivity to TTX blockade would be the same. Whether the strips were incubated with no drug, atropine to block muscarinic receptors and the responses to cholinergic stimulation, or α,β-methylene ATP to block the response to ATP release, the sensitivity to TTX was the same, and there were no differences between diabetics and controls. However, we cannot exclude the possibility that separate nerves releasing acetylcholine and ATP are present (different nerves with the same sensitivity to TTX could be present).

In separate experiments we looked at the influence of TTX on the contractile response to increasing pulse width. In the

absence of TTX, the response of strips from diabetics were significantly greater than those of the two control groups at 0.05 and 0.1 ms, widths at which the response was subsequently shown to be of neural origin. At 1.0 ms width in the presence of TTX, where the response was elicited by direct muscle stimulation, the response was also significantly greater in the strips from diabetics. This indicates that some of the diabetes-induced alterations in contractile responsiveness of the urinary bladder may result from myogenic changes, presumably related to alterations in calcium homeostasis, or second messenger systems. Previous studies from this laboratory examined the sensitivity of bladder strips from diabetics to calcium, and could find no differences in responsiveness (Longhurst *et al.*, 1992). However, studies by Belis *et al.* (1992) found changes in calcium channel activity in bladders from diabetic rats. Additionally, the non-specificity of the increased responses makes alterations in specific receptors an unlikely explanation.

Changes in membrane lipid content or type could alter the membrane properties of the bladder cells, and cause alterations in excitability as well as responsiveness to agonists. We previously showed that bladders from diabetic rats had a significantly lower lipid content than those of controls (Eika *et al.*, 1992). We could not distinguish between nerve-associated lipids or those associated with smooth muscle membranes, but theoretically alterations in neural lipids could alter nervous transmission and excitability. Additionally, synthesis and metabolism of phosphatidylinositol is known to be altered in diabetes (Greene *et al.*, 1988), which could have implications both for neural transmission and as a second messenger system for a number of agonists. To our knowledge, the influence of diabetes on either bladder phosphatidylinositol hydrolysis or lipid metabolism has not been investigated.

The experiments described in this paper included sucrose-drinking rats as controls for the effects of diuresis on bladder contractility. The bladders from these rats were significantly larger than those of controls, although smaller than those of the diabetics. In general, the responses of strips from sucrose-drinking rats were similar to those of the controls, rather than the diabetics. The shape of the response, and sensitivity to antagonists resembled the control response, as did the sensitivity to increasing voltage and pulse width. The data lead us to conclude that the increased responsiveness of bladders from diabetic rats to electrical stimulation results from the diabetic state rather than from the effects of diuresis-induced increases in bladder mass.

In conclusion, the present study suggests that bladders from diabetic rats are more excitable than those of control or sucrose-drinking rats, resulting in increased contractile responses to field stimulation. These effects on responsiveness to electrical stimulation do not seem to result from diuresis-induced effects on the bladder. The increased excitability could be the result of decreased nerve or bladder membrane lipids associated with diabetes mellitus, resulting in changes in resting membrane potential or other membrane effects. Additional possibilities include a diabetes-induced partial denervation which results in a form of postjunctional supersensitivity.

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