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The effect of streptozotocin-induced diabetes on cholinergic motor transmission in the rat urinary bladder

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1 The effect of streptozotocin (STZ)-induced diabetes on cholinergic motor transmission in the rat urinary bladder was investigated by recording contractile activity of detrusor strips *in vitro*.

2 The Ca^{2+}-channel antagonist, nifedipine, was found to be more effective in blocking the non-cholinergic motor transmission than P_2-purinoceptor desensitization by α,β-methylene ATP.

3 The neurogenic contractile responses to electrical field stimulation in the presence of nifedipine (cholinergic) were larger in the diabetic detrusor than in the non-diabetic controls. The potentiation of the cholinergic transmission was more evident at higher frequencies.

4 Concentration-response curves for acetylcholine were identical in detrusors from diabetic and non-diabetic animals, thus excluding a postsynaptic supersensitivity to acetylcholine being responsible for the potentiation of cholinergic motor transmission.

5 It is concluded that the potentiation of cholinergic motor transmission is due to enhanced release of acetylcholine in diabetic detrusor. Possible reasons for this enhancement are discussed in relation to diabetes.

Keywords: Streptozotocin; diabetes mellitus; urinary bladder; nifedipine; cholinergic transmission

Introduction

Dysfunction of urinary bladder is of common occurrence in human diabetics and is believed to be the result of diabetic autonomic neuropathy (Faerman *et al.*, 1971; Buck *et al.*, 1974; Ellenberg, 1980). Since the motor transmission of the mammalian urinary bladder, including that of man, is dual in nature comprising an atropine-sensitive (cholinergic) and an atropine-resistant (non-cholinergic) component (Ambache & Zar, 1970; Burnstock *et al.*, 1972; Hoyle *et al.*, 1989; Luheshi & Zar, 1990a), it is clearly of some interest to determine which of these two components is the principal target of diabetic autonomic neuropathy leading to the bladder dysfunction. An earlier investigation conducted on streptozotocin (STZ)-induced diabetic rats, an animal model for human diabetes, showed a marked decline in the non-cholinergic motor transmission, probably caused by a reduced release of the non-cholinergic motor transmitter in the diabetic animals (Luheshi & Zar, 1990b). Lincoln *et al.* (1984) reported that the cholinergic component of the motor transmission in rats was unaffected by diabetes. These workers had used atropine in their investigation to determine the cholinergic contribution, as have several other researchers in diabetic and non-diabetic animals (Sibley, 1984; Luheshi & Zar, 1987). The use of atropine for this purpose is based upon the tacit assumption that the two transmissions have identical time courses, so that the total nerve-mediated response represents the algebraic summation of the two components; therefore the elimination of the cholinergic by atropine, not only enables the non-cholinergic contribution to be accurately estimated but also allows the accurate assessment of the cholinergic contribution. However, there is no evidence to support this assumption. Indeed, a comparison of the records of contractions to electrical field stimulation (EFS) reveals a divergence in the time-course of the contraction in the presence of atropine from that in its absence (Luheshi, 1990; Zar *et al.*, 1990). Clearly these considerations make it imperative that a drug other than a muscarinic receptor antagonist should be employed to estimate the cholinergic element in the motor transmission of the detrusor. Two further procedures, namely purinoceptor desensitization and Ca^{2+}-channel inactivation by nifedipine are available for assessing the cholinergic transmission in the urinary bladder. Both procedures have been employed in the experiments to be described, in order to judge their suitability in estimating the cholinergic contribution of the nerve-evoked response in diabetic detrusor. Recent work (Luheshi & Zar, 1990c), has confirmed the earlier finding of Choo & Mitchelson (1980) that purinoceptor desensitization impairs but does not abolish the non-cholinergic motor transmission in rat isolated urinary bladder, thus casting doubt on the suitability of this procedure for assessing the cholinergic transmission in rat detrusor. The Ca^{2+}-channel antagonist, nifedipine, on the other hand has been demonstrated to block fully the non-cholinergic component while sparing the cholinergic component of transmission in the rat detrusor (Zar *et al.*, 1990). A similar conclusion was arrived at by Iacovou *et al.* (1990), using guinea-pig detrusor.

Methods

Animals

All experiments were performed on young adult male Wistar rats, 8 weeks old and weighing 210–240 g at the start of the experiment. The animals were randomly allocated to two experimental groups, a diabetic one and a corresponding control. The animals in both groups were maintained for a period of 12 weeks under precisely the same conditions. Both groups were kept in a temperature controlled room (22 ± 2°C), artificially lit from 06 h 00 min–18 h 00 min each day. The animals were fed standard laboratory diet and provided water *ad libitum*. After taking the initial weights, each rat was routinely weighed once a week and the weight recorded up to the end of the study (12 weeks).

Induction of diabetes

The rats to be made diabetic were fasted overnight and their blood glucose levels estimated. Diabetes was induced by administering a single intraperitoneal injection of 75 mg kg^{-1} STZ to each rat. Three days after the STZ treatment, the animals were fasted for 2 h before obtaining another blood sample from each animal in order to estimate the blood glucose level. This was to ensure that diabetes had been

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induced. Animals with blood glucose levels $\geqslant 16.6\,mmol\,l^{-1}$ were deemed to be diabetic and therefore suitable for the study. The blood glucose levels of the diabetic rats were thereafter monitored at regular intervals. The control animals were treated in an identical fashion except that saline was injected instead of STZ. Both groups of animals were then maintained for 12 weeks.

Experimental procedures

At the end of the allocated period for maintenance (12 weeks) the animals were killed by concussion and decapitation. Final blood samples were collected for estimation of their glucose contents.

Preparation of the detrusor strip The strip was prepared according to the procedure of Ambache & Zar (1970), as subsequently modified by Zar *et al.* (1990). After killing the animal the lower abdomen was opened and the bladder exposed. The urine present in the bladder was withdrawn into a 5 ml syringe and its volume recorded. The bladder was then held at its apex slightly stretched and the investing layers of serosal coat, connective tissue and accompanying blood vessels were cut away as close as possible from the outer surface of the bladder wall. The bladder was excised by a cut above the trigone and rapidly weighed. The bladder was then placed in a Petri dish and washed with several changes of Krebs-Henseleit solution. The bladder was opened by two lateral incisions and then unfolded to give a rectangular sheet of tissue. The unfolded tissue was laid on Krebs-soaked tissue paper and strips of bladder 1–1.5 cm long and 0.2 cm wide were cut with a pair of fine scissors. The strip preparation of the detrusor was suspended in a 1 ml jacketed organ bath between built-in vertical platinum electrodes, at a resting tension of 0.5 g. The resting tension was kept constant at 0.5 g throughout the experiment by appropriate adjustments whenever needed. The preparation was maintained at 37°C in Krebs-Henseleit solution gassed in the reservoir and in the organ bath with 95% O_2 plus 5% CO_2 mixture. Indomethacin 10 μM was present in the Krebs solution throughout the experiment in order to minimize the spontaneous activity of the isolated preparation. For recording the tension of the detrusor muscle, the preparation was connected to an isometric transducer and a potentiometric recorder. An equilibration period of 30 min was allowed before the start of further experimentation (see below); during this period the preparation was repeatedly washed with fresh Krebs solution.

Experiments on isolated detrusor strips In each experiment, isolated detrusor strips were used to obtain contractile responses to two types of stimuli: electrical field stimulation and acetylcholine (ACh). The two types of stimuli were not delivered to the same strip. One, or if necessary, more strips were reserved for each type of stimulus.

Electrical field stimulation The strips reserved for electrical field stimulation (EFS) were treated with either α,β-methylene ATP (α,β-MeATP) following the method of Luheshi & Zar (1990a) in order to produce purinoceptor desensitization or with nifedipine following the method of Zar *et al.* (1990). The parameters of EFS were as follows: trains of pulses: pulse duration of 0.1 ms: train duration, 10 s: frequency of 1, 2, 4, 8, 16, 32 and 64 Hz; interval between two trains of 60 s: voltage, supramaximal for evoking maximal contraction at each frequency. In experiments where the strips were treated with α,β-MeATP the maximum frequency of stimulation used was 16 Hz. The choice of parameters for EFS was based upon the satisfaction of two criteria: first, reproducibility of the response at each frequency of stimulation and second, total abolition of the response by tetrodotoxin, 0.5 μM. Fulfilment of both criteria was deemed to imply that EFS was supramaximal and the EFS-evoked responses were neurogenic. In experiments using nifedipine, EFS was started after allowing a 30 min exposure to nifedipine and during its continued presence for the rest of the experiment. The chosen concentration of nifedipine (1 μM) was based upon previous observations (Zar *et al.*, 1990) where the responses to EFS were maximally depressed by nifedipine 1 μM and nifedipine in higher concentrations was no more effective than nifedipine 1 μM.

Construction of dose-response curves to acetylcholine The object of the experiments was to compare the ability of nifedipine-treated detrusor strips from the two groups of animals, diabetic and control, to generate tension in response to ACh. The strips were exposed to nifedipine 1 μM for 30 min before obtaining concentration-response relationships for ACh. ACh was added to the bath in a concentration range of 10^{-8}–10^{-3} M, in a non-cumulative manner. Exposure to a dose of ACh was maintained until the maximal response to that concentration was obtained. The preparation was then repeatedly washed with several changes of nifedepine containing Krebs-solution. The preparation was allowed to relax fully before exposing it to the next higher concentration of ACh.

Statistics

At the end of each experiment, the bladder strip was detached from the recording set up, blotted dry and weighed. In order to render the results comparable from one strip to another and from strips of different animals, the tension generated by the strip and the weight of the strip were used to calculate the tension per 100 mg bladder tissue.

All values are expressed as mean ± s.e.mean and the statistical significance was calculated by Student's t test.

Materials

The drugs used and their sources were: nifedipine (Bayer), physostigmine sulphate (Burroughs Wellcome), tetrodotoxin (Sankyo), acetylcholine chloride, atropine sulphate, indomethacin, streptozotocin, α,β-MeATP (Sigma). Solutions of drugs were made fresh on the day of their use with the exception of tetrodotoxin the stock solution of which was stored at −20°C.

Preparation of drug solutions

Streptozotocin: this was dissolved immediately prior to its injection in 50 mM sodium citrate buffer and titrated to pH 4.5 with HCl. The drug solution was kept on ice at all times before use.

Nifedipine: this drug was dissolved in absolute ethanol to make a solution of 10^{-3} M.

Indomethacin: absolute ethanol was also used to dissolve this drug to make a solution of 10^{-2} M.

All other drugs were dissolved in distilled water.

Nifedipine is very light-sensitive, specially in solution and is degraded to inactive metabolites when exposed to light of wavelength less than 450 nm which covers daylight and ordinary fluorescent laboratory light. All experiments involving this drug were therefore carried out in a laboratory illuminated solely with a sodium vapour lamp.

Composition of Krebs-Henseleit solution

The composition in mM was: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.

Estimation of blood glucose level

Periodic routine estimation was carried out by taking a drop of blood from the tail vein of the rat and estimating its glucose level with BM-Test-Glycemie strips (Boehringer-Manheim).

Table 1 Body weight, blood glucose concentration, weight of bladder and volume of residual urine in diabetic and control rats

	Controls	*Diabetic*
Body weight (g)		
Initial	219 ± 8	215 ± 8
Final	387 ± 15	182 ± 9***
Blood glucose ($mmol\,l^{-1}$)	4 ± 0.2	27 ± 2.1***
Residual urine in bladder (ml)	0.5 ± 0.08	3.9 ± 0.3***
Bladder weight (mg)	91 ± 7	805 ± 42***

All values are means ± s.e.mean ($n = 8$). The values in diabetic animals have been compared with their corresponding controls and the level of significant difference is indicated by *** $P < 0.001$. Volume of residual urine represents the volume of urine present in the bladder immediately after exsanguination of the animal.

The final estimate of blood glucose was carried out after the animal had been killed. Blood samples and glucose standards (50 μl) were collected into ice cold 0.016% uranyl acetate (1 ml). The mixture was agitated, centrifuged at 800 g and the supernatant assayed for glucose spectrophotometrically by the standard glucose oxidase method with the 'GOD-PAP' assay kit (Boehringer-Ingelheim, London).

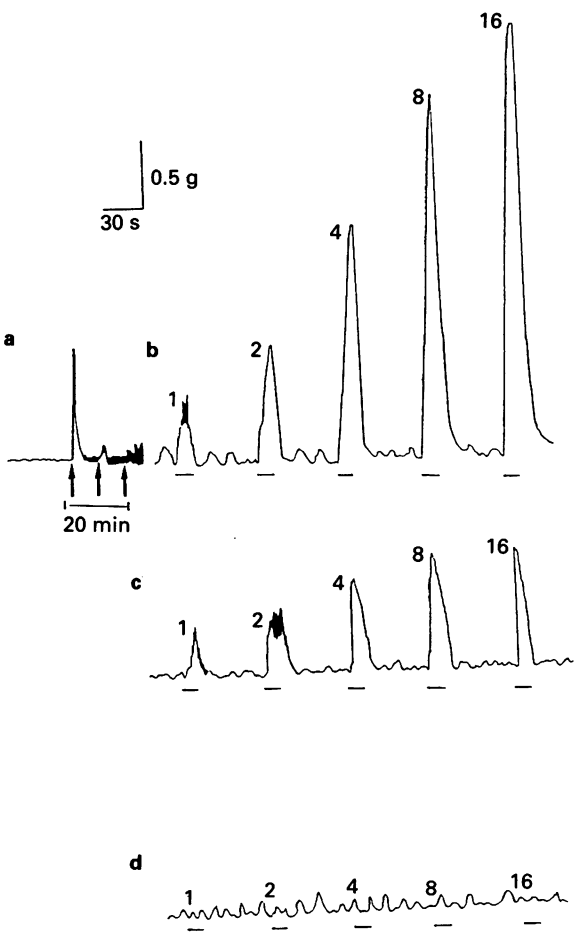

Figure 1 Isolated detrusor from a streptozotocin (STZ)-diabetic rat: a typical experiment showing responses to electrical field stimulation following desensitization to the contractile action of α,β-methylene ATP (α,β-ATP) first without any drugs (b) and then in the presence of atropine (c) followed by atropine 3 μM + tetrodotoxin (TTX) 0.5 μM (d). Atropine was introduced into the bath 20 min before (c) and TTX 5 min before (d). Electrical field stimulation: 10 s bursts of stimuli, duration indicated by bars and frequency (1–16 Hz) by subscripts. Desensitization to α,β-MeATP was obtained by three administrations of the drug (marked by arrows) in 10 μM concentrations (a).

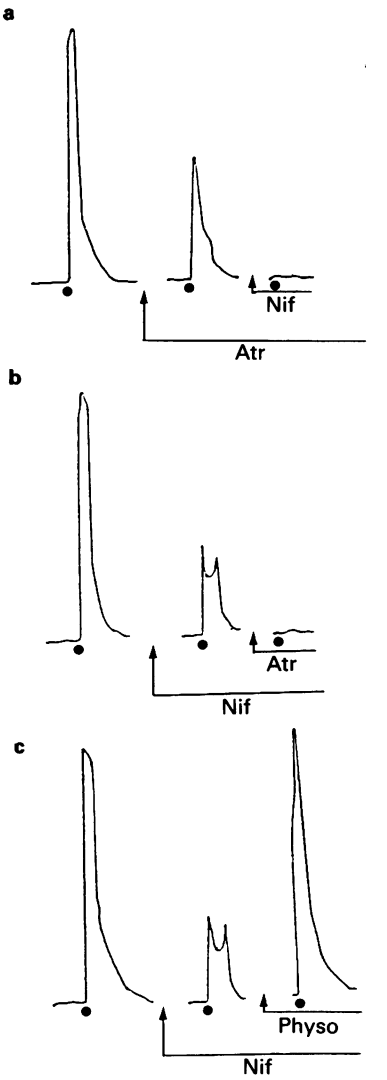

Figure 2 Isolated detrusor of diabetic rat: contractile responses of three detrusor strips, (a), (b) and (c) from the same animal to electrical field stimulation (●, 10 s bursts of stimuli at 8 Hz). In each strip, the response in the left panel represents the control response, the response in the middle panel was obtained following 30 min exposure to atropine, 3 μM (Atr) or nifedipine 1 μM (Nif) while the response in the right panel was evoked following a 30 min exposure to nifedipine (a), atropine (b) or physostigmine 1 μM (Phys; c).

Results

Body weight and blood glucose levels

The animals in the diabetic group, in contrast to their corresponding controls showed no gain in weight over the course of the investigation. The control animals made significant weight gains during the same period (Table 1).

As was to be expected, the blood glucose levels in the STZ-treated diabetic animals were extremely high when compared to the untreated control rats (Table 1) and remained so throughout the duration of the investigation.

Bladder weight and residual urine

By the end of the investigation the weight of the whole urinary bladder in the diabetic group was significantly higher than in

controls. Similarly, the volume of residual urine (VRU) i.e. the volume of urine present in the bladder after the death of the animal was also considerably higher in the diabetic animals (Table 1).

Contractile responses to electrical field stimulation

Two sets of preliminary experiments, one employing purinoceptor-desensitized detrusor strips and the second using the calcium channel antagonist nifedipine, were conducted to determine the optimal method for eliminating the non-cholinergic motor transmission.

Purinoceptor desensitized preparations In these experiments the effectiveness of purinoceptor desensitization in abolishing the non-cholinergic motor transmission of the diabetic detrusor was investigated. The record of one such experiment is shown in Figure 1. Following desensitization of the detrusor strip to the contractile action of α,β-MeATP (Figure 1a), the strip was subjected to EFS at frequencies of 1, 2, 4, 8 and 16 Hz. The field stimulation evoked slow, sustained and frequency-dependent contractions which were maintained throughout the duration of the electrical stimulus (Figure 1b). Repeating the cycle of stimulation following exposure to atropine 3 μM for 30 min and in its continued presence resulted in the partial suppression of the contractile responses but at no frequency of stimulation was the response completely abolished (Figure 1c). The responses, resistant to α,β-MeATP desensitization and to atropine were completely abolished by exposure to TTX (0.5 μM) indicating that such responses were neurogenic (Figure 1d).

Nifedipine-treated preparations In the second set of preliminary experiments, nifedipine was used to test whether it would totally abolish the non-cholinergic component of the motor transmission in bladder strips from diabetic rats, as it did in detrusor strips from normal animals (Zar *et al.*, 1990). These experiments involved stimulating the preparations with 10 s bursts of stimuli at 8 Hz. Records from a typical experiment are shown in Figure 2. The results show that nifedipine 1 μM completely abolished the atropine-resistant component of the motor transmission (Figure 2a); nifedipine partially blocked the EFS-evoked response of the non-atropinized preparation and the nifedipine-resistant component was fully blocked by atropine, 3 μM (Figure 2b). The nifedipine-resistant component was greatly augmented by physostigmine 1 μM (Figure 2c).

The effect of diabetes on cholinergic motor transmission in the rat detrusor in presence of nifedipine in order to eliminate non-cholinergic transmission

In light of the results of the foregoing preliminary experiments, the detrusor strips from diabetic and control animals were contracted with 10 s bursts of stimuli at frequencies of 1, 2, 4, 8, 16, 32 and 64 Hz in the presence of nifedipine 1 μM. The field stimulation produced responses which increased with increasing frequency in the detrusor strips taken from both diabetic as well as control rats. Figure 3 is the record of two typical experiments showing the tension generated by the detrusor strips in response to EFS from a control (a) and a diabetic (c) animal following incubation with nifedipine 1 μM. The electrical stimulus was then repeated at the same frequencies, this time in the presence of atropine (3 μM) as well as nifedipine. As can be seen from Figure 3 (b and d) the introduction of atropine completely abolished the nifedipine resistant response confirming its cholinergic nature.

When compared to the tensions generated by detrusor strips from the control animals, the nifedipine-resistant

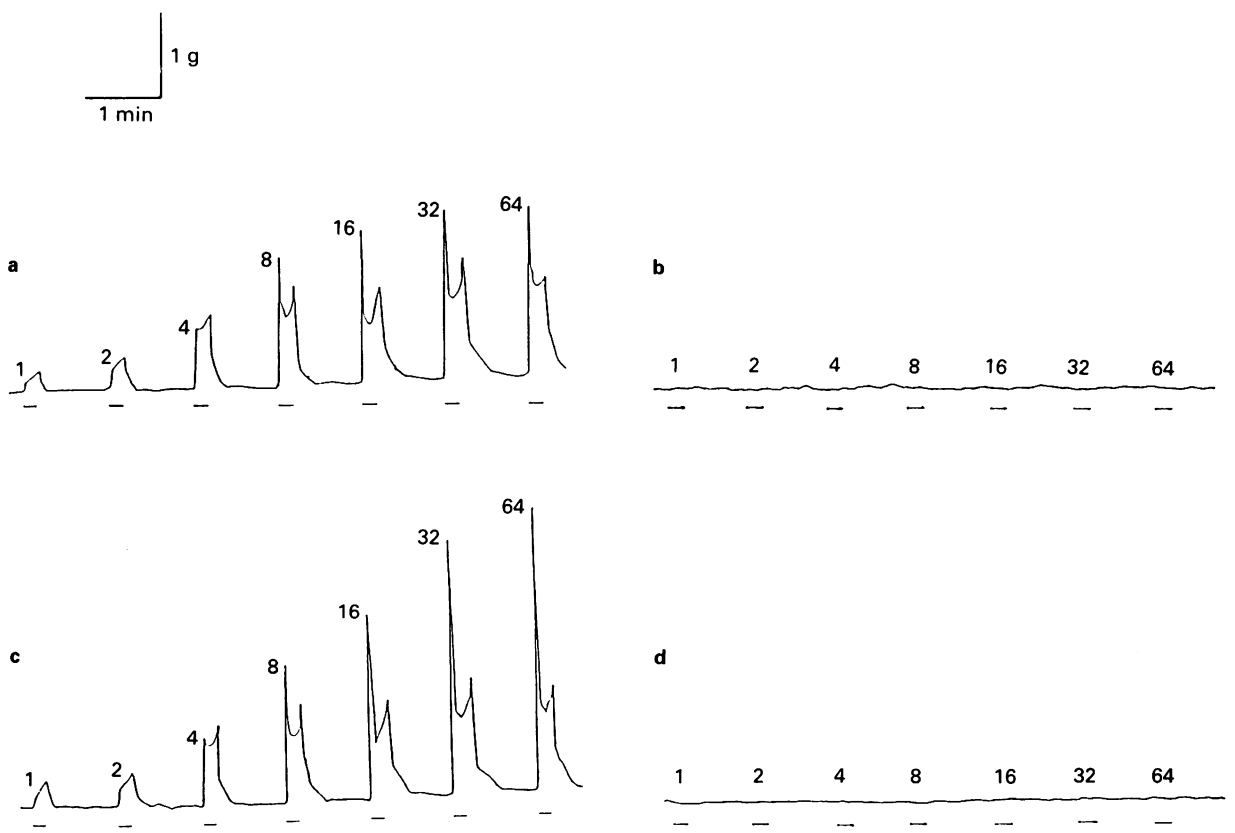

Figure 3 Isolated detrusor strips from a control (above) and diabetic (below) rat: two typical experiments showing contractile responses to electrical field stimulation in the presence of nifedipine, 1 μM. The frequency of pulses within each train is given by the subscripts (1–64 Hz) and the duration of each stimulus burst is indicated by a horizontal bar. The responses to electrical field stimuli were evoked in the absence (a, c) or presence of atropine 3 μM. Note that the peak tension generated by the 'diabetic' detrusor was substantially greater than the control detrusor and both were blocked by atropine.

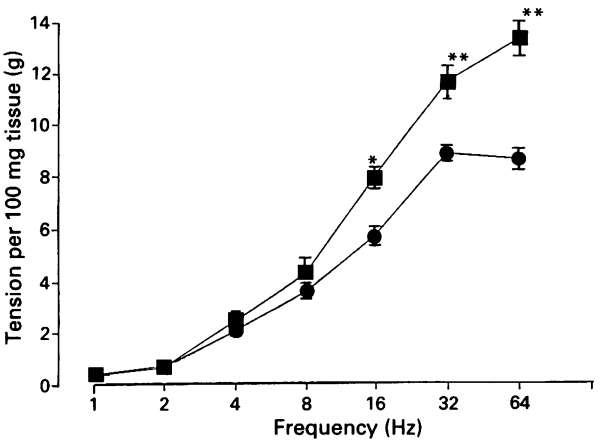

Figure 4 Cholinergic contractile responses of detrusor from diabetic (■) and non-diabetic control (●) rats to electrical field stimulation. All values are means (s.e.mean shown by vertical bars; $n = 8$). Nifedipine 1 μM was present throughout the duration of each experiment. * $P \leq 0.05$ and ** $P \leq 0.01$ indicate the degree of significant difference from the non-diabetic control value.

(cholinergic) components in strips from STZ-diabetic animals generated greater tension (Figure 3). This difference was statistically significant at frequencies of 16 Hz and above and was most evident at the highest two frequencies, 32 and 64 Hz, (Figure 4). In addition to the difference in the magnitude of tension generated, the responses of the strips from the control animals tended to peak at a frequency of 32 Hz while those produced by the strips from the diabetic animals continued to rise up to the maximum frequency of 64 Hz used in this investigation.

Contractile response to acetylcholine

ACh, 10^{-7}–10^{-3} M produced contractions that increased with increase in the dose of the drug. The tension generated by the detrusor strips from both sets of animals in response to ACh was very similar in magnitude and there was no significant difference evident between the 2 groups of animals at any concentration of ACh (Figure 5).

Discussion

There are sound reasons to believe that the animals treated with STZ in the present investigation were fully diabetic. Thus the data shown in Table 1 demonstrating the failure of the STZ-treated rats to gain weight coupled with the highly significant rise in blood glucose levels in these animals are strongly indicative of the successful induction of diabetes. Similarly the increase in bladder weight and in the volume of residual urine observed in the STZ-treated animals when compared to the controls (Table 1) is also consistent with changes observed by other workers using STZ-treated rats as animal models for human diabetes (Kolta *et al.*, 1985; Longhurst & Belis, 1986; Moss *et al.*, 1987; Santicioli *et al.*, 1987; Steers *et al.*, 1990). These changes in the bladder mass of diabetic animals parallel a rise in protein concentration (Longhurst & Bellis, 1985) and have been previously attributed to diuresis (Goss *et al.*, 1973), or to damage of sensory or motor nerves (Buck *et al.*, 1974; Ekstrom & Uvelius, 1981).

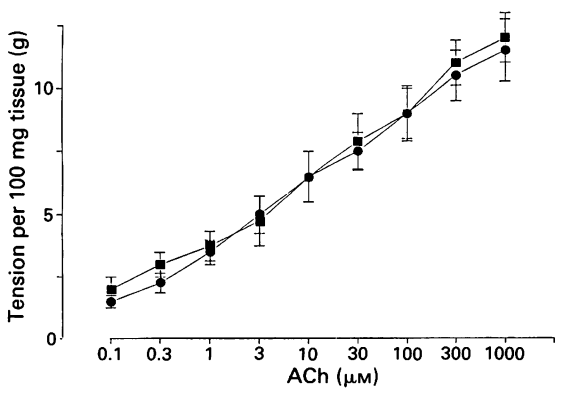

Figure 5 Concentration-response curves of acetylcholine (ACh) in detrusor strips from diabetic (■) and non-diabetic control (●) rats. All values are means and vertical bars indicate s.e.mean ($n = 8$). Nifedipine 1 μM was present throughout the duration of each experiment. Note that there is no significant difference between the two curves at any concentration of ACh.

It is quite obvious from the results of experiments with α,β-MeATP that purinoceptor desensitization as a method for isolating the cholinergic component of the motor transmission in the diabetic detrusor is less than satisfactory. The results of an earlier investigation on detrusor from non-diabetic rats had also led to a similar conclusion regarding the ineffectiveness of purinoceptor desensitization in inducing a full extinction of non-cholinergic transmission in this organ (Luheshi & Zar, 1990c). In the non-diabetic detrusor, nifedipine, unlike purinoceptor desensitization, has been shown to be highly effective in its selective inhibition of the non-cholinergic motor transmission (Zar *et al.*, 1990). From the results obtained in the present study, there seems to be little reason to doubt that the effect of nifedipine on motor transmission is the same in the STZ-diabetic detrusor as in the non-diabetic one. As was shown in the detrusor strips taken from the bladders of non-diabetic rats (Zar *et al.*, 1990), treatment with nifedipine drastically reduced the amplitude of the EFS-evoked response in the diabetic detrusor as well. Also in common with the findings in non-diabetic detrusor, the nifedipine-resistant response of the diabetic detrusor was potentiated by the anticholinesterase physostigmine and was abolished by atropine. It seems, therefore, reasonable to conclude that the nifedipine-resistant response of the diabetic detrusor to EFS was mediated entirely through cholinergic mechanisms.

The finding that the EFS-evoked cholinergic response generated significantly greater tension in diabetic detrusor compared to the control is unexpected, as well as puzzling, since clinical diabetes mellitus is frequently associated with a 'failing' detrusor function (Fagerberg *et al.*, 1967; Kahan *et al.*, 1970; Buck *et al.*, 1974; Bradley, 1980; Ellenberg, 1980). In theory, the enhanced nerve-mediated cholinergic response of the diabetic detrusor must have originated in either an augmented sensitivity of the detrusor to released ACh, or to an augmented ACh release, or to a combination of both of these factors. That this apparent 'good health' of the cholinergic response in the diabetic detrusor is not attributable to a postsynaptic exaggerated responsiveness or supersensitivity to ACh is shown by a lack of any significant change in the ACh concentration-response curve in diabetic detrusor compared to the control. This conclusion seems to be broadly in line with the findings of other workers regarding ACh responsiveness of the diabetic detrusor which have been somewhat variable, with reports of increased contractile responses (Kolta *et al.*, 1985), decreased contractile responses (Longhurst & Belis, 1986), or no change in contractile responses (Lincoln *et al.*, 1984). It seems likely that such differences in the reported ACh responsiveness might be a reflection of the state of innervation of the detrusor which in its turn would depend upon the severity and duration of experimental diabetes.

Having thus disposed of the possibility of a postsynaptic mechanism underpinning the enhanced cholinergic response to EFS, one is left with the unavoidable conclusion that the concentration of ACh in the synaptic cleft following EFS was higher in detrusor preparations from diabetic rats. The single most important factor determining the transmitter-concentration in the synaptic cleft is, without doubt, the quantum of the neurotransmitter released in response to the stimulus. Another factor contributing to a rise in transmitter-

concentration within the synaptic cleft might be a diminished rate of ACh inactivation in and around the synaptic cleft in the detrusor of diabetic animals. The latter possibility seems unlikely since tissue cholinesterase activity in diabetic bladder is reported to be higher than in controls (Lincoln *et al.*, 1984). Also the fact that the sensitivity to ACh remained unaltered, speaks against a diminution of cholinesterase activity in the diabetic detrusor. It follows therefore that the likeliest cause of the enhanced nerve-mediated cholinergic response in the diabetic detrusor is a greater than normal release of ACh in response to EFS. The results of the present investigation do not throw any light on the mechanism responsible for this enhanced release of transmitter in response to EFS. However it may be hypothesized that this phenomenon is an early sign of the onset of degenerative changes in the cholinergic nerves initiated by diabetes and that these pathological changes may cause the nerves to lose their normal control over the quantum of transmitter released in response to EFS. An alternative explanation could be that the increase in the cholinergic response is a result of a hypothetical compensatory mechanism by the cholinergic component for the progressive failure of the non-cholinergic component of the motor transmission in diabetic detrusor, evidence of which was presented earlier (Luheshi & Zar, 1990b). It seems rational therefore to assume that the cholinergic and the non-cholinergic components of the motor transmission in the detrusor act in concert with each other so that a partial failure of one (in this case the non-cholinergic) would trigger a compensatory enhancement of the other (the cholinergic).

In conclusion, the present study has shown that the Ca^{2+}-channel antagonist, nifedipine is more effective than purinoceptor-desensitization by α,β-MeATP in abolishing the non-cholinergic motor transmission of the diabetic rat detrusor. Furthermore, induction of diabetes causes a presynaptically-mediated augmentation of the cholinergic motor transmission in rat detrusor.

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