Br. J. Pharmacol. (1993), **109**, 1276–1281

Altered cardiac adrenergic neurotransmission in streptozotocin-induced diabetic rats

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1 Functional alterations of the sympathetic neuroeffector junction of the left atria were studied in rats with streptozotocin-induced diabetes.

2 Eight to 12 weeks of diabetes resulted in a marked decrease in the positive inotropic response of left atria to electrical field stimulation (EFS).

3 The overflow of [^{3}H]-noradrenaline from diabetic left atria caused by EFS was much less than that from control preparations.

4 The concentration-response curves showed no change in sensitivities of the left atria to exogenous noradrenaline and tyramine in diabetic rats. The maximum positive inotropic response to these agents were similar in diabetic and control animals.

5 The left atrial content of noradrenaline was not significantly changed in diabetic rats. The cocaine-sensitive uptake of [^{3}H]-noradrenaline was also unaltered.

6 Atropine enhanced the positive inotropic response and [^{3}H]-noradrenaline overflow induced by EFS in control left atria. Similarly, yohimbine caused an enhancement of EFS-evoked inotropic response in control atria. However, these effects of the antagonists were not observed in diabetic left atria.

7 It is concluded that the decrease in the positive inotropic response of the left atria to EFS in diabetic rats is caused by an impairment of noradrenaline release from the sympathetic nerve terminals through a calcium-dependent exocytotic mechanism. The present results also indicate that presynaptic α_2-adrenoceptors and muscarinic receptors that are linked to inhibition of the noradrenaline release during nerve stimulation may be functionally impaired in diabetic animals.

Keywords: Diabetes; heart; electrical field stimulation; positive inotropic effect; noradrenaline release; presynaptic inhibitory receptors

Introduction

Autonomic neuropathy is a significant complication of diabetes and is considered to be responsible for orthostatic hypotension, skin ulceration, arterial calcification and abnormal temperature regulation (Sharpey-Schaffer & Taylor, 1960; Moorhouse *et al.*, 1966; Goebel & Fuessl, 1983; Edomonds *et al.*, 1986). Clinical studies have revealed that there is less orthostatic increment in noradrenaline release in diabetic patients (Cryer & Weiss, 1980; Hilsted, 1982).

Ultrastructural study has revealed many abnormal, degenerated adrenergic nerve profiles in diabetic right atria (Tomlinson & Yusof, 1983). Furthermore, a decreased noradrenaline content in the heart of diabetic patients (Neubauer & Christensen, 1976) and reduced noradrenaline turnover in diabetic rat hearts (Yoshida *et al.*, 1985) have been reported. Functional studies have shown that the positive chronotropic and inotropic responses to sympathetic transmural nerve stimulation were markedly attenuated in diabetic rat atria (Sato *et al.*, 1989; Hashimoto *et al.*, 1990).

On the other hand, cardiac noradrenaline levels have been shown to increase in diabetes (Paulson & Light, 1981; Fushimi *et al.*, 1984). Ganguly *et al.* (1986) have demonstrated that cardiac noradrenaline in the diabetic rat heart is maintained at a higher level by an increased synthesis and uptake of noradrenaline in the adrenergic nerve terminals and have indicated higher sympathetic activity in the diabetic myocardium. It is possible that elevated noradrenaline levels may result in decreased density of β-adrenoceptors and defective β-adrenoceptor-adenylate cyclase coupling (Savarese & Beikowitz, 1979; Heyliger *et al.*, 1982; Smith *et al.*, 1984; Atkins *et al.*, 1985). Therefore, it remains uncertain whether the decreased response of diabetic rat atria to transmural nerve stimulation results from a decreased noradrenaline release from the sympathetic nerve terminals or from a decreased postsynaptic response to noradrenaline.

The purpose of the present study was to examine the function of the sympathetic neuroeffector mechanism in the left atria from rats with streptozotocin-induced diabetes. The aim was to determine how diabetes affects the responsiveness of cardiac muscle to endogenously released noradrenaline and to determine how diabetes affects the content, release and uptake of noradrenaline in cardiac sympathetic nerves.

Methods

Induction of diabetes

Male Wistar rats, 16 weeks old, weighing 450–520 g, were anaesthetized with diethyl ether and received a single intravenous injection of streptozotocin, 45 mg kg^{-1}, via the tail vein. Streptozotocin was dissolved in a citrate solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.5). Control rats received the citrate buffer solution alone. All rats were fed on normal rat chow and water *ad libitum* until they were used. On the day of the experiment, a blood sample was collected and serum glucose level was determined by Rapid Blood Analyzer Super using Uni-Kit (Chugai, Tokyo, Japan). All animals injected with streptozotocin were severely hyperglycaemic (range 473–865 mg dl^{-1}). The general features of diabetic rats and age-matched control animals were summarized in Table 1.

Organ bath experiments

Eight to 12 weeks after treatment, rats were anaesthetized with diethyl ether. The hearts were rapidly excised and transferred to dissection baths filled with oxygenated Krebs-

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Table 1 General characteristics of control and streptozotocin-induced diabetic rats

	Control	*Diabetic*
Body weight (g)	690 ± 14	381 ± 5***
Plasma glucose (mg dl^{-1})	183 ± 4	617 ± 16***
Heart weight (mg)	940 ± 86	660 ± 56***

All values are means ± s.e.mean ($n = 45$ for each group). The values in diabetic animals are compared with their corresponding controls and the level of significant difference is indicated by ***$P < 0.001$.

Henseleit solution at room temperature (composition mM): NaCl 143.9, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 10.0, pH 7.4. The left atrium was carefully dissected and mounted vertically in a 50 ml water-jacketed bath containing Krebs-Henseleit solution bubbled with 95% O_2 and 5% CO_2 at 33°C. The lower end of the left atrium was pinned on a pair of hook electrodes for stimulation and the other end was connected by a silk thread to a force transducer (Nihon Kohden, TB-612T). Isometric tension was recorded with a force-transducer and displayed on a pen recorder (Nihon Kohden, RTG-3026) through a preamplifier (Nihon Kohden, RP-5). The resting tension was adjusted to 2 g. The atria were electrically paced by rectangular pulses of 5 ms duration and an intensity of 1.5 times threshold current at 1 Hz frequency. The pulses were delivered from an electronic stimulator (Sanei-Sokki, 3F46) through an isolation unit (Sanei-Sokki, 5361). Each preparation was equilibrated for 60 min before the start of the experiments. Details of the experimental procedure have been published elsewhere (Hattori & Kanno, 1985; Hattori *et al.*, 1987).

Electrical field stimulation methods during the myocardial refractory period (which were described by Blinks, 1966), were used to stimulate sympathetic nerves. The stimuli for electrical field stimulation (EFS) were delivered by a second electrical stimulator (50 mA, 5 ms duration) through platinum electrodes placed on either side of the atrium. A graded stimulus-inotropic response curve was obtained by a stepwise increase in the number of pulses.

The concentration-response curves for the positive inotropic effects of noradrenaline and tyramine were determined in a cumulative manner by increasing the concentrations in steps of 0.5 log units.

[^{3}H]-noradrenaline efflux measurements

The left atria were incubated at 2 ml oxygenated Krebs-Henseleit solution containing 0.75 mg ml^{-1} ascorbic acid and 1.5×10^{-7} M (−)-[^{3}H]-noradrenaline (New England Nuclear, Boston, U.S.A., specific activity 41 Ci mmol^{-1}) for 15 min at 33°C and then transferred to a superfusion chamber. The atrium was pinned on the bottom of the chamber and superfused at a rate of 2.0 ml min^{-1} with Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2. The temperature of the perfusate was maintained at 33°C. The left atrium was electrically stimulated by rectangular pulses of 1 Hz frequency, 5 ms duration and 1.5 times threshold current, delivered by a pair of platinum wire electrodes connected to the electronic stimulator through the isolator. After a washout period of 90 min to establish a stable basal efflux of radioactivity, superfusate samples were collected at a 1 min interval. EFS was applied for 15 min through platinum field electrodes on either side of the atrium. At the end of the experiment the preparations were frozen with liquid nitrogen and weighed. Evaluation of EFS-evoked tritium overflow was made by calculating the difference between the overflow for 5 min before EFS and the overflow for 5 min after the start of EFS.

[^{3}H]-noradrenaline uptake measurements

[^{3}H]-noradrenaline uptake was determined according to the methods described by Williams (1991). Briefly, the left atria were preincubated in Krebs-Henseleit solution containing 0.75 mg ml^{-1} ascorbic acid, 3×10^{-5} M hydrocortisone and 10^{-5} M pargyline in the presence or absence of 3×10^{-6} M cocaine for 30 min at 33°C. Then (−)-[^{3}H]-noradrenaline (final concentration 1.5×10^{-7} M) was added to the incubation medium. After incubation for 60 min, the tissue was removed, immediately blotted with Whatman No. 5 filter paper, rinsed by placing the tissue consecutively into four beakers containing Krebs-Henseleit solution without radioligand, blotted again, and weighed. Subsequently, the tissue was treated with 1.0 ml tissue solubilizer (Solvable, New England Nuclear) for 3 to 6 h at 50°C. Finally, 0.1 ml 30% H_2O_2 was added and radioactivity in the solubilized tissue was determined. The difference in the [^{3}H]-noradrenaline accumulation in the absence and presence of cocaine was defined as cocaine-sensitive neuronal uptake.

Catecholamine assays

Each of the left atria obtained from control and diabetic rat hearts were frozen with clamps which had been cooled with liquid nitrogen. The frozen muscle samples were weighed and then homogenized in 2.5 ml of 0.4 N $HClO_4$ containing 2 mM EDTA by means of a micro homogenizer (Nition, NS 310E) for 30 s. The homogenate was centrifuged at 10,000 r.p.m. for 10 min at 4°C. The supernatant was collected and subjected to the determination of noradrenaline by a high performance liquid chromatographic procedure (Yui *et al.*, 1980).

Drugs

The drugs used were as follows: atropine sulphate, (−)-noradrenaline bitartrate and physostigmine sulphate (Wako, Osaka, Japan), cocaine hydrochloride (Takeda, Osaka, Japan), prazosin hydrochloride (Taito-Pfizer, Tokyo, Japan), (±)-propranolol hydrochloride, yohimbine hydrochloride, hydrocortisone, pargyline, and streptozotocin (Sigma Chemical, St. Louis, MO, U.S.A.). All compounds were dissolved in Krebs-Henseleit solution or distilled water with the exception of streptozotocin (see above).

Statistics

All data were expressed in terms of the mean ± s.e.mean. For statistical evaluation, Student's paired and unpaired t tests were used and a significant difference was assumed to exist if the P value was less than 0.05.

Results

The basal force of contraction of left atria isolated from diabetic rats (446 ± 29 mg; $n = 41$) was not significantly different from that of left atria from age-matched control animals (469 ± 40 mg; $n = 41$). Trains of 1 to 64 field pulses applied during the refractory period of the atrial muscle evoked an increase in force of contraction in both control and diabetic atria (Figure 1). Diabetic atria developed a lower force of contraction in response to EFS than control atria and the difference was statistically significant at every level of stimulation (Figure 1). In the presence of 3×10^{-7} M atropine or 10^{-6} M yohimbine, the positive inotropic response to EFS was significantly enhanced in control atria (Figure 2). On the other hand, none of the antagonists augmented the inotropic response to EFS in diabetic atria (Figure 2). In both control and diabetic left atria, pretreatment with 3×10^{-6} M propranolol completely abolished the positive inotropic response caused by EFS. After blocking α_1- and β-adre-

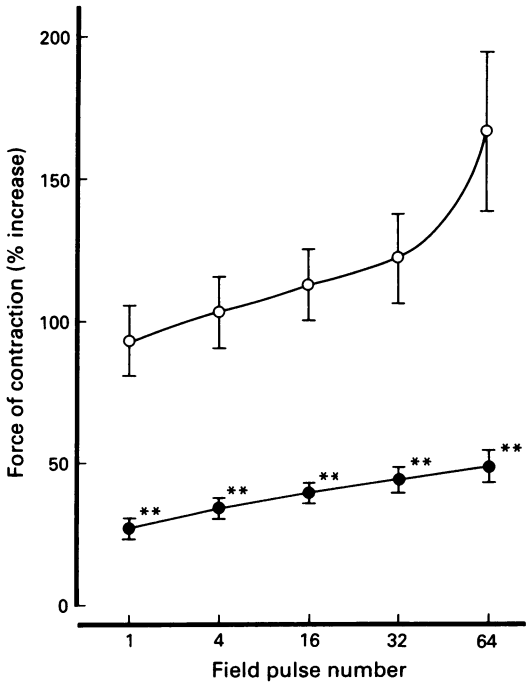

Figure 1 Positive inotropic responses to electrical field stimulation of left atria isolated from control (○, $n = 5$) and diabetic (●, $n = 5$) rats. All values are means ± s.e.mean and are expressed as % increase in basal force of contraction prior to application of field stimulation. Abscissa scale: number of pulses. $^{**}P < 0.01$ indicates a significant difference from the corresponding control values.

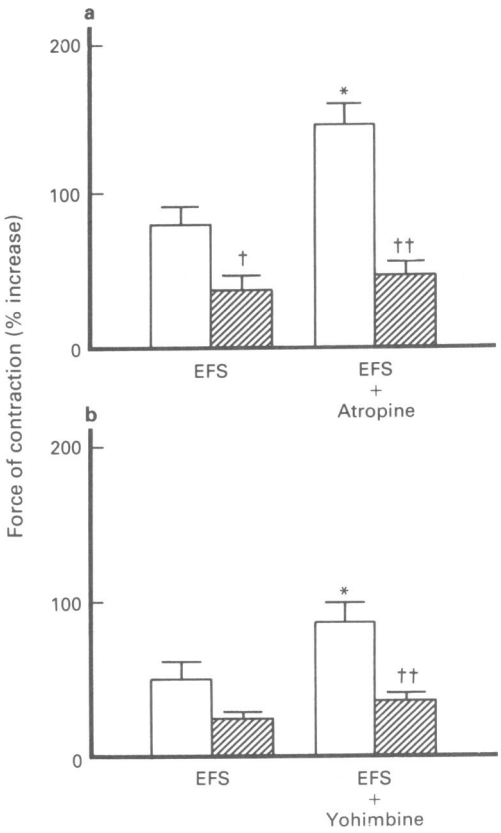

Figure 2 Influences of 3×10^{-7} M atropine (a) and 10^{-6} M yohimbine (b) on the positive inotropic response to electrical field stimulation (EFS) of left atria isolated from control (open columns) and diabetic rats (hatched columns). EFS was by application of a current of 50 mA and 5 ms duration. Values are means ± s.e.mean of five experiments and expressed as % increase in basal force of contraction prior to application of EFS. $^{*}P < 0.05$ indicates a significant difference from EFS in the absence of the antagonists: $\dagger P < 0.05$; $\dagger\dagger P < 0.01$ indicate significant differences from the corresponding control values.

noceptors with 10^{-6} M prazosin and 3×10^{-6} M propranolol, EFS did not produce a decrease in force of contraction even in the presence of 10^{-4} M physostigmine in control and diabetic atria.

Noradrenaline and tyramine produced a positive inotropic effect in a concentration-dependent manner in both control and diabetic atria (Figure 3). The maximum responses to noradrenaline and tyramine were not significantly different between control and diabetic atria. The pD_2 values for

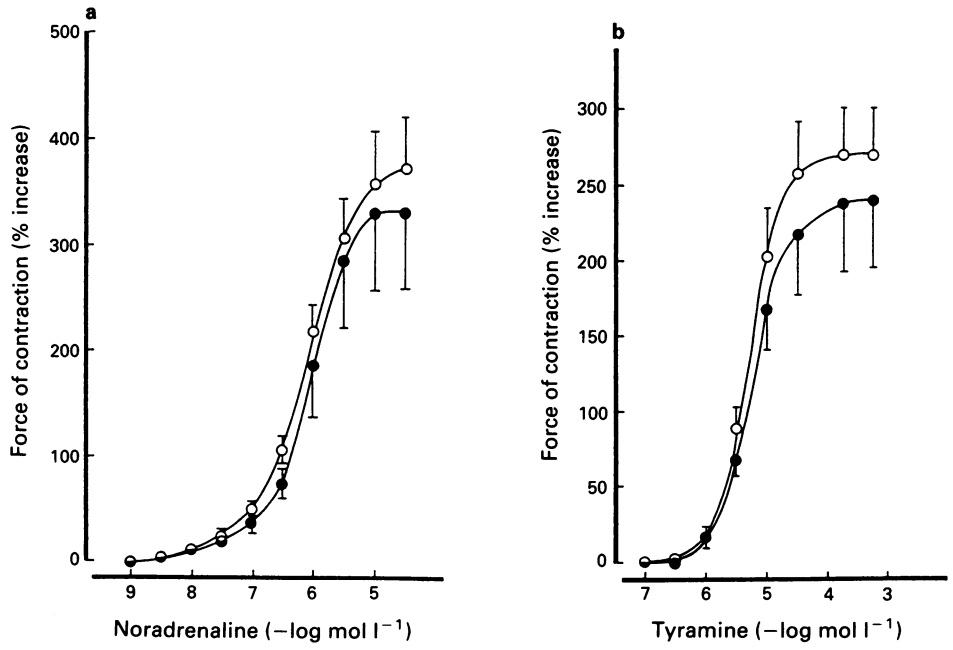

Figure 3 Concentration-response curves for the positive inotropic effects of noradrenaline (a) and tyramine (b) in left atria isolated from control (○) and diabetic (●) rats. All values are means ± s.e.mean of six to seven experiments.

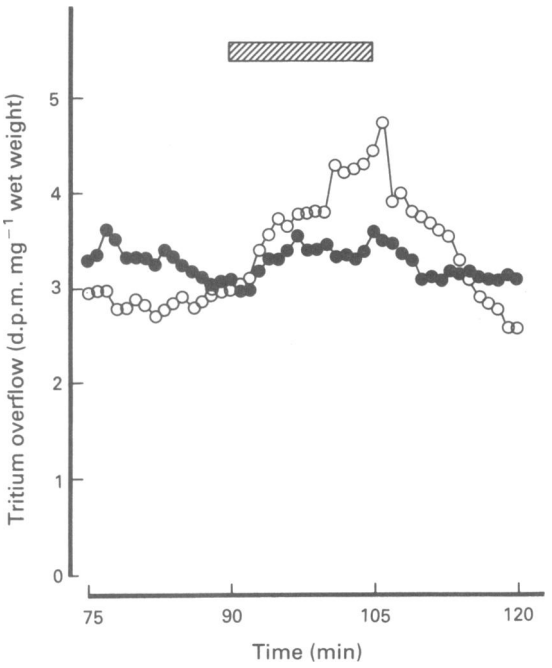

Figure 4 Representative examples of the effect of electrical field stimulation on [^{3}H]-noradrenaline overflow from left atria isolated from control (○) and diabetic (●) rats. Electrical field stimulation was by application of a current of 50 mA and 5 ms duration and is indicated by hatched bar.

noradrenaline (6.16 ± 0.07) and tyramine (5.97 ± 0.16) in diabetic atria were almost the same as the control values for noradrenaline (6.13 ± 0.07) and tyramine (5.81 ± 0.11).

The amounts of basal efflux of [^{3}H]-noradrenaline from control and diabetic atria (~75 min after superfusion) were 0.497 ± 0.06 and 0.768 ± 0.14 d.p.m. mg^{-1} wet wt. min^{-1}, respectively ($n = 5$ for each group); the difference between these values was not statistically significant. As shown in Figure 4, EFS increased the overflow of [^{3}H]-noradrenaline from control atria. However, EFS failed to increase [^{3}H]-noradrenaline efflux in diabetic atria. The efflux of [^{3}H]-noradrenaline in response to EFS was significantly greater in control atria than in diabetic atria (141.9 ± 9.4 *vs* 103.8 ± 11.8% of the basal tritium overflow, $n = 5$ for each group, $P < 0.01$). In the presence of 3×10^{-7} M atropine, the increase in [^{3}H]-noradrenaline efflux induced by EFS was markedly enhanced in control atria but not in diabetic atria (data not shown).

In the presence of hydrocortisone and pargyline, the uptake of [^{3}H]-noradrenaline was not significantly different between control and diabetic atria (2627 ± 241 *vs* 2656 ± 238 d.p.m.; mg^{-1} wet wt., $n = 5$ for each group). Cocaine inhibited the majority of the uptake of [^{3}H]-noradrenaline in both groups (1303 ± 166 *vs* 1759 ± 264 d.p.m. mg^{-1} wet wt., $n = 5$ for each group). There was also no statistically significant difference between these values. The difference in neuronal uptake of [^{3}H]-noradrenaline (defined as the difference in uptake of [^{3}H]-noradrenaline in the absence and presence of cocaine) was also insignificant between both groups.

Noradrenaline contents of the left atria were not significantly different between control and diabetic animals (633 ± 31 *vs* 632 ± 56 ng ml^{-1} wet wt., $n = 6$ for each group).

Discussion

In the present study we showed that the positive inotropic response to EFS was markedly attenuated in the left atria obtained from diabetic rat. The findings that the positive inotropic response to exogenous noradrenaline and tyramine were unaffected in the left atria from diabetic rats indicate that the attenuation of the positive inotropic response to EFS may be caused by presynaptic derangement of neurochemical transmission. The release of noradrenaline by tyramine is reported to be independent of extracellular calcium (Bönisch & Trendelenburg, 1988), whereas EFS releases noradrenaline from the stores in sympathetic nerve endings by a calcium-dependent exocytotoxic mechanism (Rubin, 1970; Brasch, 1989). The most probable explanation for the observation that the atrial response to EFS, but not tyramine, was depressed in diabetic rats is that the release of noradrenaline by calcium-sensitive exocytosis may be specifically impaired in left atria from diabetic rats.

In contrast to our findings that diabetic left atria responded normally to exogenous noradrenaline, a reduction in the inotropic response to β-adrenoceptor stimulation has been reported in ventricular myocytes (Horackova & Murphy, 1988), in spontaneously beating atria (Foy & Lucas, 1978) and in left atria (Sato *et al.*, 1989; Hashimoto *et al.*, 1990) from diabetic rats, and a decrease in the number of the β-adrenoceptors has been demonstrated (Savarese & Berkowitz, 1979; Atkins *et al.*, 1985). It has been suggested that the decline in β-adrenoceptor density may be responsible for the decreased inotropic response to β-adrenoceptor stimulation in diabetic hearts (Heyliger *et al.*, 1982). However, Durante *et al.* (1989) have found no difference in the positive inotropic response of left atria to isoprenaline between control and diabetic rats, despite the fact that β-adrenoceptor density is reduced by 64% in left atria from diabetic rats, indicating that the decrease in cardiac β-adrenoceptor density in diabetes does not necessarily result in an altered β-adrenoceptor-mediated inotropic response. Our findings are in agreement with other studies showing no change in the inotropic effect of isoprenaline in left atria and papillary muscle (McCullough & McNeill, 1983) or isolated hearts (Ingebretsen *et al.*, 1980; Vadlamudi & McNeil, 1984) from diabetic rats. Moreover, Austin & Chess-Willims (1991) have observed an enhanced sensitivity to isoprenaline in left atria and papillary muscles from diabetic rats. Thus, it seems very unlikely that changes in the number of β-adrenoceptors and β-adrenoceptor-adenylate cyclase coupling are involved in the diminished positive inotropic response to EFS in diabetic left atria.

In diabetes, the noradrenaline concentration in the myocardium has been found to increase (Paulson & Light, 1981; Fushimi *et al.*, 1984; Ganguly *et al.*, 1986), decrease (Neubauer & Christensen, 1976) or to be unchanged (Kaul & Grewal, 1980; Akiyama *et al.*, 1989). Noradrenaline turnover has been reported to be depressed in diabetic rats hearts, indicating a reduction in sympathetic nerve activity (Yoshida *et al.*, 1985; 1987). In contrast, Ganguly *et al.* (1986) have shown an increased turnover, uptake and synthesis of noradrenaline in diabetic hearts, implying an increased sympathetic activity. The apparent discrepancy in these results may be related to difference in experimental models, i.e., strain and age of animals, duration and severity of diabetes. In this study we found that the content of noradrenaline in the left atrium was not significantly changed in diabetic rats. In addition, in the presence of hydrocortisone and pargyline, which inhibit extraneuronal uptake and monoamine oxidase, respectively, the cocaine-sensitive accumulation of [^{3}H]-noradrenaline in the left atrium was not significantly different between diabetic and control atria. However the overflow of [^{3}H]-noradrenaline produced by EFS was significantly less in diabetic left atria than controls. Since the diminished overflow of [^{3}H]-noradrenaline reflects a decreased noradrenaline release rather than enhancement of its uptake or retention with the tissue, an impairment of the presynaptic functional process for releasing noradrenaline from the nerve endings could explain the decreased inotropic response to EFS in diabetic left atria.

In the left atria from control rats yohimbine significantly

enhanced the positive inotropic effect induced by EFS, which is considered to be due to blockade of presynaptic α_2-adrenoceptors which are activated by released noradrenaline (Langer, 1981; Starke, 1987). Similarly, atropine caused significant potentiation of the positive inotropic response to EFS in control left atria. It seems unlikely that this potentiating effect of atropine resulted from inhibition of a negative inotropic response to acetylcholine released from cholinergic nerve stimulation, as we did not detect a negative inotropic effect elicited by EFS even in the presence of propranolol, prazosin and physostigmine, but rather through inhibition of presynaptic inhibitory muscarinic receptors (Starke, 1977; Langer, 1981). It appears that atropine is blocking presynaptic inhibitory muscarinic receptors resulting in potentiation of the positive inotropic response to EFS, as atropine facilitated the overflow of [^{3}H]-noradrenaline induced by EFS in control left atria.

In contrast to their effects in nondiabetic atrial preparations, neither yohimbine nor atropine potentiated the positive inotropic response to EFS in diabetic left atria. It is suggested that activation of presynaptic inhibitory receptors reduces transmitter release by decreasing the availability of calcium for stimulus-secretion coupling (Starke, 1977; Langer, 1981). Therefore, if the availability of calcium necessary for stimulus-secretion coupling in the sympathetic nerve endings is primarily impaired in diabetes, actual effects of the experimental procedures of reducing calcium availability such as stimulation of presynpatic inhibitory receptors would be minimized. Alternatively, the amounts of noradrenaline and acetylcholine released from diabetic nerve endings by EFS may be too small to activate efficiently presynaptic inhibitory receptors. Moreover, we cannot exclude the possibility that the densities of presynaptic inhibitory receptors might be depressed in diabetic left atria. Whatever mechanisms are involved in the lack of effect of yohimbine and atropine on the positive inotropic response to EFS in diabetic left atria, our results indicate that regulation of noradrenaline release through presynaptic inhibitory receptors may be altered in diabetic animals.

In summary, the present study shows that the positive inotropic response to EFS is specifically depressed in left atria from diabetic rats. This appears to be due to a decrease in noradrenaline release from the sympathetic nerve terminals through calcium-dependent exocytosis. In addition, it is suggested that presynaptic inhibitory receptors on diabetic sympathetic endings may lose a functional role in modulating adrenergic neurotransmission.

The authors thank Miss R. Yamazaki for her secretarial assistance. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan and by the Akiyama Foundation.

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(Received January 25, 1993
Revised March 25, 1993
Accepted April 14, 1993)