Acetylcholine and Substance P Responsiveness of Intestinal Smooth muscles in Streptozotocin Diabetic Rats

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Abstract We found previously that in in vitro tube form preparations of isolated intestine of streptozotocin (STZ) diabetic rats, frequency of spontaneous intraluminal pressure waves was significantly reduced in duodenum when compared with normal controls. In order to elucidate further the diabetic intestinal disorders, we examined the frequency and amplitude of spontaneous length changes and contractile responses to acetylcholine (ACh) and substance P (sP) in isolated intestinal segments of normal and experimental diabetic rats. In comparison with normal controls, we could confirm the significantly decreased frequency of spontaneous length changes in isolated longitudinal and circular muscle preparations of diabetic duodenum (1 month after STZ injection). Furthermore, amplitude of spontaneous length changes was significantly decreased in circular muscle preparations of duodenum, jejunum, and ileum but not in colon nor in longitudinal muscle preparations. Dose-response curves revealed that both ACh and sP responses were significantly decreased in longitudinal and circular muscle preparations of diabetic duodenum, jejunum, and ileum but not in colon. Mechanisms of reduced contractility of diabetic intestinal smooth muscle in response to ACh and sP were discussed.

Key words: diabetic rat, streptozotocin, acetylcholine, substance P, smooth muscle.

It is well known that diabetes mellitus is frequently accompanied by gastrointestinal abnormalities, such as gastric retention, diminished gastric acid secretion, disordered small intestinal movements, colonic atony, constipation and diarrhea (KATZ and SPIRO, 1966; HOSKING et al., 1978; CLARKE et al., 1979). Animal models of diabetes are often used to elucidate these gastrointestinal disorders, which exhibit...
similar changes to human diabetics (Nelson et al., 1976; Scott and Ellis, 1980). In such diabetic animal models, defective cholinergic neuromuscular transmission in the myenteric plexus of the distal colon is revealed by electrical field stimulation (Nowak et al., 1986). On the other hand, no significant changes are reported on the gastrointestinal muscarinic receptors of diabetic rats by obtaining acetylcholine (ACh)-induced responses (Altan et al., 1987). Substance P (sP) is involved in gastrointestinal motility (Barthó and Holzer, 1985). In addition, sP content is reported to be decreased in diabetic rat intestine (Ballmann and Conlon, 1985; Belai et al., 1985). However, sP responsiveness of diabetic intestine is not clear. The purpose of our study is to determine whether or not there are observable differences in contractile responses induced by ACh and sP between normal and diabetic rat intestinal preparations. We will show that in STZ diabetic rats, both ACh- and sP-induced contractile responses are depressed both in circular and longitudinal muscle preparations, as well as the decreased frequency and amplitude of spontaneous length changes in vitro. The abstract of this study has already appeared (Liu et al., 1988).

MATERIALS AND METHODS

Age-matched Wistar rats, 7 weeks old, weighing about 200 g, were purchased from a commercial source. They were divided into two groups: one for control and the other for experimental diabetes. Rats received a single injection of STZ (60 mg/kg) dissolved in citrate buffer (50 mM, pH = 4.4), via caudal vein under light ether anesthesia. Controls were injected with only the adjusted volume of vehicle, the citrate buffer. All rats had free access to food and water. After 1 month, they were sacrificed for the experiments. After decapitation, blood from trunk was collected and plasma was prepared for the determination of blood glucose. Small and large intestine were isolated as a whole from the body cavity and kept in ice-cold Tyrode’s solution. Segments of 4–5 cm were dissected out of the duodenum, jejunum, ileum, and proximal colon (except caecum). After the contents inside of the segments were flushed out with Tyrode’s solution, they were kept again in ice-cold Tyrode’s solution until the start of the experiment. The composition of Tyrode’s solution was as follows: NaCl 138 mM, KCl 3.4 mM, CaCl₂ 1.3 mM, MgCl₂ 1.2 mM, NaHCO₃ 21 mM, NaH₂PO₄ 0.6 mM, and glucose 10 mM.

Three types of preparations were made from the isolated intestine. First, a tubular intestinal segment was used. One end of it was ligated to a glass rod and immersed in a translucent vertical tube of Magnus type, containing Tyrode’s solution. The other end of it was connected to the lever of an isotonic transducer (TD-112S and JD-1125, Nihon Kohden, Japan) through a cotton thread. A 1-g load was placed on the other end of the lever. Length changes were recorded on a chart recorder (RM-251, Nihon Kohden, Japan). The vertical tube was kept in a warm bath to keep the Tyrode’s solution inside at 37°C. Mixed gas, 95% O₂ and 5% CO₂, was bubbled from the bottom of the vertical tube. The preparations, especially of

Japanese Journal of Physiology
Table 1. Comparison of body weight and blood glucose level of normal and diabetic rats used in this study.

<table>
<thead>
<tr>
<th>Rats used for circular muscle preparations</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
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<tr>
<td>Normal</td>
<td>428 ± 17.5</td>
<td>148 ± 9.3</td>
</tr>
<tr>
<td>DM</td>
<td>261 ± 25.1</td>
<td>602 ± 123.0</td>
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<tr>
<td>(p &lt; 0.001)</td>
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<tr>
<th>Rats used for longitudinal muscle preparations</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
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<tr>
<td>Normal</td>
<td>418 ± 8.0</td>
<td>147 ± 6.0</td>
</tr>
<tr>
<td>DM</td>
<td>268 ± 40.2</td>
<td>633 ± 19.6</td>
</tr>
<tr>
<td>(p &lt; 0.05)</td>
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Mean ± S.E., n = 5.

diabetic rats, were gradually dilated and the length increased during the incubation in the vertical tube, probably due to resting tone decrease. About 30 min later, the length of them was readjusted to 3 cm by sectioning and they were again ligated to the glass rod. Thereafter, frequency and amplitude of spontaneous motile activities (length changes) of the preparation were recorded on a chart recorder and a data recorder (DFR-3415, Sony Magnescale Inc., Japan). They were analyzed online or off-line by a minicomputer, Signal Processor 7107A, loading the program No. 22B (power spectrum by fast Fourier transform) and No. 307 (amplitude analysis) (NEC-San-ei, Japan), by digitizing at 90 ms for 92 s. After that, length changes induced by ACh or sP were recorded on a chart recorder. Drugs were usually tested in increasing concentration and the preparations were washed 1–2 times in the case of ACh and 3–5 times between the tests at interval of 15–30 min in the case of sP test. Drugs were applied into the vertical tube in a droplet of various concentration so as to reach the final concentration of 1, 10, 300, 500, 1,000, 3,000, and 5,000 nM.

Second, tubular segments tested for drug-induced length changes were split in half by scissors and the half of them was removed. Thereafter, again, frequency and amplitude of spontaneous length changes were analyzed as described previously. Again, drug-induced length changes of the split half were tested. The first and second preparations were considered as longitudinal muscle ones. Length changes of half-split preparations induced by drugs were significantly larger than those of tubular ones (see RESULTS and Fig. 2).

Third, new tubular intestinal segments were slit and cut open. These sheets were further repeatedly slit 5 mm transversely, alternately from the left and right edge at 5 mm intervals. These preparations were also ligated to the glass rod and the lever of the isotonic transducer. These were considered to be circular muscle preparations. Again, frequency and amplitude of spontaneous length changes were recorded and analyzed. Thereafter, drug-induced length changes were recorded. Again, the length of circular muscle preparations were clamped to 3 cm in the
incubating tube. Body weight and blood glucose level (determined by automatic glucose analyzer, GOD-mutalotase method, Hitachi 705, Japan) of normal and diabetic rats are listed in Table 1.

RESULTS

Frequency and amplitude of spontaneous motile activity

Length changes of spontaneous motile activity were much more regular and frequent in normal than in diabetic small intestinal segments as reported previously for intaluminal pressure changes (KARAKIDA et al., 1986). Frequency of maximal power in spectral density analysis of spontaneous length changes, cycles per min, was lower in diabetic than in normal circular muscle preparations of small intestine (similar to Fig. 1 for half-split longitudinal muscle and Fig. 2C). Significant difference was found only in duodenal circular muscle preparations as previously reported (KARAKIDA et al., 1986). In longitudinal muscle preparations (Fig. 1), however, significant frequency differences between normal and diabetic ones were also found in tubular and half-split jejunum, in addition to duodenum in this study.

Fig. 1. Sample records of spontaneous length changes of normal (left panel) and diabetic half-split (right panel) duodenal preparations (longitudinal muscle preparations), recorded at the same gain. Their power spectra were obtained by digitizing 90 ms and sampling 1,024 points (92 s) by FFT (fast Fourier transform). Maximal frequency is also indicated as cycle/min. Amplitude histogram is also shown. Power spectra and amplitude histogram of normal preparations are shown at left and those of diabetic at right. Ordinate and abscissa are shown in the figures.
Maximal power frequency (frequency giving maximal power) was highest in duodenum and gradually declined to jejunum and then to ileum; known as this phenomenon is the law of Alvarez (Alvarez, 1914).

Vol. 38, No. 6, 1988
Amplitudes of spontaneous length changes of normal circular muscle preparations were similar to those of normal and diabetic longitudinal ones in duodenum, in jejunum, and in ileum. As for proximal colon, spontaneous length changes of normal and diabetic longitudinal preparations were larger than those of normal and diabetic circular ones (Fig. 2B, D). Amplitude of spontaneous length changes between normal and diabetic longitudinal muscle preparations were similar and significant differences were not found in any segments (Fig. 2B). However, in circular muscle preparations, amplitudes of spontaneous length changes were significantly smaller in diabetic than in normal duodenum, jejunum, and ileum (Fig. 2D).

Dose-response relationships of longitudinal and circular muscle preparations induced by acetylcholine and substance P

Typical dose-response curves of duodenal and colonic longitudinal muscle preparations are shown in Fig. 3A–F. In general, length changes of diabetic preparations induced both by acetylcholine (ACh) and substance P (sP) were smaller than those of normal ones. Diabetic length changes of longitudinal and circular muscle preparations were about half of normal ones. Significant differences of length changes between normal and diabetic preparations were found in duodenum, jejunum, and ileum (except colon) of both tubular and half-split longitudinal preparations and of circular muscle ones (not shown). However, in colonic longitudinal and circular muscle preparations, significant length differences between normal and diabetic preparations were rare. In longitudinal muscle preparations of duodenum, jejunum, and ileum (except colon), length changes of half-splits induced by ACh or sP were about two times larger than those of tubular preparations. However, in proximal colon, length changes of half-splits were only 1.2–1.4 times larger than those of tubular ones.

As for ACh responses, 1,000–3,000 nm induced maximal length changes both in longitudinal and circular preparations. Therefore, length changes of longitudinal tubular and circular muscle preparations induced by 3,000 nm ACh were compared. As seen in Fig. 4A, length changes of longitudinal muscle preparations were significantly larger than those of circular ones both in normal and in diabetic duodenum, ileum, and colon. On the other hand, 300–500 nm sP induced maximal length changes both in longitudinal tubular and circular muscle preparations. Comparison of length changes induced by 500 nm sP shows that those of longitudinal muscle preparations were significantly larger than those of circular ones only in normal and diabetic duodenum and in normal jejunum (Fig. 4B). In general, larger length changes were induced in longitudinal muscle preparations than in circular ones by ACh and sP (Fig. 4A, B).

Length changes induced by a given concentration of ACh and sP were similar between normal and diabetic longitudinal muscle preparations of every intestinal segment (Fig. 4C). Furthermore, they were also similar between normal and diabetic circular muscle preparations (Fig. 4D). However, length changes of normal
colonic circular muscle preparations induced by 500 nM sP were significantly larger than those by 500 nM ACh (Fig. 4D).

The 50% effective doses (ED$_{50}$s) for ACh and sP were obtained from each dose-
response curve and they were compared between normal and diabetic groups after obtaining mean ± S.E. of ED\textsubscript{50}s in each intestinal segment. However, there were no statistically significant differences between normal and diabetics, probably because of great variations in ED\textsubscript{50}s.

DISCUSSION

We previously found that both frequency and amplitude of spontaneous intraluminal pressure changes were generally reduced in diabetic rat intestines,
especially in duodenum when compared with normal controls. Furthermore, we supposed that reduced motile frequency was related to disorders of pacemaker activity and diabetic metabolic disorders (Karakida et al., 1986). In this study, we could confirm the significantly reduced frequency of spontaneous length changes of both longitudinal and circular muscle preparations of streptozotocin diabetic rat duodenum. In addition, amplitudes of spontaneous length changes were also significantly reduced in diabetic circular muscle preparations of duodenum, jejunum, and ileum (except colon). We also showed that contractile response (length changes) induced by ACh and sP of circular and longitudinal muscle preparations were significantly reduced in duodenum, jejunum, and ileum of diabetic rats (except colon; Figs. 1, 2).

We could not determine the site of action of exogenous ACh and sP. They probably acted both at the intrinsic innervation and at the contractile elements of the smooth muscle, because we did not eliminate the nerve-mediated responses by using tetrodotoxin. However, the reduced responses to drugs (Figs. 3, 4) might be due to disorders of receptors for ACh and sP such as decreased receptor affinity or to smooth muscle contractile system or reduced transduction after receptor activation or to combination of these. Threshold concentration of ACh and sP and ED₅₀ for contraction were not significantly different between normal and diabetic preparations because of large variations in this study. Therefore it was difficult to discuss the receptor affinity for ACh and sP. Less contractility of diabetic preparations than of normal ones might show the absence of intrinsic or extrinsic efferent denervation and the absence of denervation supersensitivity in experimental diabetic rat intestines (Whalen et al., 1969). Denervation hypersensitivity of intestinal motility was not demonstrated using mecholyl (McNally et al., 1969). Furthermore, neostigmine injection in humans (i.m.) demonstrated a dose-related stimulatory effect on colonic myoelectric and motor activity in both normal subjects and diabetics but differences were not found between them (Battle et al., 1980). Contractile responses to bethanechol and sP were not altered in diabetic jejunum (Mathison and Davison, 1988). However, contractile responses to ACh and sP were reduced in diabetic duodenum, jejunum, and ileum of longitudinal and circular muscle preparations (Figs. 3, 4). We also showed that spontaneous and drug-induced contractility was not significantly different between normal and diabetic preparations of colon. Schmidt et al. (1981) reported the following in diabetic animals: 1) reduced cholinesterase staining of both myenteric ganglia and bundles of axons, 2) degenerative changes in terminal autonomic axons in the colonic submucosa and muscularis, 3) degenerative pattern of enlarged unmyelinated axonal profiles of dense bodies, and multilaminar membranous bodies, 4) significant decrease in colonic and ileal activities, 5) significant decrease in choline acetyltransferase activity, and 6) deficiencies in colonic adrenergic and cholinergic innervation; however, degenerative neurons were not found in the intrinsic ganglia of the colonic wall. Moreover, they reported that ultrastructural analysis of the Auerbach’s plexus showed that lesions (axonal fibrillar degeneration) were confined
to the distal, unmyelinated axons in the diabetic Chinese hamster. On the other hand, a reduced number of Auerbach’s plexus was reported (Diani et al., 1976). If cholinergic extrinsic and intrinsic innervations are defective, cholinergic supersensitivity seems to be induced in diabetic intestinal smooth muscle. They also reported the reduction of the muscularis and the presence of excessive connective tissue in the muscular coat of the diabetics (Diani et al., 1976). Connective tissue replacement of the outer longitudinal muscle layer has also been observed in diabetic man (Berge et al., 1956). The dry weight of mucosal scrapings was significantly greater in diabetic animals at 8 days after alloxan treatment. The weight of underlying tissue remaining after scraping off the mucosa was not significantly different, localizing the growth to the mucosa (Schedl and Wilson, 1971). The relative reduction of muscle mass also may reduce the drug-induced contractility of intestine. However, increased reactivity of the vascular system to vasopressor has been demonstrated in diabetic animal models, demonstrating increased vascular responses to vasoactive agents, such as noradrenaline (Brody and Dixon, 1964; Cseuz et al., 1973; Owen and Carrier, 1980; Scarborough and Carrier, 1984; MacLeod and McNeill, 1985; Agrawal et al., 1987). These differences in sensitivity to drugs between diabetic intestine and the vascular system might be due to the differences in innervation of the autonomic nervous system. It is well known that autonomic nervous system innervates intestinal smooth muscle via the intrinsic nervous system. Auerbach and Meissner, while it directly innervates vascular smooth muscle.

REFERENCES


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