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Calmodulin content and in vitro contractility of duodenum from streptozotocin-induced diabetic rats: effects of insulin therapy and calmodulin antagonism

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Abstract

The effects of experimental diabetes and insulin treatment on the decreased reactivity of isolated rat duodenum to KCl and calmidazolium, a specific calmodulin antagonist, were examined. After 8 weeks of streptozotocin diabetes, the contractile effect of KCl and the non-competitive antagonistic effect of calmidazolium against KCl on isolated rat duodenum were decreased. Calmodulin levels, as measured by radioimmunoassay, were also found to be decreased in duodenum from streptozotocin-diabetic rats. Neither impaired reactivity to KCl nor decreased calmodulin levels in diabetic rat duodenum were corrected by treatment with insulin (10 IU/kg for 20 days). Following insulin treatment, there was only a partial correction in the antagonistic effect of calmidazolium as shown by the increase in non-competitive antagonist affinity constant.

Keywords: Diabetic rat; Streptozotocin; Ca^{2+}; Calmodulin; Calmidazolium; Insulin; Intestinal reactivity

1. Introduction

Diabetes mellitus is a metabolic disease which causes gastrointestinal complications such as dysphagia, abdominal pain, nausea and vomiting, malabsorption, fecal incontinence and diarrhea (Ogbonmaya and Arem, 1990; Yang et al., 1984). Disturbances of the gastrointestinal tract are among the first of the autonomic syndromes related to diabetes to achieve prominence, and, of these, 'diabetic diarrhoea' has attracted most attention (Bargen et al., 1936; Sheridan and Bailey, 1946). The diarrhea is often intermittent, sometimes alternating with periods of constipation. The major clinical problem concerns the conflict between diabetic diarrhea and constipation (Hosking and Hampton, 1978; Katz and Spiro, 1966; Malins and French, 1957). Another diabetic complication related to the alimentary tract is atonic dilatation of the stomach (Kassander, 1958).

Although relatively few studies have investigated the gastrointestinal manifestations in animal models of diabetes, altered contractile and relaxant responses have been reported in gastrointestinal smooth muscles from diabetic rats. Decreased relaxant responses to β-adrenoceptor agonists and contractile responses to serotonin in the gastro-intestinal tract of diabetic rats have been reported as long-term diabetic complications which are closely related to clinical manifestations of diabetes mellitus (Altan et al., 1987; Mathison and Davison, 1988; Yıldızoğlu-Arı et al., 1988; Öztürk et al., 1992a,b). These changes in gastro-intestinal β-adrenergic and serotonergic responsiveness have been attributed to a decrease in the number of β-adrenoceptors and in the affinity of serotonin receptors resulting from diabetes. Nevertheless, no specific changes have been observed in the gastro-intestinal muscarinic responses of diabetic rats (Altan et al., 1987). It has been shown that the gastro-intestinal complications related to β-adrenergic and serotonergic responsiveness are improved following in vivo insulin treatment. The decreased β-adrenergic responses,

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but not serotonergic responses, are also improved following in vitro insulin treatment (Yıldızoğlu-Arı et al., 1988; Öztürk et al., 1992a). Similar changes in the β-adrenergic responses have been observed in the gastrointestinal tract from rats with non-insulin dependent diabetes (Öztürk et al., 1990). Although hyperreactivity to Ca^{2+} has been observed in the gastric fundus from streptozotocin-diabetic rats (Aihara and Sakai, 1989), other studies have indicated a decreased sensitivity of the intestine to Ca^{2+} in rats with long-term diabetes mellitus (Öztürk et al., 1987, 1996). In relation to this observation, decreased calmodulin levels have been reported in the intestinal smooth muscle of rats with long-term streptozotocin-diabetes (Öztürk et al., 1994). These changes in Ca^{2+} responsiveness and calmodulin levels in the intestine do not seem to occur in short-term diabetic rats (Öztürk et al., 1994, 1996). However, the relationships between impaired responsiveness to Ca^{2+} and decreased calmodulin level in diabetic gastrointestinal smooth muscle are still not clear. Therefore, this study was designed to investigate the relationship between impaired responsiveness to Ca^{2+} and decreased calmodulin level in gastrointestinal smooth muscle from experimentally diabetic rats. It was also aimed to investigate the effectiveness of insulin on both impaired gastointestinal responsiveness to Ca^{2+} and decreased calmodulin level.

2. Materials and methods

2.1. Diabetic model and insulin treatment

Male Wistar rats (local inbred strain) were used (150–200 g body weight). They were housed in a room with controlled temperature (21 ± 3°C) and humidity (65–70%) and a care was taken with the day-and-night cycle. All animals received food and water ad libitum, except when indicated. The rats were fasted 18–24 h before diabetes induction. Streptozotocin was dissolved in ice-cold 0.1 M citric acid buffer adjusted to pH 4.5. Under light ether anaesthesia, diabetes was induced in the half of the rats with a single injection of streptozotocin (60 mg/kg body weight) into the lateral tail vein. The remainder of the rats were injected with the vehicle (citric acid buffer) only. The injection volume did not exceed 0.1 ml in each case. After injection with streptozotocin, the rats were divided into four groups. Five weeks after the injection of streptozotocin, rats in the first and second groups were injected subcutaneously with 10 IU/kg insulin daily for 20 days. Rats in the third and fourth groups and their age-matched controls were injected with saline solution daily for 20 days as a vehicle. Eight weeks after the induction of experimental diabetes, diabetic and insulin-treated diabetic rats and their age-matched controls were killed to measure the smooth muscle calmodulin levels and to determine KCl-induced responses in isolated duodenum as described below. Blood samples were collected from the tail vein at the time of killing and blood glucose levels were determined by the glucose oxidase method (Fales et al., 1961). Plasma insulin, total T_3 and T_4 levels were measured by radioimmunoassay (Britton et al., 1975; Malone and Root, 1981).

2.2. Isolation of intestinal tissue

Duodenal smooth muscle from diabetic and insulin-treated diabetic rats and their age-matched controls was isolated according to earlier described procedures (Altan et al., 1987, 1989; Yıldızoğlu-Arı et al., 1988). Rats were killed by stunning and decapitation. The proximal duodenum was then excised from each animal and kept in Krebs' solution with the following composition (in mM): NaCl, 118.4; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 1.9; $NaHCO_3$, 25.0; $MgSO_4$.7H2O, 1.2; KH_2PO_4, 1.2 and glucose 11.1. The duodenum was cleaned of adhering fat and connective tissue and cut about 1.5 cm long. Duodenal tissues were suspended in an isolated organ bath filled with 10 ml of Krebs' solution (pH 7.4) continuously aerated with a mixture of 5% CO_2 and 95% O_2 at 37°C. One end of the isolated duodenum was connected to a tissue holder and the other to an isotonic transducer (Ugo Basile, No.7006, Varese, Italy) connected to a two-channel pen recorder (Ugo Basile, No.7070, 'Gemini', Varese, Italy). The tissues were equilibrated by incubation in the Krebs' medium for 60 min under a resting tension of 1.0 g. During the incubation period, the duodenum was rinsed every 10 min. At the end of this period, non-cumulative concentration-response curves were obtained with KCl in the absence and in the presence of calmidazolium (compound R24571), a specific inhibitor of calmodulin (Gietzen et al., 1981; Mazzei et al., 1984; Van Bella, 1981). KCl, in the concentration range of 10–320 mM, was added into the organ bath in a non-cumulative manner until a maximal response was achieved. The dose-cycle for KCl was 5 min with 45–60 s of contact time. After a reproducible concentration-response relationship was obtained by repetition of same procedure, calmidazolium was added into the bathing medium at the concentration of 1.75×10^{-6} or 3.50×10^{-6} M and incubated with duodenums from non-diabetic, diabetic or insulin-treated diabetic rats for 30 min. The incubation period was determined experimentally in duodenums from control rats. After this incubation period, the concentration-response procedure for KCl was repeated in the presence of calmidazolium. In each experiment, only one concentration of calmidazolium was tested.

2.3. Calmodulin measurement in duodenum

The duodenal tissues from non-diabetic, diabetic and insulin-treated diabetic rats were quickly dissected after killing by cervical dislocation and were placed in ice-cold sucrose solution (0.33 M) buffered with 40 mM Tris-HCl (pH 7.40). The tissues were then homogenised in the

buffered sucrose solution using a glass-glass homogenizer (Braun, Potter-S, Germany) placed in ice. The total homogenates obtained were diluted with deionised water (dilution factor 1/10), heated in a water bath at 100°C for 3 min and centrifuged at 3500 × *g* for 5 min to remove denatured proteins. Supernatants were diluted 100-fold with calmodulin assay buffer (Öztürk et al., 1994; Perez de Garcia et al., 1980). Calmodulin assay buffer was prepared as follows: boric acid (3.1 g), sodium borate (4.8 g), EGTA (0.19 g), sodium azide (0.49 g), sodium chloride (2.2 g) and bovine serum albumin (0.1 g) were added to 100 ml deionised water and shaken slightly for 10 min. Subsequently, 0.15 ml of Tween 20 solution (10%) was added and the mixture was shaken until dissolution of the contents. The resulting mixture was then adjusted to pH 8.5 and finally deionised water was added up to a total volume of 500 ml. This buffer was also used for the preparation of standards for calmodulin measurements. Calmodulin levels in diluted supernatants were measured by a radioimmunoassay procedure using calmodulin labelled with ^{125}I as standard and double antibodies which were purchased from New England Nuclear (Boston, MA, USA). Radioactivity was determined with a 5-channel gamma counter (Isodata, Model 105, Costa Mesa, CA, USA) coupled to an IBM-AT compatible computer with integrated software supplied by the manufacturing company (Van Eldik et al., 1980; Van Eldik and Watterson, 1981). Heated calmodulin standard was used for the calibration, since smooth muscle homogenates were heated during the preparation as described above. To check recovery in the radioimmunoassay procedure, known quantities of calmodulin were added to a duodenal extract to give 9.0 ng/ml and 'spiked' and normal samples were then assayed at the dilution of 1:100. Calmodulin amounts in the duodenal tissues obtained from non-diabetic, diabetic and insulin-treated diabetic rats were expressed as μg/g tissue wet weight.

2.4. Analysis of data and statistics

At the end of each organ bath experiment, the tissue wet weight was determined and the contractile responses were expressed in g/g of tissue. To evaluate the actions of KCl and calmidazolium on isolated rat duodenums, pD_2 (apparent affinity constant for agonist), α^E (intrinsic activity) and pD'_2 (apparent antagonist affinity constant for antagonist) values were calculated for each experimental group (Ariëns and Van Rossum, 1957; Ariëns and Simonis, 1964). In addition, regression analysis was applied in order to examine the parallelism between the non-cumulative concentration-response curves obtained in the absence and in the presence of calmidazolium (Finney, 1978).

Radioactivity was expressed as percentage B/B_0 calculated from the following equation: percentage $B/B_0 = B \times 100/B_0$. B and B_0 are specific binding of the standards and specific binding of the blank (both cpm), respectively.

All data are presented as means ± S.E.M. (n = number of rats). The statistical significance of differences between the mean values obtained from non-diabetic, diabetic and insulin treated diabetic rats was evaluated by one-way analysis of variance (ANOVA) followed by Scheffé's multiple range test (Finney, 1978). P values < 0.05 were regarded as significant.

2.5. Materials

Boric acid, calcium chloride, hydrochloric acid, magnesium sulphate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium borate, sodium hydroxide and sucrose were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (fraction V), calmidazolium (compound R24571), EGTA, glucose, sodium azide, streptozotocin, Tris-HCl and Tween 20 were bought from Sigma (St. Louis, MO, USA). Insulin NPH was supplied from Organon (Istanbul, Turkey). Radioimmunoassay kits for insulin, T_3 and T_4 determination were purchased from Diagnostic Products (Los Angeles, CA, USA). The calmodulin assay kit was bought from New England Nuclear.

3. Results

3.1. Experimentally induced diabetes and insulin treatment

Streptozotocin-treated animals exhibited characteristic qualitative signs of diabetes such as polyphagia, polydipsia, polyuria, lethargy and discoloration of fur, etc. Some of the streptozotocin-treated rats were found to develop cataracts after 8 weeks. Decreased rate of growth, impaired insulin and thyroid hormone secretion and elevations in blood glucose were also observed in the experimentally diabetic rats relative to their age-matched controls (Table 1). In insulin-treated diabetic rats, almost all of the metabolic parameters were found to be normalized, suggesting the effectiveness of insulin administration on the metabolic deficits of streptozotocin-induced diabetes.

Table 1
The effects of streptozotocin-diabetes and insulin treatment on the blood glucose, plasma insulin, T_3 and T_4 levels in the rats

	Control rat	Diabetic rat	Insulin-treated diabetic rat
Blood glucose (mM)	6.75 ± 0.44 (n = 14)	21.21 ± 0.49 [a] (n = 8)	7.12 ± 0.35 (n = 7)
Plasma insulin (μIU/ml)	39.13 ± 4.12 (n = 15)	4.43 ± 1.84 [a] (n = 8)	Not determined
Plasma total T_3 (nM)	1.69 ± 0.16 (n = 15)	0.59 ± 0.07 [a] (n = 8)	1.49 ± 0.04 (n = 7)
Plasma total T_4 (nM)	70.84 ± 5.29 (n = 16)	21.16 ± 3.99 [a] (n = 8)	58.46 ± 5.04 (n = 7)

[a] $P < 0.001$ significance relative to controls by ANOVA.

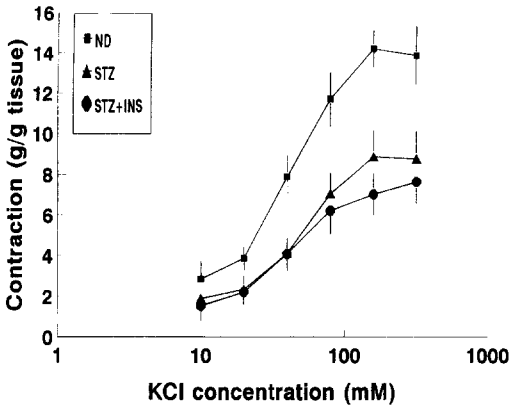

Fig. 1. Effect of streptozotocin-diabetes and insulin treatment on KCl-induced contractile responses of isolated rat duodenum (ND, non-diabetic; STZ, streptozotocin-diabetic rats; STZ-INS, insulin-treated diabetic rats).

3.2. Effect of KCl on rat duodenal smooth muscles

In a concentration range of 10–320 mM, non-cumulatively applied KCl was found to cause concentration-dependent contractions in duodenums from non-diabetic, streptozotocin-diabetic and insulin-treated diabetic rats. Neither the non-diabetic rat duodenum nor the duodenal tissues from diabetic and insulin-treated diabetic rats exhibited any tachyphylaxis or desensitization phenomena on the dose cycle used in the present study. As can be seen in Fig. 1, streptozotocin-induced diabetes caused a decrease in the responsiveness of rat duodenum to KCl and insulin treatment was found to be ineffective on the decreased responses. pD_2 values calculated for contractile effects of KCl on the rat duodenum were found to be unchanged in streptozotocin-diabetic and insulin-treated diabetic rats (Table 2). In contrast, a statistically significant decrease was observed in the α^E value for the effect of KCl on the diabetic rat duodenum, when compared to controls. A similar decrease was also detected in the α^E value calculated for the KCl-induced contractions of duodenums from insulin treated diabetic rats (Table 2).

3.3. Effect of calmidazolium on KCl-induced contractions of rat duodenum

Calmidazolium, applied into the organ bath at the concentration of 1.75×10^{-6} or 3.50×10^{-6} M, inhibited KCl-induced contractions of duodenums from non-diabetic, diabetic and insulin-treated diabetic rats (Fig. 2). According to the results of regression analyses, these inhibitions were found to be non-competitive. The pD'_2 value for the antagonistic effect of calmidazolium against KCl was found to be slightly increased in the diabetic rat

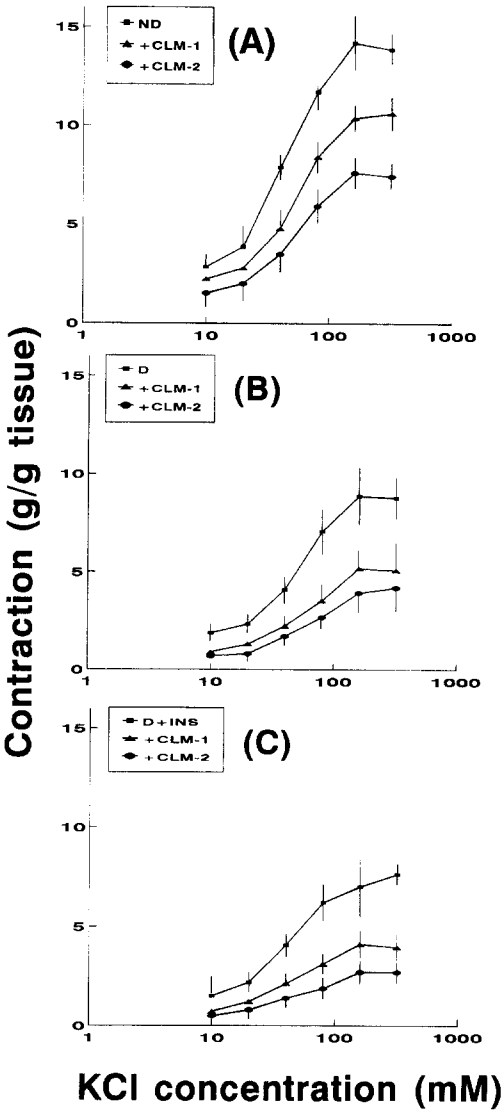

Fig. 2. Antagonistic effects of calmidazolium at the concentration of 1.75×10^{-6} M (CLM-1) and 3.5×10^{-6} M (CLM-2) against KCl in non-diabetic (A),streptozotocin-diabetic (B) and insulin-treated diabetic (C) rat duodenums.

Table 2
Apparent affinity constants (pD_2 values) and intrinsic activities (α^E values) calculated for the contractile effect of KCl on duodenums from non-diabetic, streptozotocin-diabetic and insulin-treated diabetic rats

	Control rat	Diabetic rat	Insulin-treated diabetic rat
pD_2 values	1.52 ± 0.04 ($n = 7$)	1.54 ± 0.01 ($n = 7$)	1.53 ± 0.03 ($n = 7$)
α^E values	1.00 ± 0.02 ($n = 7$)	0.64 ± 0.02 [a] ($n = 7$)	0.55 ± 0.03 [a] ($n = 7$)

[a] $P < 0.001$ significance relative to controls by ANOVA.

Table 3
Apparent antagonist affinity constants (pD'_2 values) calculated for the antagonistic effect of calmidazolium against KCl in duodenums from non-diabetic, streptozotocin-diabetic and insulin-treated diabetic rats

	Control rat	Diabetic rat	Insulin-treated diabetic rat
pD'_2 values	5.27 ± 0.03 ($n = 7$)	5.59 ± 0.01 [a] ($n = 7$)	5.71 ± 0.01 [b] ($n = 7$)

[a] $P < 0.05$ and [b] $P < 0.001$ significance relative to controls by ANOVA.

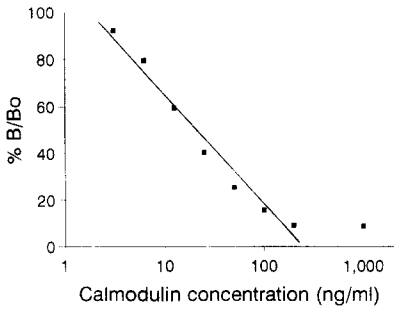

Fig. 3. Typical standard curve of calmodulin radioimmunoassay. B: specific binding (cpm) of standard; B_0: specific binding (cpm) of the blank.

Table 4
The effects of streptozotocin-diabetes and insulin treatment on the calmodulin levels (μg/g tissue wet weight) of rat duodenal tissue

	Calmodulin levels
Control	578.27 ± 9.44 ($n = 9$)
Diabetic rat	53.07 ± 1.07 [a] ($n = 7$)
Insulin-treated diabetic rat	39.80 ± 0.80 [a] ($n = 7$)

[a] $P < 0.001$ significance relative to controls by ANOVA.

duodenum. This slight, but significant increase in the pD'_2 value for calmidazolium was further increased in the duodenum from insulin-treated diabetic rats (Table 3).

3.4. Calmodulin measurement in rat duodenum

In the concentration range of 3.1–200 ng/ml, the standard curve of the calmodulin radioimmunoassay exhibited good linearity (Fig. 3) which was also verified by linear regression analyses ($r = 0.930$, $P < 0.00$). Recovery, after substraction of endogenous calmodulin, was 91%. The calmodulin content was found to be decreased in the duodenal smooth muscle from rats with 8-week diabetes. Insulin treatment for 20 days was ineffective on the decreased calmodulin levels of diabetic rat duodenum (Table 4).

4. Discussion

Experimental diabetes has been reported to cause a decreased sensitivity to Ca^{2+} in gastrointestinal smooth muscles as a long term complication (Öztürk et al., 1987, 1996). In this study, the responsiveness of duodenal smooth muscle to KCl was found to be decreased due to the experimental diabetes. KCl causes contractile responses in smooth muscle by changing Ca^{2+} channel kinetics (Spedding, 1985) and Ca^{2+} mobilization from intracellular stores (Triggle, 1983). Therefore, the contractile responses of duodenal smooth muscle reflect its responsiveness to Ca^{2+}. Since streptozotocin-induced diabetes caused a decrease in the KCl-induced contraction of rat duodenum, it seems logical that the experimental diabetes causes a decreased sensitivity of the gastrointestinal smooth muscle to Ca^{2+}. Furthermore, in the present study, an increased reactivity to calmidazolium was observed in the diabetic rat duodenum. The pD'_2 value calculated for the antagonistic effect of calmidazolium in the diabetic duodenum was significantly higher than that in non-diabetic rat duodenum, suggesting an increased affinity of calmodulin due to experimental diabetes. It seems logical to suggest that the decreased Ca^{2+} responsiveness of duodenal smooth muscle may be related to a defect in the calmodulin-dependent contractile machinery and the affinity of calmodulin may be increased to compensate for the decreased calmodulin level in duodenal smooth muscle. In fact, a recent report by our group has revealed that the decreased sensitivity to Ca^{2+} may be related to a decreased calmodulin level in the gastrointestinal smooth muscle from streptozotocin-diabetic rats (Öztürk et al., 1994).

The present study confirms previous reports on calmodulin levels in non-diabetic and diabetic tissues. The calmodulin content of duodenal smooth muscle from non-diabetic rats was also in general agreement with the results of earlier studies in which the smooth muscle calmodulin levels in the range of 400–600 mg/kg wet tissue had been reported (Walsh et al., 1980). The maintenance of smooth muscle function in diabetic rats however seems to remain adequate in spite of such a dramatic decrease in tissue calmodulin contents (Table 2). There are a number of other Ca^{2+}-binding proteins such as leiotonin, α-actinin, parvalbumin, p70, calpactin, calponin, caldesmon, lipocortins, endonexin I, endonexin II, calelectrin, calretinin, visinin, cristalins, oncomodulin, calcineurin B, calbindin-D28 K etc. (Schachter and Kowarski, 1985; Burgoyne and Geisow, 1989; Heizmann and Hunziker, 1991). Some of these proteins are known to exist in various smooth muscles and their functions in these tissues have not yet been fully elucidated (Adelstein and Eisenberg, 1980). Recent studies have also revealed that some of these proteins, for example calponin and caldesmon, may play an important role in smooth muscle contraction (Haeberle and Hemric, 1994; Gerthoffer and Pohl, 1994; Carmichael et al., 1994) and smooth muscles possess at least one contractile mechanism which is not dependent on myosin phosphorylation (Zhang et al., 1994). It is also interesting that the release and biosynthesis of these proteins are inducible by various endocrine stimuli (Flower and Rothwell, 1994). Hence, it seems likely that at least one of the other Ca^{2+} binding proteins may contribute to the contractile process as a regulatory function and its level may be increased as a compensatory mechanism for the maintenance of vital functions in the deficient smooth muscles from diabetic animals. Unfortunately, there is no experimental evidence to support the above suggestion.

As an alternative possibility, it may be suggested that

diabetic changes in the responsiveness of duodenum to Ca^{2+} are due to irreversible glycosylation of calmodulin. Increased glycosylation of proteins, which is a common feature in both clinical and experimental diabetes may cause long-term changes in the elasticity and integrity of smooth muscle (Rosenberg et al., 1979; Brownlee et al., 1986). Such glycosylation may decrease the affinity of calmodulin to actomyosin, leading to decreased contractile responses of diabetic rat duodenum to Ca^{2+}. Glycosylation of calmodulin may also result in lower affinity to the antibodies used for its determination by radioimmunoassay. Again, experimental evidences for the presence of glycosylated calmodulin in diabetes have not yet been obtained.

Another interesting observation from the present study is the lack of effect of insulin treatment to normalize the diabetic changes in the duodenal tissue. Neither decreased calmodulin levels nor decreased responsiveness of duodenum to KCl were corrected in insulin-treated diabetic rats (Tables 2 and 4). The dose employed in this study was higher than those reported to reverse the diabetic changes in gastrointestinal, vascular (MacLeod, 1985; Takiguchi et al., 1989; Karasu and Altan, 1993) and cardiac (Makino et al., 1987; Karasu et al., 1990; Özüari et al., 1993; Lafçi-Erol et al., 1994) muscles. In contrast, insulin was found to cause an increase in the antagonist affinity constant (pD'_2 value) for the effect of calmidazolium against KCl on diabetic duodenum, suggesting a beneficial effect of insulin in diabetic gastrointestinal smooth muscle. Overall, the findings presented herein strongly suggest that experimental diabetes causes an impairment in the Ca^{2+}/calmodulin-dependent contractile process of gastrointestinal smooth muscles which seems to be resistant to insulin therapy.

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