Altered calcium signaling in colonic smooth muscle of type 1 diabetic mice

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Touw K, Chakraborty S, Zhang W, Obukhov AG, Tune JD, Gunst SJ, Herring BP. Altered calcium signaling in colonic smooth muscle of type 1 diabetic mice. Am J Physiol Gastrointest Liver Physiol 302: G66–G76, 2012. First published October 6, 2011; doi:10.1152/ajpgi.00183.2011.—Seventy-six percent of diabetic patients develop gastrointestinal symptoms, such as constipation. However, the direct effects of diabetes on intestinal smooth muscle are poorly described. This study aimed to identify the role played by smooth muscle in mediating diabetes-induced colonic dysmotility. To induce type 1 diabetes, mice were injected intraperitoneally with low-dose streptozotocin once a day for 5 days. Animals developed hyperglycemia (>200 mg/dl) 1 wk after the last injection and were euthanized 7–8 wk after the last treatment. Computed tomography demonstrated decreased overall gastrointestinal motility in the diabetic mice. In vitro contractility of colonic smooth muscle rings from diabetic mice was also decreased. Fura-2 ratiometric Ca²⁺ imaging showed attenuated Ca²⁺ increases in response to KCl stimulation that were associated with decreased light chain phosphorylation in diabetic mice. The diabetic mice also exhibited elevated basal Ca²⁺ levels, increased myosin phosphorylation targeting subunit 1 expression, and significant changes in expression of Ca²⁺ handling proteins, as determined by quantitative RT-PCR and Western blotting. Mice that were hyperglycemic for <1 wk also showed decreased colonic contractile responses that were associated with decreased Ca²⁺ increases in response to KCl stimulation, although without an elevation in basal Ca²⁺ levels or a significant change in the expression of Ca²⁺ signaling molecules. These data demonstrate that type 1 diabetes is associated with decreased depolarization-induced Ca²⁺ influx in colonic smooth muscle that leads to attenuated myosin light chain phosphorylation and impaired colonic contractility.

As many as 76% of diabetic patients develop gastrointestinal (GI) symptoms, such as dysphagia, vomiting, constipation, diarrhea, or fecal incontinence, that have been linked to poor glycemic control, rather than duration of the disease (3, 8). Of these, constipation resulting from impaired colonic motility is the most common symptom and affects ~60% of patients (8, 24). Animal studies have shown that diabetes can lead to accelerated or delayed GI motility, depending on the animal model used and the specific parts of the GI tract tested. Several studies have used streptozotocin (STZ) to cause specific loss of pancreatic β-cells creating type 1 diabetes-like animal models. STZ-induced diabetic rats have been reported to exhibit increased small intestine smooth muscle mass and increased colon contractility (10, 26). The spontaneous contractile activity in STZ-induced diabetic rat colon smooth muscle was increased (12) without a change in intracellular Ca²⁺ handling (11), while, in the ileum, intracellular Ca²⁺ handling was decreased (11). Conversely, in STZ-induced diabetic mice, the colon smooth muscle contractile response to carbachol stimulation was weaker compared with control animals at 4 and 8 wk following STZ treatment (42). In these mice, this was also associated with delayed gastric emptying and increased intestinal transit time (1). Diabetic db/db mice also show slower gastric emptying and prolonged whole gut transit time compared with wild-type mice (45). Studies in these mouse models are consistent with human studies that demonstrated impaired colonic smooth muscle contractility in diabetic patients (4).

Most studies attribute GI motility changes to autonomic neuropathy (34, 35, 39). A study using nonobese diabetic, STZ-induced diabetic, and db/db mice models demonstrated that nonobese diabetic and STZ mice develop autonomic neuropathy, while chronically diabetic db/db mice fail to develop neuritic dystrophy (33). STZ-induced diabetic rats have been reported to show decreased number of enteric neurons in colon and stomach after only 7 days of hyperglycemia (13, 15). Human studies have demonstrated a loss of enteric neurons in colons from diabetic patients (4). Type 1 and type 2 diabetes have also been shown to lead to decreased numbers of interstitial cells of Cajal, leading to altered motility (17, 43, 45). However, few studies have explored the possibility that there are also defects in GI smooth muscle itself, or shown how these defects progress during development of the diabetic state. These studies have been largely restricted to stomach smooth muscle, in which impaired contractility has been reported to occur due to alterations in muscarinic receptor coupling through GTP-binding proteins in STZ-induced diabetic mice and db/db diabetic mice (37). Similarly, in diabetic BB/W rats, a decreased contractile response of stomach smooth muscle has been reported to result from altered intracellular signal transduction through inositol-trisphosphate (IP₃) and PKC pathways (38). In some severe diabetic cases in human patients, where hyperglycemia is controlled poorly, gastroparesis is associated with gastric smooth muscle myopathy (25). It has been shown that reduction in insulin/IGF-I in diabetic mice causes decreased stem cell factor production, resulting in smooth muscle atrophy that eventually leads to depletion of the interstitial cells of Cajal (20). These results suggest that myopathy may play a more central role in diabetic gastroenteropathies than previously recognized.

The main goal of the present study was to determine the effects of diabetes on colon smooth muscle structure and function in a type 1 diabetic mouse model induced by low-dose STZ. Using this model, mice develop hyperglycemia and hypoinsulinenia rapidly and maintain these changes for several weeks. We observed that STZ-induced diabetic mice have decreased colonic contractility and motility, which develops rapidly within 1 wk and is maintained for more than 7 wk. The decreased contractility is likely a result of impaired Ca²⁺ handling within the colonic smooth muscle cells.

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**MATERIALS AND METHODS**

**STZ-induced diabetic mice.** Nine- to twelve-week-old C57BL6 mice were injected intraperitoneally with freshly prepared STZ (Sigma, S0130) at dose of 55 mg/kg for 5 consecutive days. Mice developed hyperglycemia (>200 mg/dl) within 1 wk after injection and were euthanized 1 wk or 7–8 wk following the last injection of STZ. Blood glucose levels were measured using an Accu-Check Aviva glucose measuring kit (Roche). All animal procedures were approved by the Indiana University School of Medicine IACUC committee.

**Computed tomography.** For in vivo GI motility measurements, mice were fasted over a 12-h period, and then 100 μl of the oral contrast agent Gastrografin (Bracco Diagnostic), diluted with 300 μl saline, was given through oral gavage. Computed tomography (CT) images were subsequently acquired at 3, 5, 9, and 15 h after administration of the contrast agent. For all scans, an X-ray voltage of 90 kVp and an anode current of 100 mAs were utilized. CT imaging was performed with a high-speed clinical CT scanner, a Siemens Somatom Sensation-16, capable of obtaining a mouse volumetric image within 1 min. The image voxel resolution was 300 μm × 300 μm × 625 μm. A three-dimensional volume rendering was generated using Siemens’ image processing workstation equipped with the “Syngo” software.

**Contractility measurements of colon rings.** Colonos were dissected and cut into 0.5-cm-long circular rings. Eight rings cut from along the length of the colon (see Fig. 2A) were placed in Krebs buffer in an organ bath (Kent Scientific) and attached to isometric force transducers. An optimal resting tension of 1.5 g was determined empirically following stimulation with 60 mM KCl. Colon rings from control and STZ-induced diabetic animals were equilibrated at optimal resting tension in Krebs buffer for 1 h and contracted using 60 mM KCl. In some experiments, rings were treated with STZ (0.69 mg/ml), diltiazem (1 μM), or tetrodotoxin (1 μM) before KCl stimulation. After contraction, rings were washed for 30 min, and the contraction repeated. Samples from each experiment were paired, and data are expressed as percentage of the KCl-induced contraction.

**Ca²⁺ imaging.** The ratiometric imaging of fura-2 AM fluorescence was employed to monitor intracellular Ca²⁺ changes in colon strips. Ratiometric imaging eliminates the artifacts associated with the tissue movement during KCl applications. One-millimeter-wide colon strips were dissected free of epithelia and loaded with fura-2 AM for 5–14 h at room temperature in phosphate buffer solution containing 0.901 mM Ca²⁺, 0.493 mM Mg²⁺, 5.56 mM glucose, and 0.327 mM pyruvate, 40 μm fura-2 AM, and 0.1% BSA. The Fura-2 AM-loaded colon strips were washed in standard external solution containing 145 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 5.5 mM glucose, and 0.327 mM pyruvate, 0.8 liter oxygen/min. Between each scan, mice were awake and received regular water and food. The GI motility was visualized by comparing images at different time points.

**Immunohistochemical staining.** Tissues were collected from control and diabetic mice and either fixed in 4% paraformaldehyde solution for 24 h and processed for paraffin embedding, or processed for cryosections, as described previously (19). After embedding, 6-μm sections were cut and stained with hematoxylin and eosin or with neurofilament 200 antibody (Sigma, 1:80).

**Myosin light chain phosphorylation.** The middle portion of the colon was isolated, cut in 0.5-cm circular rings, and hung in an organ bath, as described above for contractility measurements. Tissues were flash frozen in the basal noncontracted state or at the peak of contraction initiated by 60 mM KCl stimulation. Myosin light chain (MLC) phosphorylation levels were measured by Western blotting of proteins separated on urea/glycerol gels, as described previously (16).

**RESULTS**

**STZ-induced diabetic mice have decreased overall GI tract motility.** In this study, we used a low-dose STZ model of type 1 diabetes. This model resulted in elevated blood glucose levels measured 1 wk (374 ± 94 mg/dl compared with 134 ± 15 mg/dl, n = 23) or 7–8 wk (426 ± 83 mg/dl compared with 134 ± 15 mg/dl, n = 21) after the last STZ injection. The model has the advantage that it avoids the major metabolic changes associated with the genetic diabetic models, such as ob/ob mice. Moreover, the drug can be given to a genetically homogeneous population of mice, thus decreasing animal-to-animal variability. We also found that direct acute treatment of colonic rings with STZ (0.69 mg/ml for 1 h), in vitro, did not affect contractile responses, suggesting that STZ itself does not
Table 1. Primers used for quantitative RT-PCR analysis

<table>
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<th>Primers</th>
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<td>CTTCTCTGCAAGAAGAAC</td>
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<tr>
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<tr>
<td>Egr1</td>
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PMCA, plasma membrane Ca\(^{2+}\)-ATPase; TRPC, canonical transient receptor potential; SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; RyR, ryanodine receptor; IP1, inositol-trisphosphate; NCX2, sodium-calcium exchanger 2; HPRT, hypoxanthine phosphoribosyltransferase; iNOS, inducible nitric oxide synthase; PAR2, protease-activated receptor 2; IL-1ß, interleukin-1ß; TNF-α, tumor necrosis factor-α; Egr1, early growth response protein 1.

have any direct toxic effects on colonic ring contractility (data not shown). As secondary complications of diabetes usually develop slowly during the progression of the disease, we initially analyzed mice 7 wk after completion of the STZ injections. CT was used to assess the rate of food transit through the GI tract of control and diabetic animals. At the earliest time point analyzed (0 h) following oral gavage of contrast reagent, the contrast was found mainly in the stomach and was starting to enter the small intestine in both mouse groups (data not shown). Within 3 h, although some contrast agent reached the rectum in both control and diabetic mice (Fig. 1), there was more residual contrast left in the stomach and small intestine of the diabetic animals. After 5 h, contrast agent was seen primarily in the colon and rectum of control mice, while, in diabetic mice, a significant amount of contrast was still visible throughout the whole of the small and large intestine. In control mice, the contrast agent was completely excreted by 9 and 15 h postgavage. In contrast, in diabetic mice, large amounts of contrast material still remained in the small and large intestine, even 15 h after gavage. The CT scan data show that overall GI motility in diabetic animals is decreased, leading to increased GI transit time. These results correlate with patient data showing significant constipation in large proportion of the diabetic human population (8, 24).

**STZ-induced chronic diabetic mice show decreased colon contractility.** To better understand the mechanisms responsible for the decreased motility in diabetic mice, we analyzed the contractility of colon rings ex vivo. We analyzed the ability of the rings to contract in response to direct depolarization induced by 60 mM KCl. This approach bypasses the neuromuscular synaptic transmission pathway and thus allows us to directly assess the contractility of the colonic smooth muscle itself. Complete blockade of KCl-induced contractions by the L-type calcium channel inhibitor diltiazem (1 μM) confirmed that KCl depolarization acts primarily by opening L-type voltage-gated calcium channels (data not shown). To determine whether there were localized or global changes in colonic contractility, the colon was divided into eight segments, and the contractility of each segment was measured separately (Fig. 2A). In diabetic mice, rings from the proximal part of the colon (P1 to P3) did not show any statistically significant changes in contractility compared with that in control animals (Fig. 2, B and C). In contrast, the more central and distal P4 to D2 parts of the colon showed 30–70% decreases in contractility in diabetic mice compared with control mice, with the most pronounced decrease (70%) found in the D4 portion (Fig. 2, B and C). Similar attenuated contractility was observed in colonic segments pretreated with tetrodotoxin to block all neural activity (Fig. 2D). These data indicate that the colons from STZ-treated diabetic mice displayed a myogenic dysmotility. After combining data from all colonic segments, the colons from diabetic mice showed a statistically significant decrease in overall colonic contractility compared with that in control mice (Fig. 2E).

**Diabetic mice have increased basal intracellular Ca\(^{2+}\) levels and decreased Ca\(^{2+}\) response to KCl stimulation.** To begin unravel the causes of the contractility defects, we examined changes in intracellular calcium in response to KCl stimulation. Strips of colonic smooth muscle were obtained from the most significantly affected central region of the colon (P4-D2 region in Fig. 2A) and loaded with fura-2 for ratiometric calcium imaging. Ratiometric measurements showed a large increase in the basal Ca\(^{2+}\) levels in strips obtained from long-term diabetic mice compared with control mice (Fig. 3, A and B). These levels calibrated to ~200 mM [Ca\(^{2+}\)] in control mice and 600 mM in diabetic mice (data not shown). In addition, the calcium transient generated in response to KCl stimulation was ~60–70% lower in STZ-induced diabetic mice compared with control mice (Fig. 3, A and C). Despite the elevated basal intracellular calcium levels seen in the diabetic mice, there was no significant change in the basal MLC phosphorylation levels in these mice (Fig. 3D). This result
suggests that the diabetic muscle has been desensitized to the elevated calcium levels. Calcium desensitization can occur when MLC phosphatase activity is increased. This can occur by decreased expression of inhibitory molecules, such as CPI-17, or inhibition of Rho kinase-mediated phosphorylation of CPI-17 or the MYPT1 subunit of the MLC phosphatase. In contrast to mice with inflammatory bowel disease (27, 32) in which CPI-17 levels decreased, we did not observe a significant decrease in total CPI-17 levels (Fig. 3G) or phosphorylation of CPI-17 (Fig. 3E) in the STZ-induced diabetic mice. Similarly, we saw no change in the phosphorylation of MYPT1 (Fig. 3F) or the expression of MLC kinase (MLCK) (Fig. 3G), although we did observe a small but statistically significant increase in the level of MYPT1 in the diabetic mice (Fig. 3G).

**Diabetic animals show significant changes in levels of calcium-handling proteins.** To determine the mechanisms that may lead to the alteration in basal and KCl-stimulated Ca^{2+} levels, we performed quantitative RT-PCR and Western blot analysis of a wide panel of proteins known to be important for regulating intracellular calcium levels (Fig. 4). This analysis revealed an approximately twofold increase in SERCA2b and IP3R2 mRNA levels in diabetic mice compared with control mice (Fig. 4A). The L-type calcium channel (Cav1.2b), PMCA4, ryanodine receptor 2, and sodium-calcium exchanger 2 mRNA levels also showed a trend toward an increase in diabetic mice; however, due to the variability among animals, these changes did not show statistical significance (Fig. 4A). In contrast, SERCA2b and IP3R2 protein levels decreased 20–40% in diabetic mice (Fig. 4B). Cav1.2b protein levels were not changed in diabetic mice, whereas PMCA4 protein levels increased by 20% in diabetic mice (Fig. 4B). In addition, we did not observe any significant changes in smooth muscle contractile (myosin, actin) or regulatory (caldesmon, SM22a, telokin, MLCK, or CPI-17) proteins at either the mRNA or protein levels (Figs. 3G and 4A and data not shown), with the exception of MYPT1, which increased in diabetic mice (Fig. 3G).

**Contractility in short-term diabetic mice is decreased due to an attenuated intracellular Ca^{2+} response and decreased MLC phosphorylation.** The complex and somewhat confounding changes in expression of calcium-handling proteins described above suggest that, after 7 wk of hyperglycemia, adaptive changes may have occurred in the diabetic mice in an attempt to compensate for an initial defect. To try to identify which changes may represent the initial defect, we analyzed diabetic mice that had been hyperglycemic for a much shorter time (<1 wk). Mice that have been hyperglycemic for <1 wk (euthanized 1 wk after the last STZ injection) also showed decreased contractile responses to KCl in the middle portion of the colon, in either the presence or absence of tetrodotoxin (Fig. 5A). Ratiometric measurements of intracellular calcium showed no changes in the basal Ca^{2+} levels, but a lower Ca^{2+} increase in response to KCl in these diabetic mice compared with controls (Fig. 5B and C). Collectively, the data from the 7- to 8-wk and 1-wk hyperglycemic mice suggest that an initial defect in depolarization-stimulated Ca^{2+} entry is later confounded by an elevation in basal intracellular Ca^{2+} that occurs following prolonged hyperglycemia. Despite the attenuated calcium response, no changes in protein and mRNA levels of most of the channels, receptors, and pumps involved in Ca^{2+} signaling were observed; only PMCA4 protein levels showed a small but statistically significant decrease (Fig. 5E). Consistent with the intracellular calcium measurements, there was no differences in the basal MLC phosphorylation levels between diabetic and control mice (Fig. 5F), although KCl-stimulated MLC phosphorylation levels were lower in diabetic mice compared with controls (Fig. 5F).

**Diabetic mice do not display altered smooth muscle structure, neural density, or any evidence for colonic inflammation.** To rule out the possibility that the attenuated contractility of the colons from diabetic mice is due to a thinner smooth muscle layer, we examined hematoxylin- and eosin-stained cross sections. Smooth muscle layer thickness (Fig. 6A) and nuclear density (data not shown) were not different between diabetic and control mice. Similarly immunohistological anal-
ysis of neurons in the colon did not reveal any gross differences in neural density between control and STZ-induced diabetic mice (Fig. 6B). As inflammatory cytokines are known to attenuate GI smooth muscle contractility (23), we examined the possible contribution of elevated cytokines in the attenuated contractility observed in the colons from diabetic mice. Quantitative RT-PCR analysis did not reveal any significant changes in inflammatory cytokines in the colons of mice 1 wk after STZ treatment (Fig. 6C). In contrast, we observed a fourfold increase in inducible nitric oxide synthase (iNOS) mRNA levels, a 50% decrease in interleukin (IL)-1β levels, and no significant changes in TNF-α levels in colon smooth muscle tissues from diabetic mice 7 wk after STZ treatment (Fig. 6D). These data would suggest that there is not a marked inflammatory response in the colon at either 1 or 7 wk following STZ injection, as this would be expected to result in an elevation of several cytokines.

**DISCUSSION**

Previous studies have demonstrated that diabetes affects GI motility through damaging the neuronal regulation of the GI tract. For example, studies in STZ-induced diabetic rats showed a neuronal defect in the duodenum and colon following a short-term hyperglycemic exposure for 7 days (14, 15). However, few studies have explored possible effects of diabetes directly on colonic smooth muscle. We observed that mice with hyperglycemia for 7–8 wk exhibited a similar impairment in the whole gut transit time to that reported previously in mouse and human studies (Fig. 1) (21, 44, 45). These findings are consistent, with constipation being the most common GI disorder observed in human diabetic patients. Results from our ex vivo contractility measurements further demonstrate that, in this type 1 diabetic mouse model, hyperglycemia results in a direct impairment of colonic smooth muscle contractility, in-
the contractile response along the length of the colon from smooth muscle (Fig. 4A). The smooth muscle similar to that seen in injured vascular smooth muscle (Fig. 4A and data not shown).

Perhaps surprisingly, we observed regional differences in the contractile response along the length of the colon from STZ-induced diabetic mice. The decreased contractile response to KCl in STZ-treated mice was most pronounced in the middle portion of the colon (P4–D2 in Fig. 2). These observations are consistent with previous studies that showed that only the middle portion of the colon exhibited impaired contractility in a sodium dextran sulfate-induced colitis model (40). These data suggest that the middle portion of the colon is particularly sensitive to environmental changes. Although we can only speculate about possible reasons for this regional variation, in the mouse, the middle portion of the colon exhibits a sharp bend as it is positioned in the abdominal cavity. This region also exhibits a steep decrease in its diameter compared with the more proximal part. It is likely that these combined anatomical features provide increased resistance to the movement of fecal matter through this region. By analogy to the vascular system, where similar regions of increased wall stress and turbulent flow are more prone to pathological remodeling, it is possible that these increased stresses also sensitize this portion of the colon to pathological changes. It is unlikely that differences in the neuronal innervation of the proximal (vagal) and distal (pelvic) portions of the colon are directly contributing to the myopathic changes in the diabetic mice, as these changes were also evident in the presence of neurotoxins, such as tetrodotoxin (Figs. 2D and 3A). However, the P4–D4 region of the colon marks a change in the overall effect of neural input from stimulatory to inhibitory (data not shown). This transition could be further contributing to the sensitivity of this region to pathological changes.

Our observations that the impaired colonic contractility in diabetic mice is associated with an impairment in the depolarization-induced intracellular calcium increase is consistent with previous studies in diabetic rats, which also exhibited impaired calcium handling (11, 12). Moreover, our temporal data suggest that a primary defect in depolarization-induced calcium increases eventually leads to an elevation in basal intracellular calcium levels following more long-term exposure to hyperglycemia (Figs. 3B and 5, B and C). To determine the molecular mechanism that accounts for the altered calcium responses in diabetic mouse colon, we examined the expression of the major proteins that regulate intracellular calcium.

Fig. 3. Basal levels of intracellular Ca-sup+ are increased, whereas the Ca-sup+ response to 60 mM KCl is decreased in diabetic mice. A: representative averaged recordings of intracellular Ca-sup+ measured using fura-2 in a middle part of colonic smooth muscle strip isolated from an STZ-treated mouse (7 wk) or a control saline-injected mouse. B: Δbasal levels of intracellular Ca-sup+ in STZ-treated mice compared with control mice. C: Δintracellular Ca-sup+ in response to 60 mM KCl in STZ-treated mice compared with control mice. For A–C, each tracing/bar represents the mean ± SE obtained from 6–8 different mice. For B and C, intracellular Ca-sup+ levels of control mice are set to 1. *P < 0.05. D: parallel colonic smooth muscle samples were used to measure myosin light chain (MLC) phosphorylation levels (n = 4 mice). MLC phosphorylation is expressed as MLCphosphorylated/MLCtotal. Values are means ± SE. E: CPI-17 phosphorylation levels were measured in colonic smooth muscle tissue samples (n = 9). CPI-17 phosphorylation is expressed as CPI-17phosphorylated/CPI-17total. Values are means ± SE. F: myosin phosphatase targeting subunit 1 (MYPT1) phosphorylation levels were measured in colonic smooth muscle tissue samples (n = 8). MYPT1 T696 phosphorylation is expressed as MYPT1phosphorylated/MYPT1total ± SE. G: CPI-17, MYPT1, and MLC kinase (MLCK) expression levels were quantitated by Western blotting and compared between control mice and mice that were hyperglycemic for 7–8 wk. Means ± SE (n = 8) following normalization to meta-vinculin or β-actin as an internal control are shown. *P < 0.05.
levels. Mice that were hyperglycemic for less than 1 wk exhibited a statistically significant but small decrease in PMCA4 protein levels, but no significant changes in the expression of any of the other calcium-handling proteins examined (Fig. 5, D and E). Although statistically significant, the decrease in PMCA4 is not likely to account for the attenuated calcium response. A decrease in PMCA would result in decreased Ca\(^{2+}\) extrusion from the cell, thus causing an elevation in cytoplasmic Ca\(^{2+}\) levels. As basal Ca\(^{2+}\) levels were not altered, this may suggest that the small change in PMCA expression was not sufficient to significantly perturb calcium homeostasis. The lack of changes in expression of calcium-handling proteins would suggest that posttranslational changes in the activity of proteins, such as the L-type calcium channel, are most likely causing the attenuated calcium response following short-term hyperglycemia. In support of this possibility, the diabetic state can lead to several different posttranslational modifications of proteins, including nitration and O\(^{-}\)-glycosylation (5, 7). Hyperglycemia, in particular, leads to increased O\(^{-}\)-glycosylation of proteins through increased flux through the hexosamine pathway. Increased global O\(^{-}\)-glycosylation in cardiac and skeletal muscles has been shown to decrease Ca\(^{2+}\) sensitivity in these tissues and attenuate contractility (6, 18, 29). Previous studies have also shown that diabetes can lead to oxidative stress in smooth muscle (2, 9, 28), and studies with mouse inflammatory bowel models have shown that oxidative stress-related nitration of L-type calcium channels leads to their decreased activity (22, 31). Thus far, we have been unable to demonstrate either O\(^{-}\)-glycosylation or nitration of L-type calcium channels in colon smooth muscle from diabetic mice (data not shown). However, these negative results may simply reflect the limited sensitivity of the reagents we have utilized and hence do not completely rule out a role for these modifications. Further detailed studies will be required to determine if L-type calcium channels are posttranslationally modified in smooth muscle cells of diabetic mice to diminish their activity.

In contrast to mice that were diabetic for less than 1 wk, mice that were hyperglycemic for 7–8 wk exhibited an elevated basal calcium level in addition to the attenuated KCl-induced calcium increase. As L-type calcium channels are known to exhibit calcium-dependent inactivation, it is possible that the elevated basal calcium levels may be directly attenuating the activity of the channels, resulting in decreased KCl-stimulated calcium influx under these conditions. The change in basal calcium levels was associated with changes in expression of several calcium-handling proteins. mRNA levels of SERCA2b and IP\(_3\)R2 were significantly upregulated and mRNA encoding Cav1.2b, PMCA4, ryanodine receptor 2, and sodium-calcium exchanger 2 trended toward being elevated (Fig. 4). In contrast, Western blotting revealed a downregulation of IP\(_3\)R2 and SERCA2 proteins. These inconsistencies between mRNA and protein levels could be explained by compensatory transcriptional mechanisms, occurring in response to downregulated protein levels, resulting from protein posttranslational modifications. In support of this, previous studies have shown that diabetes-induced oxidative stress can lead to nitration of SERCA (41). Nitration, in turn, can attenuate SERCA activity and lead to its degradation, resulting in
impaired Ca\textsuperscript{2+} uptake into the SR. This could be contributing to the downregulation of SERCA2b protein levels and subsequent elevated basal Ca\textsuperscript{2+} levels observed in colon smooth muscle of long-term diabetic mice. In contrast, the slightly elevated PMCA4 protein levels would be expected to increase calcium extrusion from the cell, thereby lowering intracellular Ca\textsuperscript{2+} levels. The elevated PMCA4 expression may thus be another compensatory mechanism that the cell has activated in an attempt to lower basal intracellular Ca\textsuperscript{2+} levels.

Although we observed an increase in basal Ca\textsuperscript{2+} levels in the colon from diabetic mice that were hyperglycemic for 7–8 wk, these changes did not lead to increased basal MLC phosphorylation (Fig. 3D). This result would suggest that the MLC phosphorylation pathway has been desensitized to the calcium signal, possibly through activation of the MLC phosphatase or inhibition of the MLCK in these mice. We did not observe any changes in the expression or phosphorylation of CPI-17 (Figs. 3, E and G), a protein that inhibits MLC phosphatase activity (36). Similarly, we did not observe any changes in the phosphorylation of MYPT1, suggesting that decreased Rho kinase-mediated phosphorylation of CPI-17 or MYPT1 likely does not account for the decreased calcium sensitivity of MLC phosphorylation seen in the tissues from diabetic mice. In contrast, the observed increase in MYPT1 expression levels in the diabetic mice, without any changes in MLCK levels (Fig. 3G), could explain the normal levels of basal MLC phosphorylation in the presence of elevated basal calcium concentrations. It also remains possible that other modifications of the

Fig. 5. Contractility in mice that were hyperglycemic for <1 wk is decreased due to attenuated intracellular Ca\textsuperscript{2+} responses and decreased MLC phosphorylation. One week after becoming hyperglycemic, mice were euthanized, and a middle part of colons was isolated for contractility measurements, Ca\textsuperscript{2+} imaging, mRNA and protein analysis, and MLC phosphorylation measurements. A: colon rings from the central portion of the colon of control (open bars) and 1-wk diabetic mice (STZ; solid bars) were equilibrated at optimal resting tension in Krebs buffer for 1 h and contracted using 60 mM KCl. Following washing, rings were then incubated with tetrodotoxin (1 \textmu M) for 5 min and then stimulated with 60 mM KCl. Values are expressed as percentage of KCl-induced contraction obtained from colon of control mice. *Force that were significantly different from 100% are indicated: * \textit{P} < 0.05 (n = 6–13). B: basal levels of intracellular Ca\textsuperscript{2+} measured in the middle part of the colon obtained from 5–6 control and STZ-treated mice. *P < 0.05. C: mean ± SE relative change in intracellular Ca\textsuperscript{2+} levels in response to 60 mM KCl stimulation in control and STZ-treated mice (n = 5–6 mice). *P < 0.05. D; RNA was isolated from the middle to distal part of colon (P4–D2) obtained from control or STZ-treated (1 wk) mice, and transcripts were quantitated as in Fig. 4. Each bar represents the mean ± SE values obtained from 4–5 different mice. *P < 0.05. E, left: immunoblots of proteins obtained from parallel samples to those shown in D. Molecular mass markers (kDa) are indicated at the left of the blots. Right: quantification of immunoblots following normalizing to vinculin. Each bar represents the mean ± SE of 8 different mice. *P < 0.05, **P < 0.005. F: colon rings obtained from control and STZ-treated (1 wk) mice were hung in a muscle bath, and MLC phosphorylation levels were determined under resting conditions (n = 7 mice) and at the peak of a 60 mM KCl-induced contraction (n = 8 mice). MLC phosphorylation is expressed as MLCphosphorylated/MLCtotal. Values are means ± SE. *P < 0.05.
MYPT1 or posttranslation modification of the MLCK are also contributing to calcium desensitization in the colon smooth muscle of long-term diabetic mice.

Studies have shown that, in diseases such as colitis, inflammatory cytokines, such as TNF-α, can lead to decreased smooth muscle contractility in the gut (23). However, the influence of cytokines in altering GI motility in diabetes has not been previously examined. In our studies, we did not find any elevation of TNF-α mRNA, and in fact observed a decrease in IL-1β mRNA levels in long-term diabetic mice (Fig. 6, C and D). Together these data suggest that the increased iNOS expression likely results from long-term oxidative stress rather than from an intestinal inflammatory response, which would be expected to also increase TNF-α and IL-1β levels. This elevated iNOS could also be contributing to the impaired contractility in the long-term, but not short-term, diabetic mice through increased production of nitric oxide that can directly promote relaxation or promote nitration or nitrosylation of contractile proteins.

In summary, our results show that STZ-induced diabetic mice develop decreased colon contractility as early as 7 days after developing hyperglycemia. The decreased contractility is...
caused by an attenuated depolarization-induced intracellular Ca\(^{2+}\) increase, which leads to decreased MLC phosphorylation and decreased contractility. The primary molecular defect that results in the attenuated intracellular Ca\(^{2+}\) increase is most likely an alteration in the activity of L-type Ca\(^{2+}\) channels, possibly through posttranslational modifications, such as nitration or O-glycosylation. In mice that were diabetic for 7–8 wk, we also observed increased basal intracellular Ca\(^{2+}\) levels that could be due to a decrease in SERCA2b levels, leading to decreased Ca\(^{2+}\) uptake to the sarcoplasmic reticulum. Together, these data demonstrate that, in addition to any effects it may have on enteric neural innervation, diabetes also has a direct effect on colonic smooth muscle cells, impairing their contractility.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


