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The responses to manipulation of extracellular and intracellular calcium are altered in the streptozotocin-diabetic rat colon and ileum

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Abstract

Studies were performed to see if alterations in Ca^{2+} homeostasis underlie the gastrointestinal motility complications seen in many diabetic patients. Experiments were performed on colonic and ileal tissues taken from streptozotocin-induced diabetic and control rats. Diabetes caused alterations in the responses of the tissues to Ca^{2+} manipulation but these differed between the colon and ileum. In the colon a small but not significant increase in contractile responses to $CaCl_2$ was observed in diabetic tissues, whereas the responses of the ileum were depressed relative to those of the controls. In contrast, responses of the diabetic ileum to the Ca^{2+} channel agonist Bay K8644 were greater than those of the controls, whilst the agonist failed to contract the colon. Similarly, the Ca^{2+}-ATPase inhibitors, thapsigargin and cyclopiazonic acid, produced contractions which were greater in diabetic ileal tissues. Thus, alterations in the responses of the diabetic gut to Ca^{2+} manipulation are complex, and also tissue-specific.

Keywords: Streptozotocin-diabetes; Colon; Ileum; Calcium; Motility, (rat)

1. Introduction

Gastrointestinal alterations have been reported in up to 76% of the diabetic patient population (Mathison and Davison, 1988). These alterations affect most of the gastrointestinal tract, and include oesophageal dysmotility (Stewart et al., 1976), gastroparesis (Clarke et al., 1979), constipation (Katz and Spiro, 1966) and diarrhoea (Malins and French, 1957), although constipation and/or diarrhoea are the most commonly reported symptoms (Hosking et al., 1978; Quigley, 1997).

It has been suggested that diabetic constipation and diarrhoea are the result of altered gastrointestinal motility, and both increased and decreased intestinal transit have been measured in diabetic rodent models (Chang et al., 1995; Anjaneyulu and Ramarao, 2002). No definite cause for diabetic gastrointestinal symptoms has been established, although neuropathy and alterations in the interstitial cells of Cajal have been recognised (Katz and Spiro, 1966; Ordog et al., 2000; He et al., 2001).

It has also been postulated that there are smooth muscle alterations in the diabetic state and increased contractile responses to muscarinic agonists (Carrier and Aronstam, 1990; Talubmook et al., 2003), prostaglandins (Talubmook et al., 2003), and potassium chloride (Aihara and Sakai, 1989) have been reported. Furthermore, there have been limited reports of altered calcium (Ca^{2+}) sensitivity in the diabetic gastrointestinal tract. However, the reported changes in response to Ca^{2+} in the gastrointestinal tract are contradictory, with Zhu and Sakai (1996) reporting unaltered responses in 8-week streptozotocin-diabetic rat gastric fundus, whilst reduced responses in the 8-week alloxan-diabetic rat intestine were reported by Ozturk et al. (1996). There appear to be no studies relating to the effect of diabetes on Ca^{2+} homeostasis within the rat colon.

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The aim of this study was to characterise any alterations in response to Ca^{2+} in both the ileum and colon taken from 8-week diabetic rats.

2. Methods

Male, Wistar rats weighing 200–350 g were used in the present study. Animals were assigned into one of two groups: diabetic, or age-matched controls. Housing conditions and all experimental work were conducted in accordance with the Animals (Scientific Procedures) Act 1986 under project licence number PPL 70 4649 with a project title of Gastrointestinal Research.

2.1. Induction of diabetes

Diabetes was induced with a single intraperitoneal injection of streptozotocin (65 mg kg^{-1}, 1 ml 100 g^{-1} body weight) freshly dissolved in 20 mM citrate buffer solution (pH 4.5). Age-matched controls were injected with an equal volume of citrate buffer. The rats were housed two per cage (one control, one streptozotocin-treated) and provided with 2% sucrose water for the first 48 h after injection to prevent hypoglycaemia. Diabetes was verified by a blood glucose level of $\geq$300 mg dl^{-1}, measured using an Accu-Chek active blood glucose testing kit (Roche Diagnostics Ltd) on blood taken from the tail vein. Approximately 10% of the rats injected with streptozotocin failed to become diabetic.

2.2. Tissue preparation

Eight weeks after injection, control and streptozotocin-diabetic rats were killed by carbon dioxide asphyxiation, and the body weight and blood glucose levels were measured. The abdominal cavity was opened via a midline incision and the ileum and colon were removed and immediately immersed in Krebs solution (composition (mM): NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; K_2HPO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25, and D-glucose, 11.1), gassed with 95% O_2/5% CO_2.

Pieces of colon approximately 1 cm in length were removed from the proximal end and opened out, the contents removed with the aid of a cotton bud and segments of tissue cut (~5 mm×2 mm) and mounted on the circular axis in 30 ml organ baths filled with gassed Krebs solution and maintained at 37 °C. Pieces of whole distal ileum approximately 2 cm in length were flushed through with buffer and mounted in the longitudinal axis in 30 ml organ baths filled with gassed Krebs solution and maintained at 37 °C.

The tissues were attached via a thread to force displacement transducers (Dynamometer UF1) which were connected to a chart recorder (Lectromed 5041) via a preamplifier (Lectromed 3552) to record changes in isometric tension. A tension of 1 g was applied to each tissue, and tissues were allowed to equilibrate for at least 30 min before the experiments were commenced.

Whilst the tissues equilibrated, spontaneous activity was observed to develop in both the ileum and colon. This activity was recorded, and the frequency and amplitude of the waves measured as the change in tension above baseline levels when a plateau of activity had been achieved. Measurements taken in the presence of drugs were made in a similar manner.

Cumulative concentration–effect curves to the Ca^{2+} ionophore A23187 (5-(Methylamino)-2-[[2*R*,3*R*,6*S*,8*S*,9*R*,11*R*)-3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid; 10 nM–10 μM) or Bay K 8644 (1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridinecarboxylic acid, methyl ester; 1 nM–1 μM) were performed at half log-unit intervals, each response being allowed to plateau before the next concentration was added.

For cumulative concentration–effect curves to $CaCl_2$ (10 μM–100 mM), the tissues were immediately submerged in Ca^{2+}-free buffer after removal from the animals. Concentration–effect curves to $CaCl_2$ were then performed at half log-unit intervals, as usual. Again each response was allowed to plateau before the next concentration was added. It should be noted that all spontaneous activity was abolished by incubation in the Ca^{2+}-free buffer.

Single concentrations of the Ca^{2+}-ATPase inhibitors thapsigargin (1 μM) or cyclopiazonic acid (1 μM) were added to the organ bath and any response allowed to plateau, both in terms of spontaneous activity and increased tension from baseline levels.

The concentration–effect curves to A23187, Bay K 8644 and $CaCl_2$ were expressed as a change in tension at each concentration. Results are shown as a mean±S.E.M., where *n* indicates the number of tissues used (maximum two tissues per animal). Differences between means (at each concentration) were determined using two-way analysis of variance (ANOVA) with interactions followed by the Bonferroni modified *t*-test for multiple comparisons. Probability levels of less than 0.05 ($P<0.05$) were taken to indicate statistical significance.

Responses to single concentrations of thapsigargin or cyclopiazonic acid were measured either as an increase in tension from baseline levels, or as a reduction in spontaneous activity as appropriate. Again, results are shown as a mean±S.E.M., where *n* indicates the number of tissues used (maximum two tissues per animal). Differences between means were determined by Student's *t*-test for unpaired data. As before, probability levels of less than 0.05 ($P<0.05$) were taken to indicate statistical significance.

2.3. Drugs

Nifedipine, *N*-methylnitrocarbamyl-D-glucosamine (streptozotocin, STZ) and Bay K 8644 were obtained from

Sigma, Poole, UK. Cyclopiazonic acid, thapsigargin and A231287 were purchased from Tocris, Avonmouth, UK. Calcium chloride ($CaCl_2$) was obtained from Fischer Chemicals, Loughborough, UK.

3. Results

3.1. Concentration–effect curves to calcium chloride

As previously shown, a concentration-dependent inhibition of the spontaneous activity of both the distal ileum and proximal circular colon was caused by the L-type Ca^{2+}-channel blocker nifedipine, with abolition of this activity at 10 μM (Forrest and Parsons, 2003). In this study, this observation was confirmed since no spontaneous activity was observed to develop in either tissues when Ca^{2+} was omitted from the Krebs buffer bathing the tissue (data not shown).

Concentration–effect curves to calcium chloride ($CaCl_2$; 10 μM–1 mM, half log unit intervals) were established in the proximal circular colon (n=12 (control); n=13 (streptozotocin)) and distal whole ileum (n=10 (control); n=9 (streptozotocin)). In the colon, contractile responses to $CaCl_2$ were higher in diabetic tissues compared to the controls at the two highest concentrations, although these differences failed to reach statistical significance (P>0.05; Fig. 1A). When the data was expressed as a percentage of the maximum response, the EC_{50} values for the concentration effect curves were approximately 300 μM in both control and diabetic tissues (Fig. 1B). The contractile responses to $CaCl_2$ in the distal ileum segments (n=10 (control); n=9 (streptozotocin)) were significantly lower in the diabetic tissue preparations at the three intermediate concentrations (P<0.05), but were unaltered at the lowest and highest concentrations (P>0.05; Fig. 2).

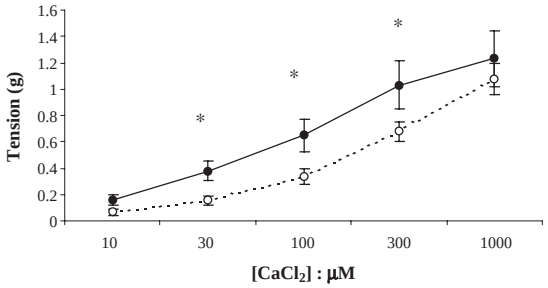

Fig. 2. Cumulative concentration–effect curves to $CaCl_2$ (10–1000 μM) expressed as developed tension (g) above basal tone in control (●, n=10) and diabetic (○, n=9) rat distal whole ileum (mean±S.E.M.). *P<0.05 significantly different from controls.

3.2. Concentration–effect curves to A23187 and Bay K 8644

A23187 (1 nM–10 μM; half log units) produced a concentration-dependent contraction of the rat distal ileum that was not significantly different between control and diabetic preparations at any concentration (n=10; P>0.05, Fig. 3A). The EC_{50} values for the control and diabetic concentration–effect curves were approximately 100 nM. Bay K 8644 also produced a contractile response in the

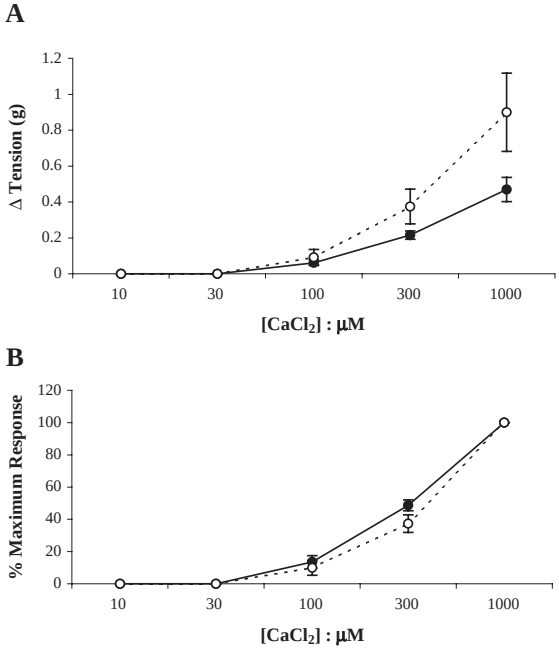

Fig. 1. Cumulative concentration–effect curves to $CaCl_2$ (10–1000 μM) expressed as (A) developed tension (g) above basal tone, and (B) percentage of the maximum response in control (●, n=12) and diabetic (○, n=13) rat proximal circular colon (mean±S.E.M.).

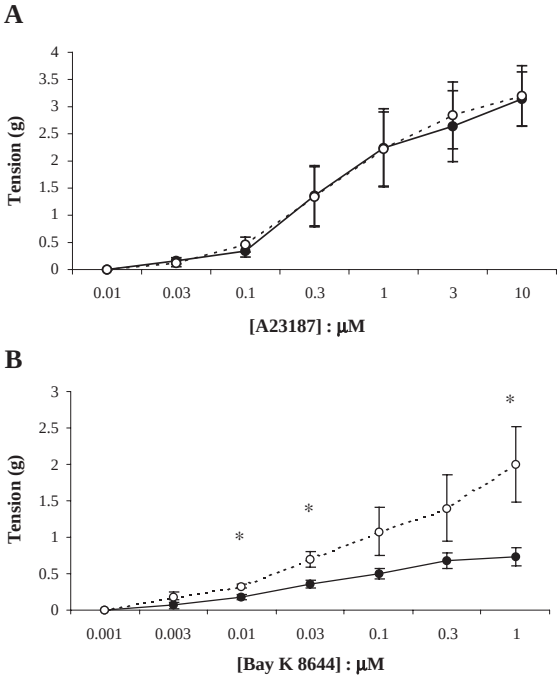

Fig. 3. Cumulative concentration–effect curves to (A) the calcium ionophore A23187 (0.001–10 μM, n=10 and (B) Bay K 8644 (0.001–1 μM, n=5) in control (●) and diabetic (○) rat distal whole ileum (mean±S.E.M.). *P<0.05 significantly different from controls.

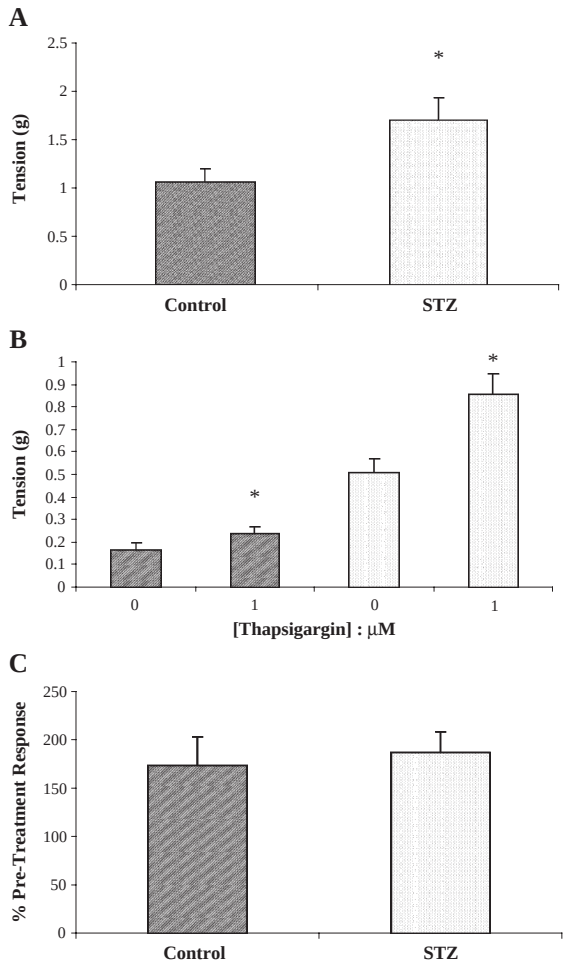

Fig. 4. (A) Contractile responses to thapsigargin (1 μM; n=14), and the effect of thapsigargin (1 μM) on the spontaneous activity of control and diabetic rat distal ileum expressed as (B) tension (g), and (C) percentage of the pre-treatment response (mean±S.E.M.) in control (▨) and diabetic (▩) rat distal whole ileum. *P<0.05 significantly different from controls.

ileum, but these contractions were significantly greater in diabetic tissues at the 0.01 μM, 0.03 μM and 1 μM concentrations compared to control tissues (n=5; P<0.05, Fig. 3B). The EC_{50} values for the control and diabetic concentration–effect curves were approximately 30 nM and 100 nM, respectively.

Neither A23187 nor Bay K 8644 produced contractions of the colon, and the amplitude of spontaneous activity was unchanged at all concentrations of A23187 and Bay K 8644 in both control and diabetic tissues (data not shown).

3.3. The effect of thapsigargin and cyclopiazonic acid

Thapsigargin (1 μM) was applied to distal ileum segments, and produced sustained contractions of the ileum preparations that were significantly larger in the diabetic tissues than in control tissues (n=14; 1.05±0.13 g (control); 1.70±0.23 g (streptozotocin); P<0.05, Fig. 4A). In addition to the contractile effect observed in the presence of thapsigargin, a significant increase in the amplitude of both the control and diabetic spontaneous activity was also observed (0.16±0.03 g vs. 0.24±0.03 g (control); 0.51±0.06 g vs. 0.86±0.09 g (streptozotocin); P<0.05, Fig. 4B). However, when the data was expressed as a percentage of the pre-treatment response, there was no difference in the level of increase of spontaneous activity in response to thapsigargin between control and diabetic tissues (173.07±29.21% (control) vs. 186.67±21.14% (streptozotocin); Fig. 4C). Cyclopiazonic acid (1 μM) produced sustained contractions of the ileum preparations that were not significantly different between control and diabetic tissues (n=6; 3.46±0.73 g (control); 3.38±0.46 g (streptozotocin); P>0.05, data not shown).

No response to either thapsigargin or cyclopiazonic acid was obtained in the ileum in the absence of exogenous Ca^{2+} (n=6), although both compounds produced a contractile response in the presence of nifedipine (10 μM; n=12 and n=6, respectively). It is interesting to note that nifedipine caused a potentiation of the contractile responses to thapsigargin (n=12; control: 1.05±0.13 g vs. 2.28±0.21 g; streptozotocin: 1.70±0.22 g vs. 2.85±0.19 g, P<0.05;

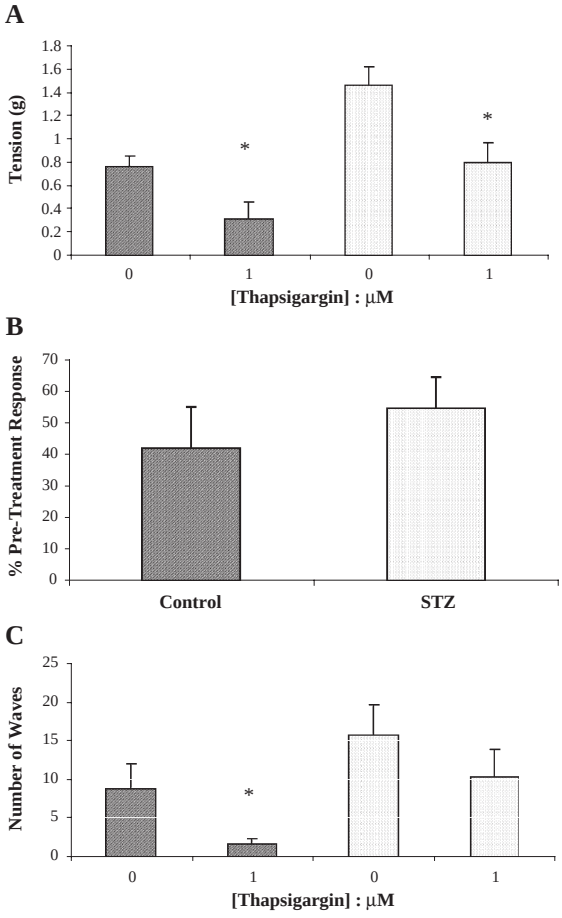

Fig. 5. The effect of thapsigargin (1 μM) on the amplitude expressed as (A) tension (g), and (B) percentage inhibition, and (C) the frequency of contractions as measured in five min of control (▨) and diabetic (▩) rat proximal circular colon spontaneous activity (mean±S.E.M., n=12). *P<0.05 significantly different from pre-treatment values.

data not shown), although the responses to cyclopiazonic acid were unaffected by the presence of nifedipine.

When thapsigargin was applied to colonic tissues it produced a significant inhibition of the amplitude of the spontaneous contractions of both control and diabetic colon with no alteration in baseline tension (n=12; control: 0.76±0.10 g vs. 0.32±0.14 g; streptozotocin: 1.46±0.16 g vs. 0.80±0.17 g, P<0.05; Fig. 5A). However, there was no difference in the levels of inhibition produced in control and diabetic tissues when the data was expressed as a percentage of the pre-treatment response (control: 42.10±13.03%; streptozotocin: 54.74±9.86%; P>0.05, Fig. 5B). The frequency of the spontaneous contractions measured over 5 min was significantly reduced in control tissue, but not in diabetic tissue (control: 8.83±3.21 waves vs. 1.67±0.66 waves, P<0.05; streptozotocin: 15.75±3.87 waves vs. 10.25±3.67 waves, P>0.05; Fig. 5C).

Similarly, cyclopiazonic acid produced a significant inhibition of the amplitude of the spontaneous contractions of both the control and diabetic colon with no alteration in baseline tension (control: 0.61±0.04 g vs. 0.48±0.03 g, P<0.05; streptozotocin: 1.66±0.06 g vs. 1.12±0.07 g, P<0.05; n=12; Fig. 6A). When expressed as a percentage inhibition of the amplitude of basal spontaneous activity, no difference in the level of inhibition was observed between control and diabetic tissues (p>0.05, Fig. 6B). Likewise, the frequency of the spontaneous contractions measured in 5 min was unaffected by cyclopiazonic acid in both control and diabetic tissue (control: 10.81±1.58 waves vs. 9.36±2.09 waves, P>0.05; streptozotocin: 9.09±1.39 waves vs. 12.72±1.42 waves, P>0.05; Fig. 6C).

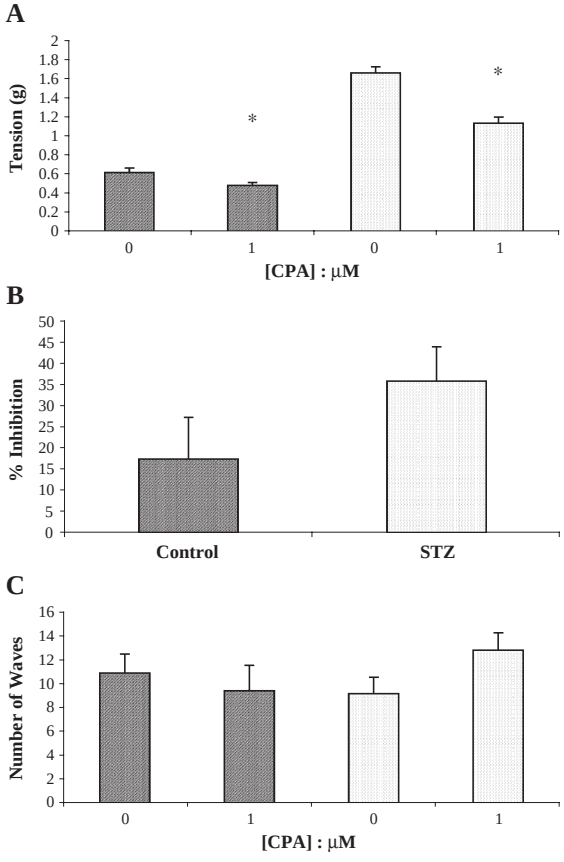

Fig. 6. The effect of CPA (1 μM) on the amplitude expressed as (A) tension (g), and (B) percentage inhibition, and (C) frequency of contractions as measured in five min of control (▨) and diabetic (▩) rat proximal circular colon spontaneous activity (mean±S.E.M., n=12). *P<0.05 significantly different from pre-treatment values.

4. Discussion

The current study was undertaken to investigate the role of calcium in the control of motility of two types of normal intestinal tissue (ileum and colon) and to compare the results with tissues taken from chronically diabetic animals.

Both ileum and colonic tissues displayed spontaneous activity in vitro. The electrical activity underlying the spontaneous mechanical activity recorded in these experiments is thought to originate from networks of interstitial cells of Cajal (ICC) with moderation by the enteric nervous system (Huizinga, 1999; Sanders, 1996). The current study shows that in both normal and diabetic tissues this spontaneous activity ceases upon removal of extracellular calcium. We have previously demonstrated that the spontaneous contractions are markedly increased in amplitude in the diabetic ileum and colon compared to normal tissues, and can be specifically and concentration-dependently inhibited by the L-type calcium channel blocker nifedipine in a similar way to control tissues (Forrest and Parsons, 2003). These results are evidence that the flow of extracellular calcium through L-type channels is involved in the generation of spontaneous activity, but that this increased activity is not directly due to changes in L-type calcium channels as both normal and diabetic tissues were equally sensitive to nifedipine.

The control of this spontaneous activity was investigated by examining a possible role for intracellular calcium stores. Thapsigargin and cyclopiazonic acid (CPA) are blockers of the endoplasmic reticulum calcium pump. In both control and diabetic ileum, these Ca^{2+}ATPase inhibitors actually increased the amplitude of the spontaneous activity, indicating that the intracellular stores are not essential for the generation of this activity. In the colonic tissues, these agents did produce some reduction of this activity but the data again suggests that intracellular Ca^{2+} does not play a major controlling role. In the ileum, both thapsigargin and CPA also produced a sustained baseline contraction which was abolished by the removal of extracellular calcium but not by nifedipine which caused a potentiation of the contractile response. These results are consistent with leakage of calcium from intracellular stores which are replenished by extracellular calcium through capacitive calcium entry (Gibson et al., 1998). The contraction produced by thapsigargin and the increase in spontaneous

activity were larger in diabetic than in normal ileum, indicating a more efficient utilisation of calcium from intracellular stores by diabetic tissue. The fact that the response to CPA was not greater in diabetic tissues may reflect known differences between the two Ca^{2+} ATPase inhibitors. For example, in studies on rat arterial rings, it has been reported that CPA raised tension in the tissue but thapsigargin had no effect (Shima and Blaustein, 1992).

As stated previously, ileal and colonic tissues stabilised in calcium-free salt solution displayed no spontaneous activity, but contracted concentration-dependently in response to addition of extracellular calcium. However while the diabetic colon showed a tendency towards larger responses to calcium in terms of maximum response produced, the diabetic ileum was significantly less sensitive to calcium than normal tissue. The latter result is consistent with those from the alloxan model of diabetes in the rat duodenum (Ozturk et al., 1996). The results indicate that in the rat at least, the colon and ileum are differentially affected by chronic diabetes and that in the ileum the increased response to thapsigargin in diabetic tissues is unlikely to be due to an increased sensitivity to calcium. To further examine whether changes in calcium access to the cell were underlying the altered responses to extracellular calcium in diabetic tissues the non-specific calcium ionophore A23187 (Borle and Studer, 1978) was used to obtain direct access of extracellular calcium to the cytoplasm. Unlike the situation with exogenously applied $CaCl_2$ where there was a reduction of the response of the diabetic ileal tissue, there were no significant differences between control and diabetic tissues in response to A23187. This suggests that the decrease in the response to calcium in the ileum induced by diabetes is due to a change in access of extracellular calcium to the contractile apparatus.

In the presence of normal extracellular calcium, the specific L-type calcium channel opener BaY K 8644 caused a concentration-dependent contraction of the ileum which was larger in the diabetic tissues than controls. Enhanced responses of diabetic tissues to BaY K 8644 have been reported before. In vascular smooth muscle cells from streptozotocin-induced diabetic rats, the responses of L-type voltage-dependent Ca^{2+} channels to BaY K 8644 were significantly greater than controls (Wang et al., 2000). However the results with BaY K 8644 in the present study taken together with the non-specific ionophore data suggests that diabetes may be affecting calcium access by a mechanism not involving L-type calcium channels.

It is clear from these studies that the rat proximal colon behaves very differently compared to the ileum in response to calcium manipulation, in that neither of the Ca^{2+} ATPase inhibitors caused contractile responses, nor did A23187 or BaY K 8644. These observations are difficult to explain but may reflect differences in the mechanisms of calcium utilisation within the tissues.

Overall the results show that diabetes affects both spontaneous and evoked contractile responses but there are marked differences in the effect of calcium manipulation between the colon and the ileum. Further experiments directly investigating intracellular Ca^{2+} signalling will be necessary to clarify the situation.

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