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Involvement of enteric neurones in the response of guinea-pig ileum preparations to metoclopramide

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1 The role of myenteric neurones in mediating the stimulant effects of metoclopramide *in vitro* in the guinea-pig ileum has been investigated using the non-ionic surfactant Triton X-100.

2 Histological examination of the ileum 30 days after application of Triton X-100 to the serosal surface demonstrated a marked reduction in the number of ganglion cells and nerve elements in the myenteric plexus.

3 Longitudinal muscle-myenteric plexus (LM-MP) preparations from Triton X-100-treated animals were unresponsive to dimethylphenylpiperazinium and responded poorly or not at all to electrical field stimulation.

4 Metoclopramide (30 μM) elicited small contractions in LM-MP preparations from control and sham-operated animals but failed to contract Triton X-100-treated tissues. However, tissues responded in a similar manner to exogenous acetylcholine (ACh).

5 These results demonstrate the importance of a prejunctional site of action for metoclopramide in this tissue and suggest that contractile responses to the drug are mediated indirectly, probably by increased release of ACh from myenteric neurones.

Introduction

The mechanism whereby metoclopramide increases gastrointestinal (GI) motility is not clear but appears not to involve dopamine receptors directly (see review by Fernández & Massingham, 1985; Costall *et al.*, 1984; Gunning & Naylor, 1985). Many observations concur that the drug facilitates spontaneous and stimulation-evoked release of acetylcholine (ACh) but a possible postjunctional sensitization of the smooth muscle cells to this neurotransmitter remains controversial (Beani *et al.*, 1970; Bianchi *et al.*, 1970; Fontaine & Reuse, 1972; 1973; Bury & Mashford, 1976; Anderson *et al.*, 1977; Fosbraey & Johnson, 1980; Fosbraey *et al.*, 1980; Kilbinger *et al.*, 1982; Zar *et al.*, 1982; Massingham *et al.*, 1985).

Fox *et al.* (1983) have recently demonstrated that surfactants can selectively ablate enteric neurones in the rat jejunum. The present studies were carried out in guinea-pigs receiving a local application of the non-ionic surfactant Triton X-100 to the ileum. This simple technique should be useful in studying the role of myenteric nerves in the response of the ileum to metoclopramide and for identifying the major site of action of this drug. A preliminary account of this work has been presented to the British Pharmacological Society (Bou *et al.*, 1985).

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Methods

Experiments were performed on 50 male Tricolour guinea-pigs weighing 350 ± 50 g, 12 animals were treated with Triton X-100, 12 animals were sham-operated and the remainder served as untreated controls.

Treatment of animals with Triton X-100

The technique used was basically that described by Fox *et al.* (1983) for the rat. Guinea-pigs, allowed free access to food and water, were anaesthetized with pentobarbitone (40 mg kg^{-1} i.p.), the abdomen shaved and washed with an antiseptic solution. Following laparotomy, a portion of the ileum was brought outside the peritoneal cavity and an 8–10 cm segment identified using 2 suture tags. Triton X-100 (1% solution in 0.9% sterile saline) was then applied to the serosal surface of the intestine between these tags every 5 min for 30 min (6 applications). Sham-operated

animals received similar applications of a 0.9% sterile saline solution. After treatment, the serosal surface of the treated tissues was thoroughly rinsed with sterile saline and returned to the peritoneal cavity. Animals were housed singly in cages with wire-mesh bottoms, allowed free access to food and water and were given antibiotic treatment for 4 days postoperatively. Thirty days after the operation animals were killed by stunning and cutting both carotid arteries. Tissues from treated, sham-operated and untreated control animals were removed, cleaned and quickly placed in warm Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $CaCl_2$ 2.6, glucose 11.1. Tissues were then used for histological examination or for mechanical studies as described below.

Histological studies

For low power (× 32) microscopic examination, longitudinal muscle-myenteric plexus (LM-MP) preparations were prepared according to the method described by Paton & Vizi (1969). Tissues were then stained with a 1% methylene blue buffer solution for 10 min, washed thoroughly with 0.9% NaCl and stretch-mounted on microscope slides. For higher power (× 125) examination, the whole ileum was fixed in 4% formaldehyde solution, embedded in paraffin wax and 5 μm thick longitudinal sections cut and stained with haematoxylin-eosin.

Mechanical studies

LM-MP preparations, length 3–4 cm, from untreated (control), sham-operated and Triton X-100-treated animals were mounted in 30 ml organ baths containing Krebs solution maintained at 37°C and aerated with 95% O_2, 5% CO_2. The Krebs solution routinely contained propranolol (3 μM). Responses of the tissues were measured isometrically using Letica force transducers (Type TRI-010) and 4 channel Leticograph-4000 chart recorders. Tissues were permitted a 30 min equilibration period then, using a 1 min contact time and 5 min time cycle, were maximally contracted several times with ACh (1–10 μM) before commencing one of the following protocols.

Protocol 1 Following stable responses to ACh, tissues were exposed 2 or 3 times to dimethylphenylpiperazinium (10 μM) for 1 min followed by 10 min washing before constructing a full concentration-response curve to ACh (0.01–1 μM).

Protocol 2 Following stable responses to ACh, a full concentration-response curve was constructed (ACh, 0.01–10 μM). Fifteen minutes later field stimulation was carried out using parallel platinum electrodes and Grass S-88 stimulators; single square wave pulses (0.5 ms), supramaximal voltage (80 V) and a frequency of 0.2 Hz. After stabilization of the twitch, stimulation was stopped and, 10–15 min later, tissues were exposed to metoclopramide (30 μM) for 5 min.

Protocol 3 Following stable responses to ACh, a full concentration-response curve was constructed to ACh (0.01–1 μM) and 15 min later the ACh curve repeated in the presence of metoclopramide (10 μM, 10 min preincubation). Experiments on tissues taken from sham-operated and Triton X-100-treated animals not exposed to metoclopramide were run simultaneously as time controls.

Drugs

The following drugs were used: metoclopramide hydrochloride (synthesized by Dr A. Vega, Department of Chemistry, Laboratorios Almirall), Triton X-100 (Merck), ACh hydrochloride (Sigma), dimethylphenylpiperazinium iodide (EGA Chemie), and propranolol hydrochloride (Sigma).

Statistical analysis

Results (expressed as mean values ± s.e.mean) were analysed by Student's 2-tailed t test. The level of significance was $P < 0.05$.

Results

Histological examination

Low power microscopic examination was carried out on LM-MP preparations obtained from 5 Triton X-100-treated and 5 sham-operated animals. This revealed an absence of the typical nerve network on the surface of the longitudinal muscle layer in Triton X-100-treated tissues. Whilst a few disrupted ganglia persisted at the extreme edges of the treated segment, in the central region they were absent and the nerve network only poorly defined. Figure 1 illustrates the marked loss of neurones in the myenteric plexus following Triton X-100 treatment (Figure 1b) compared with tissue from a sham-operated animal (Figure 1a). An almost complete loss of neurones was confirmed by higher power microscopic examination of longitudinal sections of whole ileum. These studies were conducted on tissues from 3 Triton X-100-treated and 3 sham-operated animals. At least 16 sections at various levels of each ileum segment were prepared and examined for the presence of nerve elements between the two smooth muscle layers. The surfactant, at this concentration, caused virtually complete abla-

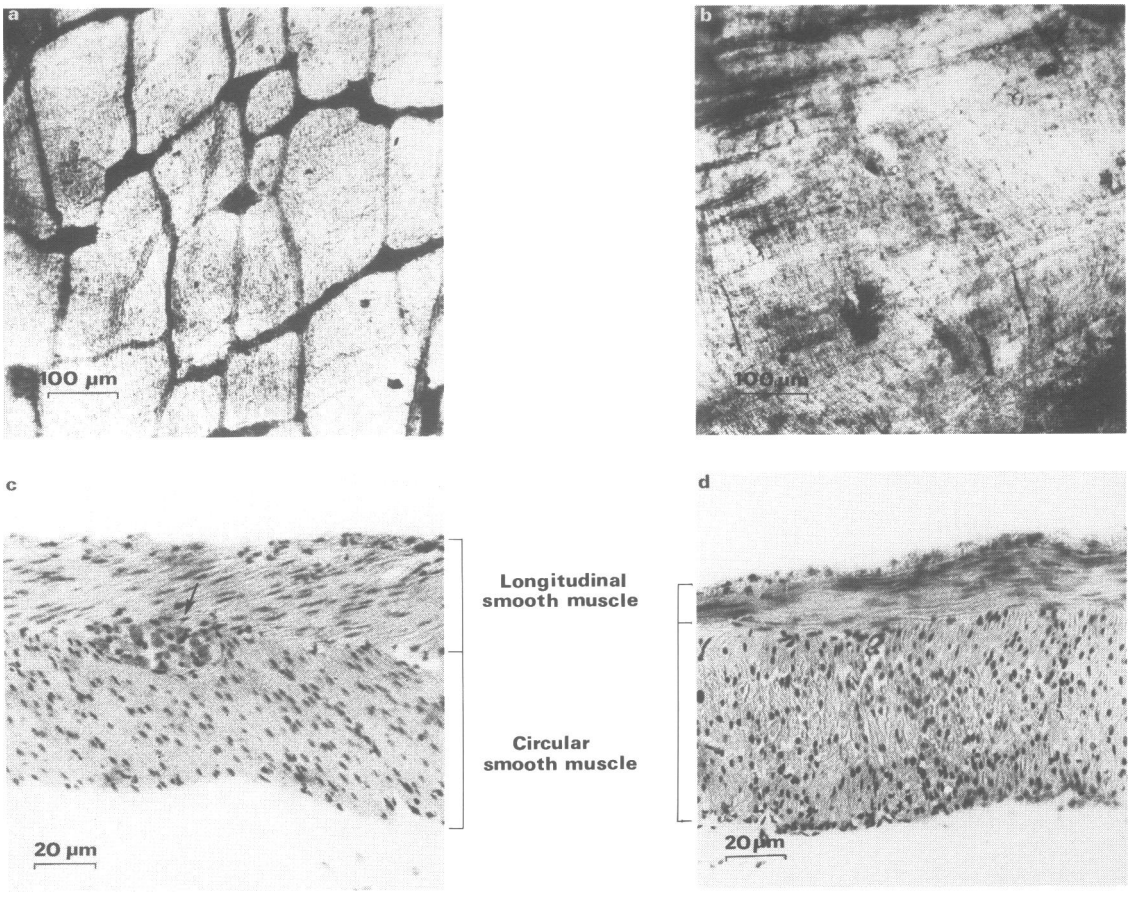

Figure 1 Typical histological findings in sham-operated and Triton X-100-treated guinea-pig ileum preparations. (a and b) Low power (×32) view of longitudinal muscle-myenteric plexus (LM-MP) preparations obtained from: (a) sham-operated animals, showing the typical network of nerves overlying the longitudinal smooth muscle layer and (b) guinea-pig receiving local application of Triton X-100 (1% solution 30 days before killing the animal; see Methods for details). Note the absence of the myenteric plexus. (c and d) Higher power (× 125) photographs of longitudinal 5 μm thick sections of ileum from: (c) sham-operated guinea-pig following haematoxylin-eosin staining, note the presence of neuronal elements and ganglion (arrow) in the myenteric plexus between the inner circular and outer longitudinal smooth muscle layers; and (d) a Triton X-100-treated animal where no ganglion cells in the area of the myenteric plexus are visible. The smooth muscle layers have a normal appearance and have not been disrupted by the surfactant.

tion of myenteric neurones and no ganglia were observed in any of the sections taken from the treated tissues (compare c and d in Figure 1).

Response to dimethylphenylpiperazinium

LM-MP preparations from Triton X-100-treated animals were always quiescent and 8 tissues from 4 treated animals failed to respond to the ganglion stimulant dimethylphenylpiperazinium (10 μM). In contrast, tissues from 5 sham-operated and 10 control animals all responded to dimethylphenylpiperazinium with brisk but transient contractions. (Figure 2).

Response to electrical field stimulation

Responses to electrical field stimulation (0.2 Hz, 0.5 ms, 80 V) were consistently reduced by Triton X-100 treatment but were not abolished in all tissues. Table 1 details these findings, responses being classified as normal, impaired or absent. An impaired response to field stimulation is shown in Figure 3

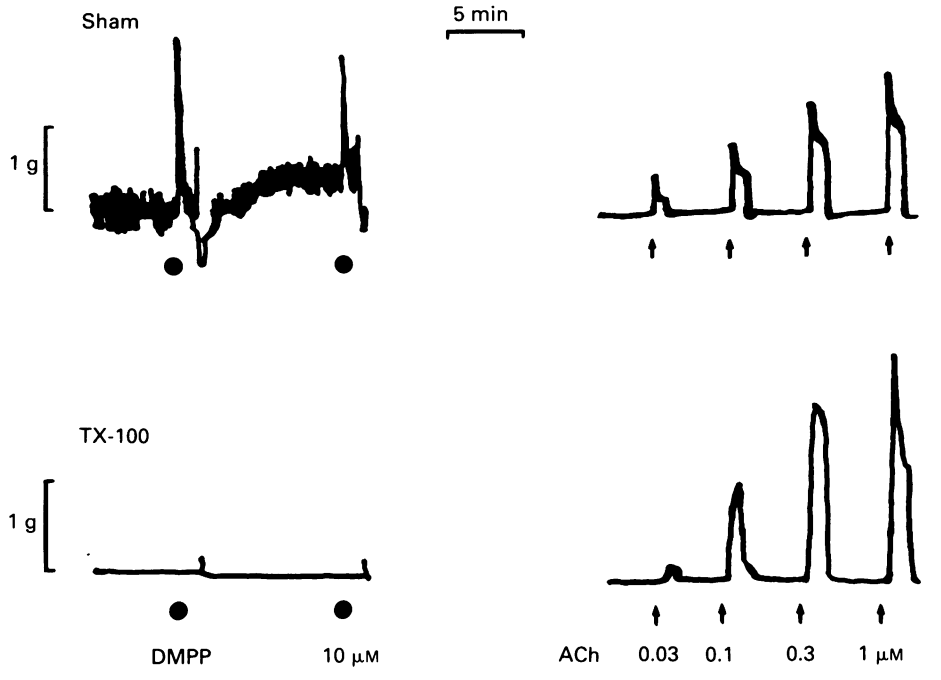

Figure 2 Typical traces showing the mechanical activity of longitudinal muscle-myenteric plexus preparations from a sham-operated (sham, upper traces) and Triton X-100-treated guinea-pig (TX-100, lower traces). Note the absence of spontaneous activity in the Triton X-100-treated preparation and the lack of response to dimethylphenylpiperazinium (DMPP, 10 μM added at the dots) compared with the tissue from the sham-operated animals. The Triton X-100-treated tissue responded normally to exogenous acetycholine (ACh; 0.03–1 μM, added at the arrows). Recorder sensitivity was constant throughout the experiment.

Table 1 Analysis of the responses elicited by (a) electrical field stimulation and (b) metoclopramide in LM-MP preparations taken from control, sham-operated and Triton X-100-treated guinea-pigs

	a Response to EFS				**b** Response to MTC			
Treatment (no. animals)	*Normal*	*Impaired*	*Absent*	n	*Normal*	*Impaired*	*Absent*	n
Control (14)	12	2	0	14	12	1	1	14
Sham (9)	17	4	2	23	16	4	1	21
TX-100 (9)	0	8	15	23	0	3	13	16

Results show the frequency and type of response observed to electrical field stimulation (EFS: 0.2 Hz, 0.5 ms, 80V) and metoclopramide (MTC: 30 μM, 5 min) in longitudinal muscle – myenteric plexus (LM-MP) in preparations from control, sham-operated (Sham) and Triton X-100 (TX-100)-treated guinea-pigs. The number of animals used in these experiments is shown in parentheses and *n* refers to the total number of observations made. Responses to field stimulation and metoclopramide have been classified as normal, impaired or absent. An impaired response to field stimulation was recorded when the stimulus failed consistently to produce a tissue response. An impaired response to metoclopramide was defined as one equal to, or less than, 12.5% of the tension produced by the tissue to 1 μM acetylcholine. See Results for further details.

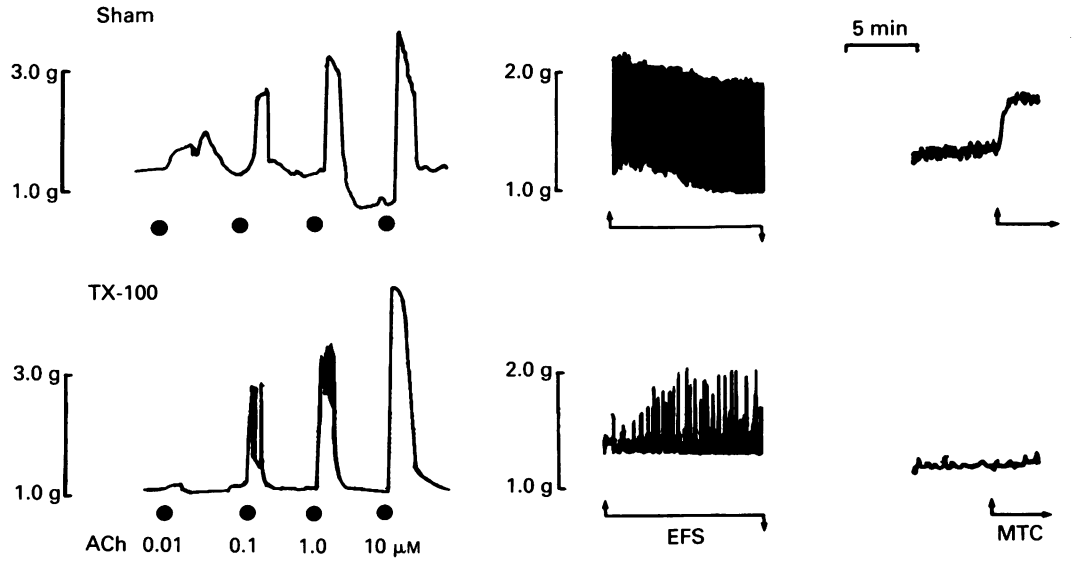

Figure 3 Typical traces obtained from longitudinal muscle-myenteric plexus preparations from sham-operated (Sham, upper traces) and Triton X-100-treated (TX-100, lower traces) guinea-pigs. The figure shows, from left to right, a non-cumulative concentration-response curve to acetylcholine (ACh; 0.01–10 μM), the response to electrical field stimulation (EFS; supramaximal voltage, 0.5 ms square wave pulses, 0.2 Hz) and the effect of adding metoclopramide (MTC; 30 μM) to the organ bath. See Methods section for additional details. The tissue from the Triton X-100-treated animal, whilst responding normally to exogenous ACh, responded only poorly to field stimulation and did not contract to metoclopramide as did the tissue from the sham-operated animal. Note the change in recorder sensitivity between the ACh concentration-response curve and field stimulation. The higher recorder sensitivity was maintained when recording responses to metoclopramide.

(lower centre trace). In such cases each electrical impulse failed to evoke consistent tissue responses, indeed on many occasions no response at all was observed. Of 14 tissues from 14 control animals, 12 responded normally and 2 had an impaired response. In the sham-operated group of 9 animals, responses to field stimulation were normal in 17, impaired in 4 and absent in 2 out of a total of 23 tissues. In contrast, not one of the 23 tissues obtained from 9 animals pretreated with Triton X-100 responded normally to field stimulation, responses were absent in 15 and impaired in 8 tissues.

Response to metoclopramide

Responses to metoclopramide (30 μM, 5 min) were not abolished in all tissues treated with Triton X-100 and were therefore classified as normal, impaired or absent. A normal response to metoclopramide can be seen in Figure 3, upper right-hand trace. In tissues obtained from 6 control guinea-pigs, ACh (1 μM) and metoclopramide (30 μM) produced peak contractions of 2.0 ± 0.2 g and 0.5 ± 0.1 g, respectively. These findings are similar to results obtained in the whole ileum (Massingham *et al.*, 1985). An impaired response to metoclopramide (30 μM) was defined as one being equal to, or less than, 12.5% of the maximum response of the tissue to 1 μM ACh. As shown in Table 1, of 14 control tissues 12 responded normally, 1 exhibited an impaired response and in a further preparation metoclopramide was inactive. A similar picture was observed in 21 tissues from 9 sham-operated animals, 16 responded normally, 4 with impaired responses and in 1 there was no response. In contrast not one of the 16 preparations obtained from 9 Triton X-100-treated animals responded normally to metoclopramide, in 13 tissues the compound had no effect and in 3 the response was impaired.

Response to exogenous acetylcholine

Five tissues from 3 sham-operated and 3 Triton X-100-treated animals responded to a similar extent, and over the same concentration range, to exogenous ACh, the respective mean EC_{50} values being 0.19 μM and 0.15 μM (see Figure 4).

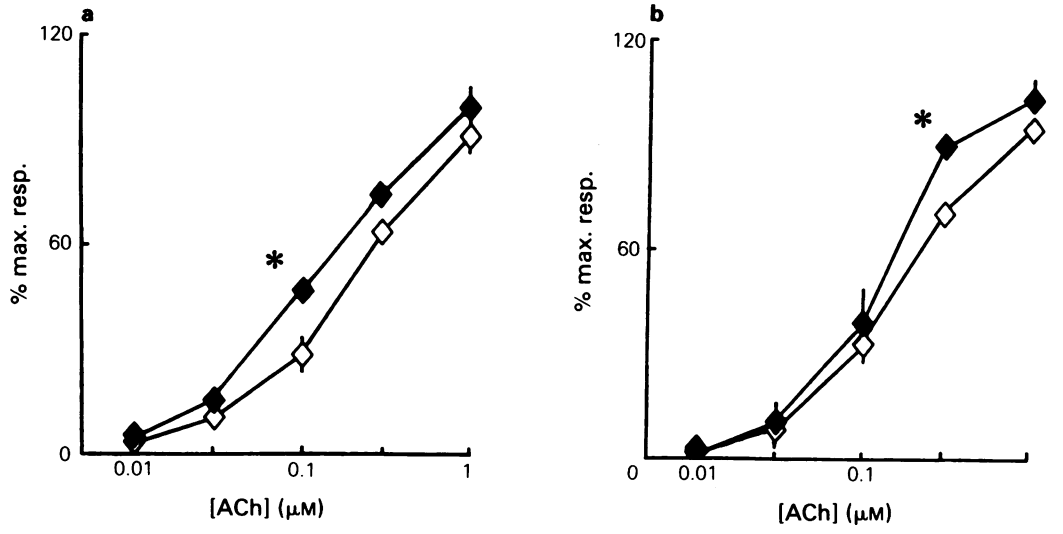

Figure 4 Concentration-response curves to exogenous acetylcholine (ACh) in longitudinal muscle-myenteric plexus ileum preparations from (a) sham-operated and (b) Triton X-100-treated guinea-pigs. Ordinates: contraction amplitude, expressed as a percentage of the maximum response (% max. resp.) to 1 μM ACh obtained in tissues from sham-operated animals during the control period in Krebs solution. Abscissae: concentrations of ACh added to the organ bath. Concentration-response curves to ACh (0.01 – 1 μM) were elicited in Krebs solution (open symbols) and in Krebs solution containing metoclopramide (10 μM, closed symbols). Initial responses to ACh in tissues from sham-operated and Triton X-100-treated animals were not statistically significantly different, maximum responses to 1 μM ACh being 1.3 ± 0.3 g and 1.7 ± 0.3 g respectively. The presence of metoclopramide significantly augmented responses to 0.1 μM ACh in sham-operated and 0.3 μM ACh in Triton X-100-treated tissues (*$P < 0.05$, Student's t test), all other responses were not significantly affected. Each point represents the mean ± s.e.mean value obtained from 5 tissues taken from at least 3 animals.

Effect of metoclopramide on responses to exogenous acetylcholine

The presence of metoclopramide (10 μM) had little or no effect on responses to ACh. In tissues from 3 sham-operated animals metoclopramide did significantly augment responses to 0.1 μM ACh and a similar effect was observed in tissues taken from 3 Triton X-100-treated animals but at 0.3 μM ACh (Figure 4). These small, inconsistent effects in the presence of metoclopramide were considered to be of minor importance.

Discussion

The major finding of this study was that metoclopramide (30 μM) failed to contract LM-MP preparations obtained from Triton X-100-treated guinea-pigs. The lack of response to metoclopramide in such tissues was not explicable in terms of a reduced end-organ sensitivity to the neurotransmitter, since concentration-response curves to exogenous ACh in sham- and Triton X-100-treated preparations were practically superimposable. Therefore the failure of metoclopramide to contract tissues following Triton X-100 treatment is most likely due to the destruction of myenteric neurones by the surfactant. Histological examination of LM-MP preparations and longitudinal sections of whole ileum directly demonstrated that, as reported previously in the rat jejunum (Fox *et al.*, 1983), the local application of Triton X-100 can ablate myenteric nerves. This, and the finding that metoclopramide (10 μM) did not modify responses to ACh in treated tissues suggests it is upon the myenteric nerves, rather than the smooth muscle elements, that metoclopramide exerts its pharmacological effects. Furthermore, these results support previous observations that metoclopramide can facilitate ACh release from intramural cholinergic neurones in a variety of GI tract smooth muscle preparations (See Massingham *et al.*, 1985 for references).

It is well documented that there is spontaneous release of ACh at rest in LM-MP preparations (Paton *et al.*, 1971) so the absence of mechanical activity following Triton X-100 is consistent with a reduced number of myenteric neurones. A similar argument may also be put forward to account for the lack of

response of treated tissues to dimethylphenylpiperazinium and field stimulation, since both procedures increase ACh release from enteric nerves. That some treated tissues responded to field stimulation probably reflects inadequate treatment with Triton X-100 rather than a resistance of certain myenteric nerves to the surfactant. In this respect, Fox *et al.* (1983) found that benzethonium chloride reduced levels of the putative neurotransmitter peptides, vasoactive intestinal peptide, enkephalin, somatostatin and substance P in the rat jejunum and Sato *et al.* (1978) showed that the same surfactant lowered acetylcholinesterase and catecholamine fluorescence in the colon and anorectum of the rat. It would therefore appear that surfactants destroy both intrinsic and extrinsic neurones. The observation that dimethylphenylpiperazinium failed to contract all Triton X-100-treated tissues suggests that enteric ganglia may be more sensitive to the surfactant than the pre-effector parasympathetic nerve endings in this preparation.

Histological examination of the ileum provided no evidence that Triton X-100 damaged the smooth muscle cells and responses to ACh were essentially similar in treated and sham-operated tissues. Nevertheless it could be argued that a potentiation of ACh responses ought to be observed in treated tissues since acetylcholinesterase is known to be present in the myenteric plexus (Ambache *et al.*, 1968). It should also be pointed out that, although EC_{50} values for ACh were similar in tissues from sham-operated and treated animals (mean EC_{50} values 0.19 μM and 0.15 μM, respectively), these values were 3–4 times higher than those normally expected under our experimental conditions (mean EC_{50} 0.05 μM). We have no adequate explanation for this finding but since sham-operated tissues responded well to metoclopramide it is unlikely that a reduced end-organ sensitivity to ACh was responsible for the lack of response of Triton X-100-treated tissues to metoclopramide. Nevertheless, additional studies are required to rule-out definitively a direct depressant effect of Triton X-100 on smooth muscle reactivity.

In conclusion, this study has demonstrated that the stimulant effect of metoclopramide in LM-MP preparations of guinea-pig ileum is indirect and probably dependent upon a prejunctional facilitation of ACh release from myenteric neurones. No evidence of an important postjunctional action of metoclopramide was obtained.

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