

In vitro contractile effects of short chain fatty acids in the rat terminal ileum

C Cherbut, A-C Aubé, H M Blottière, P Pacaud, C Scarpignato, J-P Galmiche

Abstract

Short chain fatty acids (SCFAs), produced in the gut by bacterial fermentation of carbohydrates, change intestinal motility by mechanisms as yet unknown. This study examined the mechanism(s) of action of SCFAs on contractility using isolated rat terminal ileum segments and isolated ileal smooth muscle cells. Strip contractions were recorded under isometric conditions. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured in single cells loaded with indo-1 penta-acetoxymethyl ester (indo-1 AM). SCFAs (10^{-9} to 10^{-2} mol/l) induced concentration dependent contractions. The effect was not different among the individual SCFAs. Exogenous acids (namely tartaric and citric acids) caused similar responses as SCFAs, whereas sodium acetate had no effect. The contraction was not blocked by tetrodotoxin, atropine or hexamethonium, showing that it was not mediated through a cholinergic pathway. Moreover, removal of the mucosa or addition of procaine (a local anaesthetic) to the bath did not change the SCFA induced contraction, while verapamil (a calcium-channel antagonist) completely suppressed it. In addition, application of SCFAs to isolated ileal myocytes evoked peaks in $[Ca^{2+}]_i$ inhibited by D 600 (a blocker of voltage dependent calcium channels). Taken together, these results suggest that the contractile response stimulated by SCFAs in the rat terminal ileum could result from an acid sensitive calcium dependent myogenic mechanism.

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Short chain fatty acids (SCFAs) are produced by bacterial anaerobic fermentation of carbohydrates in the forestomach of ruminants and in the hindgut of monogastric mammals. They accumulate in concentrations up to 150 mmol/l in the human colon, where they represent the major organic anions. Acetate, propionate, and butyrate account for more than 85% of formed SCFAs and are produced in nearly constant molar ratio 60:25:15.¹ These acids are also present in other parts of the gut, but their concentration seldom exceeds 1 to 5 mmol/l in the small intestine and stomach.^{2,3} SCFAs are quickly absorbed by intestinal mucosa,⁴ are readily metabolised by intestinal epithelium and liver,^{5,6} exhibit

various physiological effects, and may be implicated in some gastrointestinal disorders. Among their biological actions, SCFAs stimulate water and electrolyte absorption,⁷ potentiate proliferation of epithelial cells and colonocytes,⁸ are a differentiating agent in various cancer cell lines in vitro,⁹ and influence gastrointestinal motility.¹⁰

SCFAs could inhibit motility of the rat colon,¹¹ whereas they stimulate contractions in the terminal ileum of dogs and humans.^{12,13} Moreover, they shorten stomach to caecum transit time in the rat,¹⁴ ileal emptying in the dog,¹⁵ and increase parietal tone of the human ileum.¹⁶ Intravenous injection of SCFAs also elicits ileal contractions in anaesthetised rats.¹⁷ It has been suggested that motor effects of SCFAs may protect the ileum against colonic-ileal reflux.¹³

The mechanism(s) of the action of SCFAs in the ileum are as yet unknown. SCFAs influence ileal motility by local reflexes, which do not require systemic control, and which are not abolished by either adrenergic or cholinergic blockade.^{14,18} However, neither the specificity of action of individual SCFAs, nor the role of luminal pH are clearly established. Furthermore, it is not defined whether SCFAs act through the enteric plexus or directly on the intestinal smooth muscle cells.

In this study we investigated the effect of individual SCFAs on contractility of isolated ileal segments. In addition, the action of SCFAs on calcium movements in isolated ileal smooth muscle cells was investigated to gain new insights on their mechanism(s) of action.

Methods

MEASUREMENTS OF MUSCLE CONTRACTION

Animals and apparatus

Male Wistar rats (250–300 g) were killed by cervical dislocation. One to 1.5 cm long segments of the terminal ileum (2 cm before the ileocaecal valve) were quickly removed, opened along the mesenteric border, and cleaned of intraluminal contents in a Krebs-bicarbonate solution (pH 7.4) composed of (mmol/l): 128 NaCl, 4.5 KCl, 2.5 CaCl₂, 1.18 MgSO₄, 1.18 KH₂PO₄, 125 NaHCO₃, 5.55 D-Glucose. Either whole thickness ileal strips (1 cm × 3 mm in the longitudinal axis) or strips whose mucosa was scraped off, were prepared, then suspended under 1 g tension in a 10 ml organ bath containing continuously oxygenated (5% CO₂, 95% O₂) Krebs-bicarbonate solution. Preparations were allowed to equilibrate for 60 minutes. Isometric longitudinal mechanical

Human Nutrition
Research Centre,
INRA, Laboratory of
Nutrition and Applied
Technology, Nantes,
France
C Cherbut
H M Blottière

Laboratory of
Digestive Function and
Nutrition, North
Hospital, Nantes,
France
A-C Aubé
C Scarpignato
J-P Galmiche

Laboratory of
Physiology, University
of Bordeaux, France
P Pacaud

Correspondence to:
Dr C Cherbut, INRA, BP
1627, 44316 Nantes cedex
03, France.

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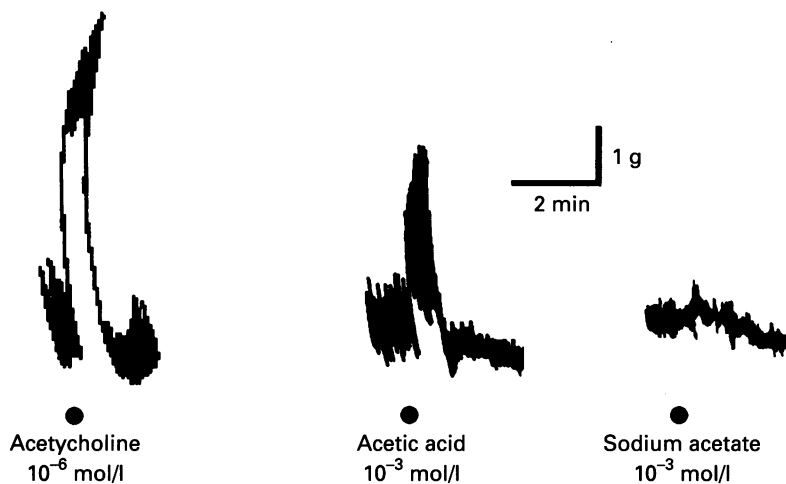


Figure 1: Representative tracing showing the contractions evoked by application of acetylcholine (10^{-6} mol/l), acetic acid (10^{-3} mol/l) or sodium acetate (10^{-3} mol/l) to rat isolated ileal strips recorded under isometric conditions.

activity of the segments was then recorded using a force transducer (Basile no 7005, Comerio, VA, Italy) as previously detailed.¹⁹ At the beginning of each experiment, acetylcholine chloride (Ach, 10^{-6} mol/l) was applied as control. Tested substances were then applied on each preparation with repeated washings of 20 to 30 minutes between each concentration. Viability of each preparation was confirmed at the end of each experience by control of spontaneous mechanical activity and response to Ach. Results are expressed as percentage of maximal response recorded with Ach.

Experimental design

In the first series of experiments, individual SCFAs: acetic acid, propionic acid, butyric acid, and a mixture of the three SCFAs (acetic 60%, propionic 25%, butyric 15%) that represents the ratio of individual SCFAs found in the rat large intestine²⁰ were applied to the bath in non-cumulative increasing concentrations (10^{-9} to 10^{-2} mol/l). Their effects were compared with a sodium salt, sodium acetate solution, applied to the bath in non-cumulative increasing concentrations (10^{-7} to 10^{-2} mol/l).

In the second series of experiments, the effect of SCFAs was compared with two exogenous organic (namely tartaric acid and citric acid) and inorganic (namely hydrochloric acid) acids applied in non-cumulative increasing concentrations (10^{-9} to 10^{-2} mol/l).

Finally, we investigated the effect of agents either affecting neural transmission or blocking endogenous receptors involved in the physiological control of gastrointestinal motility on the motor action of SCFAs. The drugs used were procaine hydrochloride ($4 \cdot 10^{-4}$ mol/l), tetrodotoxin (TTX, 10^{-6} mol/l), atropine sulphate (10^{-6} mol/l), hexamethonium bromide (10^{-4} mol/l), naloxone hydrochloride (10^{-5} mol/l), and [D-Arg¹, D-Trp^{7,9}, Leu¹¹]-substance P (spantide, 10^{-5} mol/l). In addition, blockade of cyclooxygenase by indomethacin (10^{-6} mol/l) was tested to rule out any interference of endogenous prostanoids. We also evaluated the involvement of extracellular calcium in the SCFA induced response by testing

the effect of verapamil hydrochloride (10^{-6} mol/l). These drugs were added three minutes (atropine, hexamethonium, verapamil), five minutes (naloxone, spantide, procaine, TTX), or 20 minutes (indomethacin) before application of acetic acid (10^{-3} mol/l). The concentration of these drugs was based on their ability to antagonise more than 80% of the response produced by their respective agonist in concentration of 10^{-6} mol/l.

All the tested substances were applied in volume not exceeding 1% of the bath volume, pH of the bath solution being measured after each application.

MEASUREMENTS OF INTRACELLULAR CALCIUM ($[Ca^{2+}]_i$)

Cell preparation

Male Wistar rats (250–300 g) were killed by cervical dislocation and the terminal ileum removed. Smooth muscle cells were isolated from the ileum longitudinal muscle using the method of Bitar *et al.*,²¹ with little modifications. The muscle was cut in small pieces and incubated at 37°C for three successive 30 minute periods in Ca^{2+} free phosphate buffer solution (PBS) containing 2% bovine serum albumin, 0.1% collagenase, and 0.1% soybean trypsin inhibitor. The pieces were then washed in enzyme free PBS and cells allowed to disperse under gentle trituration with pipette.

Calcium measurements

Smooth muscle cells were loaded with 1 μ mol/l-Indo-1 penta-acetoxymethyl ester (Indo-1 AM) by incubation in PBS solution for 25 minutes at room temperature. $[Ca^{2+}]_i$ was estimated from Indo-1 fluorescence as described in detail by Loirand *et al.*²² $[Ca^{2+}]_i$ was estimated from fluorescence ratio (405/480 nm) using a calibration for Indo-1 determined within cells. It was measured before and after application of acetic acid (10^{-3} mol/l) to the isolated smooth muscle cells, with or without addition of D 600 (10^{-5} mol/l) to the bath. D 600 is a blocker of voltage dependent calcium channels.

ANALYSIS OF DATA

All data are presented as mean (SEM). Significance among data was tested by using one way analysis of variance (ANOVA), followed by Scheffe *F* test. EC_{50} and 95% confidence intervals were calculated from the regression lines of the concentration response curves of each individual acid. All the calculations were performed using the StatView SE+ Graphics (Abacus concepts, CA, USA) running on a Mac computer.

CHEMICALS

SCFAs, drugs, and chemicals were purchased from Sigma Chemical (L'Isle d'Abeau Chesnes, St Quentin Fallavier Cedex, France),

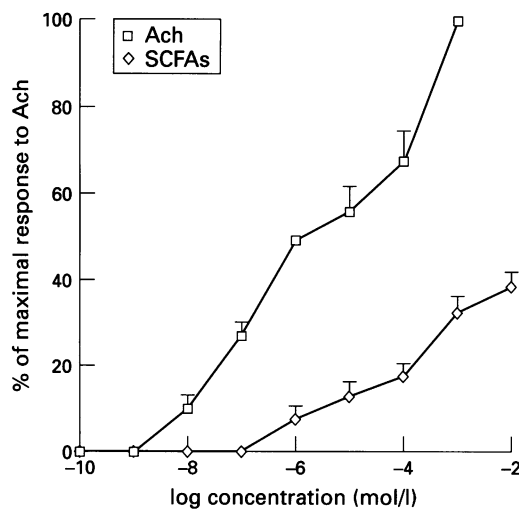


Figure 2: Concentration response curves for the effect of acetylcholine (Ach) and a mixture of SCFAs on rat isolated terminal ileum obtained under isometric recording conditions. Each point represents the mean of the values obtained for 12 measurements. Vertical bars are standard errors of the mean.

and enzymes were purchased from INC (Orsay, France).

Results

EFFECTS OF SCFAS, SODIUM ACETATE, AND EXOGENOUS ACIDS

Whole thickness segments of the rat terminal ileum showed regular spontaneous phasic contractions. Application of SCFAs evoked a tonic contraction that was not sustained but faded rapidly to reach the base line in 43.4 (10.7) s, regardless of the concentration tested (Fig 1). The occurrence of the effect was concentration dependent with a threshold concentration of about 0.1 $\mu\text{mol/l}$ and a mean EC_{50} of 64 $\mu\text{mol/l}$ (95% confidence intervals: 2, 59 $\mu\text{mol/l}$). The maximal amplitude of the contraction was registered after application of SCFAs at the concentration of 10 mmol/l, and represented about 40% of the maximal response elicited by Ach (10^{-3} mol/l (Fig 2)). There was no significant difference among the four different SCFA solutions (Table). Both organic (weak) and inorganic (strong) acids induced concentration dependent contractions similar to those produced by SCFAs (Table), whereas sodium acetate had no effect (Fig 1), regardless of the concentration (10^{-7} to

Effective concentrations (EC_{50} , $\mu\text{mol/l}$) causing 50% of the maximal contractile response of isolated ileal strips to different SCFAs and exogenous acids

	EC_{50} ($\mu\text{mol/l}$)	95% Confidence intervals ($\mu\text{mol/l}$)	
		Lower	Upper
SCFAs			
Acetic	81	1	77
Propionic	59	3	30
Butyric	67	3	85
Mixture	50	1	43
Exogenous acids			
<i>Organic</i>			
Tartaric	20	2	26
Citric	17	0.8	26
<i>Inorganic</i>			
Chlorhydric	73	3	87

10^{-2} mol/l). Increasing concentrations of SCFAs, added to the bath, provoked a stepwise decrease of pH of the bathing solution, which dropped by about 1.5 unit after reaching the concentration of 10^{-2} mol/l (Fig 3). In muscle strips whose mucosa had been removed, application of SCFAs also evoked a contraction that was virtually identical to the response elicited in the whole ileum preparation (Fig 4).

EFFECTS OF DRUGS ON SCFA INDUCED CONTRACTION

The SCFA induced contraction was not inhibited by hexamethonium (blocker of nicotinic receptors), spantide (substance P antagonist), naloxone (opiate receptor blocker), and indomethacin (cyclooxygenase inhibitor). Muscarinic blockade with atropine reduced the response, though not significantly ($p=0.291$ (Fig 5)). Moreover, neither procaine, a local anaesthetic, nor TTX, which blocks nervous transmission, affected the contraction pattern. On the contrary, a calcium channel antagonist, verapamil, was able to completely abolish the SCFA induced contraction (Fig 5).

EFFECTS OF SCFAS ON INTRACELLULAR CALCIUM

Bath application of acetic acid (10^{-3} mol/l) on isolated ileal smooth muscle cells increased $[Ca^{2+}]_i$ from a resting concentration of 124 (5) nmol/l to a peak value of 277 (7) nmol/l ($p<0.01$). Addition of D 600 (a blocker of voltage dependent calcium channels, 10^{-5} mol/l) into the bath significantly inhibited the SCFA induced response, the incremental $[Ca^{2+}]_i$ being reduced to 29 (3) nmol/l (Fig 6).

Discussion

It is well known that SCFAs are able to stimulate ileal motility in vivo. In conscious animals, infusion of SCFAs into the ileal lumen induces

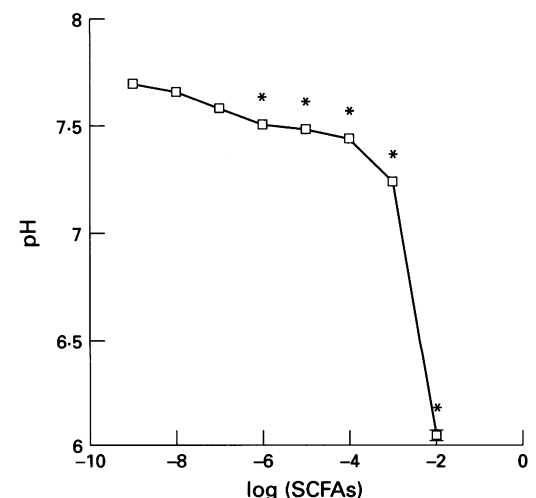


Figure 3: Effect of increasing concentrations of SCFAs on pH of the bathing solution. Each point represents the mean of the values obtained for 48 measurements. Vertical bars are standard errors of the mean. * $p<0.05$.

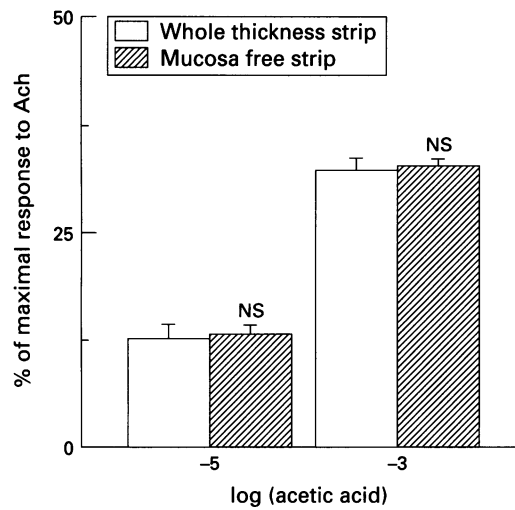


Figure 4: Contractile responses to acetic acid (10^{-5} and 10^{-3} mol/l) of rat ileal strips either with ($n=12$) or without mucosa ($n=8$). Each column represents the mean of the values obtained for 12 measurements. Vertical bars are standard errors of the mean. NS: non-significant.

bursts of phasic contractions that rapidly migrate aborally,¹² and shorten ileal emptying in the dog¹⁵ as well as oro-caecal transit time in the rat.¹⁴ The stimulatory effect of SCFAs in the ileum is also seen in healthy human subjects.¹³ In these studies, the degree of stimulation depended on acid concentration and was maximal at the concentrations found in the colon (100–150 mmol/l).

In this investigation, we have shown for the first time that applications of SCFAs, in their acidic form, evokes a concentration dependent

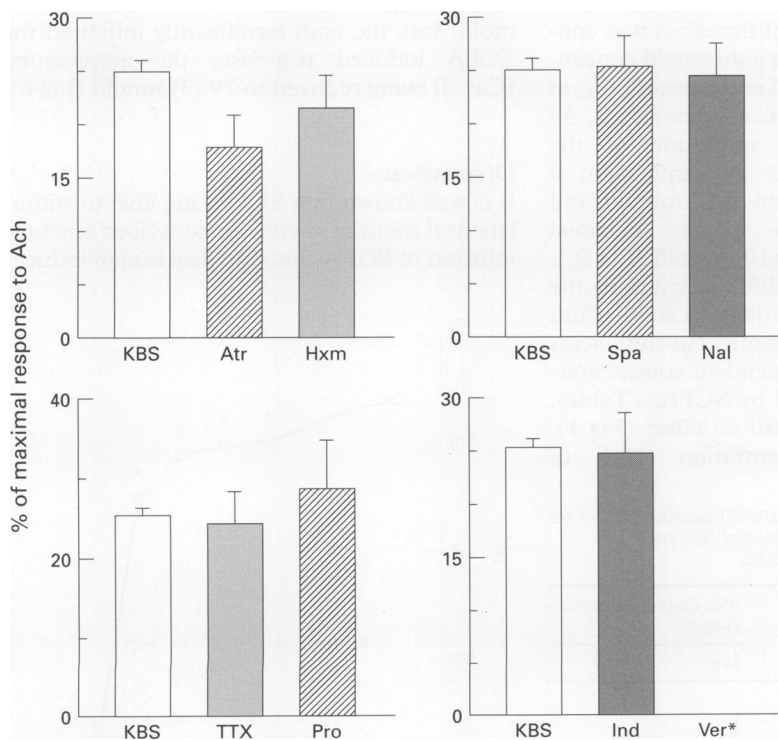


Figure 5: Contractile responses to acetic acid (10^{-3} mol/l) applied to rat isolated ileum pretreated with Krebs' solution (KBS), atropine sulphate (Atr, 10^{-6} mol/l), hexamethonium bromide (Hxm, 10^{-4} mol/l), spantide (Spa, 10^{-5} mol/l), naloxone (Nal, 10^{-5} mol/l), tetrodotoxin (TTX, 10^{-6} mol/l), procaine hydrochloride (Pro, $4 \cdot 10^{-4}$ mol/l), indomethacin (Ind, 10^{-6} mol/l), and verapamil hydrochloride (Ver*, 10^{-6} mol/l). Each column represents the mean of the values obtained for eight measurements. Vertical bars are standard errors of the mean. * $p < 0.001$.

contraction in the rat terminal ileum in vitro, an effect that was not blocked by atropine or TTX, but was abolished by blocking voltage dependent calcium channels. Although the motility of isolated strips could represent stationary, propulsive or retropropulsive patterns, the in vivo effects seen suggest that the SCFA induced contractions are of propulsive type.

Conversely from Kamath *et al*,¹² we tested 'physiological' concentrations of SCFAs, which ranged from 1 μ mol/l to 10 mmol/l. These concentrations reflect the amounts of SCFAs measured in the peripheral blood²³ and ileum of humans²³ as well as ileal contents of rats (Cherbut, unpublished results). Although in vivo studies have shown the potency of SCFAs to be inversely related to the chain length, acetic acid being more potent than butyric one,¹² in our in vitro experimental conditions the contractile activity of the different SCFAs was virtually the same. The difference of potency among the individual SCFAs seen in vivo might reflect a difference in their epithelial metabolism: enterocytes indeed oxidise butyrate preferentially than acetate.⁵ It could be therefore speculated that, as SCFAs were applied to the mucosal side in the dog ileum, butyrate was trapped within the epithelium, while acetate was excreted into the mucosal circulation. A smaller amount of butyrate may have thus reached the smooth muscle, explaining its lower potency compared with acetate. This cannot of course occur in vitro, as both mucosal and serosal sides were bathed with the same butyrate solution.

While SCFAs stimulate ileal motility, they do not affect the motor pattern of the upper small intestine. In vivo, either intraduodenal²⁴ or intravenous¹⁷ administration of SCFAs did not change both duodenojejunal motility and transit time. Similarly, motility of isolated jejunal segments in vitro was not affected by SCFAs.²⁵

Conflicting results have been reported concerning the SCFA effect on the large intestine. Indeed, while Yajima²⁶ reported a contracting activity of SCFAs on isolated rat colon, Squires *et al*¹¹ actually found an inhibitory action of these compounds at physiological concentrations. In agreement with the second of these results, infusion of SCFAs into the rat colon significantly decreased myoelectrical activity in vivo (Cherbut *et al*, unpublished results). As expected, suppression of SCFA concentration by oral administration of antibiotics, is followed by a stimulation of colonic activity.²⁷ Conversely from consistent results obtained in the ileum, the contrasting effect seen in the colon could rely on the experimental model adopted – that is, strips versus whole preparation. It is, however, worth mentioning that the results obtained in the whole colonic preparation (that used by Squires *et al*), which is more relevant to the in vivo motility, are in good agreement with the inhibitory effects seen in the conscious rat. Taken together, these findings suggest that regional differences in the response to intraluminal SCFAs may exist along the gastrointestinal tract.

The presence of chemoreceptors sensitive to SCFAs was first proposed by Kamath *et al*¹³ to

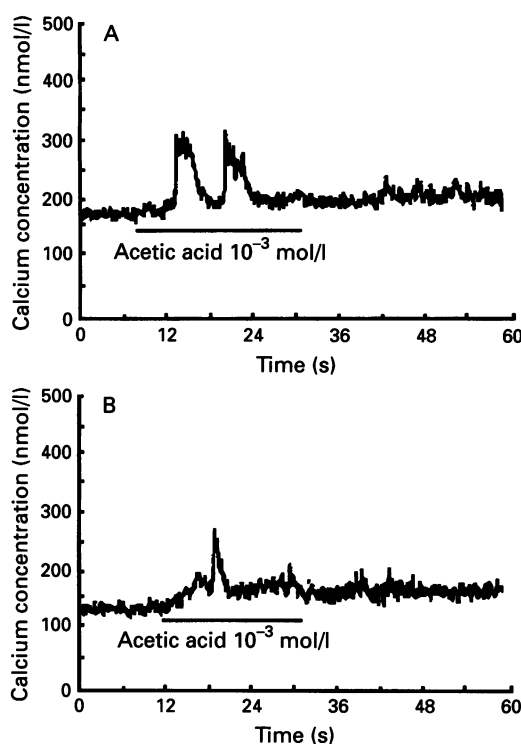


Figure 6: Representative tracing showing the effect of acetic acid (10^{-3} mol/l) applied to isolated ileal smooth muscle cells on intracellular calcium concentration. The bathing solution did not contain (A) or contained (B) D 600 ($10 \mu\text{mol/l}$) to inhibit calcium entry through voltage, dependent calcium channels.

explain the effect of these compounds on ileal motility. Such receptors, detected in the epithelium of the ruminant reticulorumen, are localised close to sensory vagal fibres.²⁸ Similar receptors may exist in the rat colonic mucosa.²⁶ However, such a receptorial mechanism could not explain the ileal motor effect of SCFAs seen in vitro. Indeed, neither the application of procaine, nor the absence of mucosa changed the contractile response to SCFAs in the ileal strips.

In our experimental conditions, TTX, atropine or hexamethonium, failed to affect the motor stimulating activity of SCFAs, suggesting that cholinergic pathways are not involved. This is consistent with results obtained in dogs where adrenergic, cholinergic or serotonergic blockade do not affect the ileal stimulatory effect of SCFAs.¹⁸ In addition, neither indomethacin nor naloxone affected the in vitro ileal response to SCFAs. These data are in agreement with results obtained in humans¹³ but differ from those of dog experiments,¹⁸ where indomethacin stimulated ileal motility and naloxone inhibited the contractions evoked by SCFAs.

It was recently shown that SCFAs can release peptide YY from endocrine cells. This is the case with isolated and perfused rabbit colon²⁹ and of in situ colon (Rozé, personal communication) where instillation of SCFAs increases circulating concentrations of peptide YY. As peptide YY plays an important paracrine part in the regulation of gastrointestinal motility,³⁰ it could participate in the ileal motor action of SCFAs. Blood concentrations of peptide YY are unchanged, however, after a bolus of SCFAs is injected into the

human ileum.¹³ In any event, a SCFA induced release of peptide YY or other peptides from the epithelium cannot explain the contractile response of the isolated ileum, as removal of the mucosa containing L cells did not change the response. All these data, taken together, strongly suggest that SCFAs could act directly on the ileal smooth muscle without involving any kind of neural pathway or paracrine regulation.

Contractions induced by SCFAs in the isolated terminal ileum were completely suppressed when the calcium channel antagonist, verapamil, was added to the bath. Furthermore, application of acetic acid on isolated ileal smooth muscle cells induced a twofold increase in $[\text{Ca}^{2+}]_i$, an effect that was significantly inhibited by D 600, a blocker of voltage dependent calcium channels. The myogenic effect of SCFAs could therefore rely on the entry of extracellular calcium into the cytoplasm.

The similarity of action of the different SCFAs and of exogenous acids (both organic and inorganic) as well as the lack of effect of sodium acetate are consistent with the idea that these compounds affect ileal motility through pH changes. Reduction of intracellular pH (pH_i) might be responsible for the $[\text{Ca}^{2+}]_i$ movements. Indeed, the addition of weak acids to isolated vascular and uterine smooth muscles induces a concentration dependent decrease of the pH_i ,³¹ which is associated with smooth muscle relaxation. What we saw, however, after addition of SCFAs to ileal strips was a contraction. This is not surprising considering the interactions between the H^+ ions and Ca^{2+} in the cytoplasm and their subsequent effect on contraction in the intestinal smooth muscle are not completely understood and may differ from those operating in the vascular and uterine muscle cells.

In summary, results of this study show that SCFAs, which are produced in the gastrointestinal lumen by bacterial fermentation of carbohydrates, stimulate ileal motility in a concentration dependent fashion. The SCFA induced contraction seems to entail neither a neural pathway nor paracrine regulation. A calcium dependent myogenic mechanism could be responsible for this motor effect.

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