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Actions of cysteinyl leukotrienes in the enteric nervous system of guinea-pig stomach and small intestine

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Abstract

Conventional intracellular microelectrodes, neuronal tracer injection techniques and immunohistochemistry were used to study the actions of cysteinyl leukotrienes (CysLTs) on electrical and synaptic behavior of enteric neurons in guinea-pig stomach and small intestine. Bath application of leukotriene C_4, leukotriene D_4 or leukotriene E_4 evoked a slowly activating depolarizing response in most of the myenteric and submucous plexus neurons in the small intestine while no effect was observed in gastric neurons. The depolarization evoked by cysteinyl leukotrienes in intestinal neurons was associated with increased input resistance and enhanced excitability. Suppression of hyperpolarizing after-potentials occurred in AH type neurons. The depolarizing action of cysteinyl leukotrienes was resistant to tetrodotoxin and cyclooxygenase inhibitors. Neither the $CysLT_1$ receptor antagonists (*E*)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid (MK 571), 1-[2-hydroxy-3-propyl-4-[4-(1*H*-tetrazol-5-yl)butoxy]phenyl}-ethanone (LY 171883) and α-pentyl-3-(2-quinolinylmethoxy)-benzenemethanol (REV 5901), nor the dual $CysLT_1$/$CysLT_2$ receptor antagonist 6(*R*)-(4′-carboxyphenylthio)-5(*S*)-hydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid (BAY u9773) significantly altered the depolarizing action of the cysteinyl leukotrienes. Neurotransmission was unaffected by the cysteinyl leukotrienes. The results suggested involvement of cysteinyl leukotrienes in enteric immuno-neural communication through excitatory actions on enteric neurons. The receptor mediating these effects was distinct from currently recognized cysteinyl leukotriene receptor subtypes ($CysLT_1$ and $CysLT_2$ receptors) and may represent a new receptor subtype.

Keywords: Intestine; Stomach; Enteric nervous system; Immune system; Inflammation; Leukotriene; Myenteric plexus; Submucosal plexus

1. Introduction

The cysteinyl leukotrienes, leukotriene C_4, leukotriene D_4 and leukotriene E_4, belong to a distinct family of arachidonic acid metabolites formed via the 5-lipoxygenase pathway. These substances exhibit a wide range of biological actions that are compatible with a role in immune/inflammatory processes (Samuelsson, 1983). For example, the cysteinyl leukotrienes recruit eosinophils (Spada et al., 1986), increase vascular permeability (Drazen et al., 1980), stimulate the excretion of mucus (Marom et al., 1982) and cause smooth muscle contraction (Hanna et al., 1981; Samuelsson, 1983). The cysteinyl leukotrienes have been implicated as important contributors in the pathophysiology of several inflammatory disorders, most notably, asthma, psoriasis, rheumatoid arthritis and inflammatory bowel disease (IBD) (Samuelsson, 1983; Henderson, 1994).

The effects of cysteinyl leukotrienes are mediated via specific plasma membrane receptors belonging to the superfamily of G protein-coupled receptors. At least two classes of cysteinyl leukotriene receptors exist ($CysLT_1$ and $CysLT_2$ receptors) and are classified by their sensitivity to receptor antagonists. The $CysLT_1$ receptor is sensitive to the classic cysteinyl leukotriene receptor antagonists (also called the $CysLT_1$ receptor antagonists) such as zafirlukast, montelukast, pranlukast, and (*E*)-3-[[[3-[2-(7-chloro-2-quinolinyl) ethenyl]phenyl][[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid (MK-571). The $CysLT_2$ receptor is resistant to these antagonists and is blocked by 6(*R*)-

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(4′-carboxyphenylthio)-5(*S*)-hydroxy-7(*E*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid (BAY u9773), a mixed $CysLT_1$ and $CysLT_2$ receptor antagonist (Coleman et al., 1995; Metters, 1995). Recent molecular cloning and functional studies of both the human and mouse $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999; Maekawa et al., 2001) and $CysLT_2$ receptor (Heise et al., 2000; Hui et al., 2001) offered new insight for studies of the functional pharmacology of leukotrienes. The pharmacological profile of cloned cysteinyl leukotriene receptors showed a rank order potency of leukotriene D_4>leukotriene C_4>leukotriene E_4 for the $CysLT_1$ receptor and leukotriene C_4 = leukotriene $D_4 \gg$ leukotriene E_4 for the $CysLT_2$ receptor (Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000; Hui et al., 2001; Nothacker et al., 2000), which corresponded to what had been previously determined pharmacologically (Coleman et al., 1995). Both $CysLT_1$ and $CysLT_2$ receptors are coupled to $G_{q/11}$ protein and are linked to mobilization of intracellular Ca^{2+} (Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000; Hui et al., 2001). The expression patterns for these two receptors are variable among different tissues and are suggestive of distinct roles in mediation of leukotriene actions in different sites.

The literature suggests that there might be additional classes of as yet unrecognized cysteinyl leukotriene receptors. Tudhope et al. (1994) found that contractions evoked by leukotriene C_4 and leukotriene D_4 in guinea-pig lung strips were partially resistant not only to the classic antagonists, but also to BAY u9773. A similar pattern of contraction that was resistant to all the currently available receptor antagonists was also observed for porcine and human pulmonary artery (Jonsson et al., 1998; Back et al., 2000a;b). These data suggest the existence of a third class of cysteinyl leukotriene receptor ($CysLT_3$ receptor) that is distinct from the currently recognized cysteinyl leukotriene receptor subtypes $CysLT_1$ and $CysLT_2$.

Although precise identification of the cell types responsible for leukotriene synthesis in the gastrointestinal tract remains to be established, the immune/inflammatory cells in the lamina propria, such as mast cells, neutrophils and macrophages, are among the most likely sources of leukotriene production (Perdue and McKay, 1994). Numerous studies have suggested roles for cysteinyl leukotrienes in modulation of gastrointestinal functions, including motility and secretion. The contractile effects of cysteinyl leukotrienes on gastrointestinal smooth muscles vary considerably not only between species but also between different regions of the gastrointestinal tract. In vitro studies of the guinea-pig ileum show that leukotriene C_4, leukotriene D_4 and leukotriene E_4 evoke contraction in segments of ileum or strips of ileal longitudinal muscle (Sirois et al., 1981; Oliva et al., 1994; Tudhope et al., 1994; Back et al., 1996). Leukotriene C_4 and leukotriene D_4 are much more potent than histamine in this respect (Sirois et al., 1981). Nevertheless, these compounds do not evoke contractions in the rat ileum (Goldenberg and Subers, 1982) and do contract the rat stomach and distal colon (Goldenberg and Subers, 1983). A similar paradox has been reported for the human intestine where leukotriene D_4 is found to evoke contraction of the longitudinal muscle of the ileum (Percy et al., 1988), while causing transient relaxation of both the longitudinal and circular muscle layers of the distal colon (Percy et al., 1990a).

The effects of cysteinyl leukotrienes on intestinal secretion are well established. Leukotriene C_4, leukotriene D_4 and leukotriene E_4 increase short-circuit current in Using chamber studies, which is an action attributed to increased active chloride secretion in the mucosa of small and large intestines of rat, rabbit, guinea pig and pig (Smith et al., 1988, 1990; Jett et al., 1991; Hammerbeck and Brown, 1993; Hyun and Binder, 1993; Traynor et al., 1993; Frieling et al., 1997). The stimulatory effect of cysteinyl leukotrienes on ion secretion is implicated in the diarrheal symptoms associated with intestinal inflammation. Cysteinyl leukotriene-evoked increases in short-circuit current were suppressed by the neuronal conduction blocker tetrodotoxin, and by atropine and hexamethonium (Hammerbeck and Brown, 1993; Frieling et al., 1997), which suggests that cysteinyl leukotrienes act to enhance mucosal secretion by elevating excitability of cholinergic secretomotor neurons in the colonic submucosal plexus (Hammerbeck and Brown, 1993; Frieling et al., 1997).

Interest in the potential role of lipoxygenase metabolites in the pathogenesis of inflammatory bowel disease is increasing. Enhanced mucosal formation of cysteinyl leukotrienes was reported in intestinal anaphylaxis (Samuelsson, 1983), in patients with active ulcerative colitis and Crohn's disease (Peskar et al., 1986; Fox et al., 1990), and in experimental models of colitis (Zipser et al., 1987). Furthermore, drugs that are capable of inhibiting 5-lipoxygenase activity are effective in treatment of inflammatory bowel disease (Rask-Madsen et al., 1994). As a result, there is a hypothesis that lipoxygenase metabolites are critical mediators of inflammation and thus may be important in the pathogenesis of abdominal distress and diarrhea associated with intestinal inflammation, in general, and inflammatory bowel disease, in particular.

Functions of the gastrointestinal tract are largely controlled by the enteric nervous system, which is sometimes called the "little brain" in the gut (Wood, 1994; Gershon, 1998). The enteric nervous system controls and coordinates motility, secretion and blood flow in the gut via intrinsic neural networks. Mediators released in paracrine fashion from non-neuronal cells can overlay the synaptic microcircuitry of the enteric nervous system and thereby modify the output of the circuits to the effector systems (i.e., secretory epithelium, musculature and blood vasculature). Immune/inflammatory cells are sources of paracrine mediators that influence the function of the synaptic networks in the enteric nervous system. Earlier work in our laboratory showed that various inflammatory mediators such as histamine, prostaglandins, bradykinin and cytokines changed the electrical and synaptic behavior of the neurons that form the enteric neuronal microcircuits (Nemeth et al., 1984; Hu et al., 1998, 1999; Xia et al., 1999). Effects of cysteinyl

leukotrienes in the enteric nervous system have not been studied systematically; although, Frieling et al. (1997) reported that exposure to leukotriene C_4 enhanced excitability of ganglion cells in the guinea-pig colon. The aim of the present study was to investigate the cellular neuropharmacology of cysteinyl leukotrienes on electrical and synaptic behavior of morphologically and neurochemically identified gastric and small intestinal neurons and to characterize the response pattern, ionic mechanism, and subtypes of receptors involved.

2. Materials and methods

2.1. Tissue preparation

Adult male Hartley-strain guinea pigs (400–600 g) were stunned by a blow to the head and exsanguinated from the cervical vessels according to procedures approved by the Ohio State University Laboratory Animal Care and Use Committee. The stomach and a 2–5 cm segment of ileum that was 15–20 cm proximal to the ileocecal junction were removed and placed in ice-cold Krebs solution. Preparations of the myenteric and submucosal plexuses for electrophysiological recording were microdissected as described earlier (Wood and Meyer, 1978; Schemann and Wood, 1989; Tack and Wood, 1992; Zafirov et al., 1993). The preparation was mounted in a 2.0-ml recording chamber that was perfused at a rate of 10–15 ml/min with Krebs solution warmed to 37 °C and gassed with 95% O_2/5% CO_2 to buffer at pH 7.3–7.4. The composition of the Krebs solution was (in mM) NaCl, 120.9; KCl, 5.9; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 14.4; $CaCl_2$, 2.5; and glucose, 11.5. The Krebs solution contained nifedipine (1 μM) and scopolamine (1 μM) to prevent smooth muscle movements from dislodging the intracellular electrode.

2.2. Electrophysiological recording

The myenteric and submucosal ganglia were visualized with differential interference contrast optics and epilumination. Ganglia selected for study were immobilized with 100-μm-diameter L-shaped stainless steel wires placed on either side of the ganglion. Transmembrane electrical potentials were recorded with conventional intracellular microelectrodes filled with 2% biocytin in 2 M KCl containing 0.05 M Tris buffer (pH 7.4) and having resistances of 80–140 MΩ. The same electrodes were used to inject the neuronal tracer biocytin by passing hyperpolarizing current into the impaled neurons. The preamplifier (M-767, World Precision Instruments, Sarasota, FL) was equipped with a bridge circuit for injecting current into the cell through the recording electrode. Fast excitatory postsynaptic potential (EPSPs), slow EPSPs and slow inhibitory postsynaptic potential (IPSPs) were evoked by electrical shocks (0.1–20 Hz) applied focally to interganglionic connectives with 20 μm diameter Teflon-insulated Pt wire electrodes connected through stimulus-isolation units (Grass SIN5) to Grass S48 stimulators (Astro-Med, Grass-Telefactor Division, West Warwick, Rhode Island). Chart records were made on Astro-Med thermal recorders (Astro-Med). The amplitude of the spikes in some of the recordings was blunted by the low frequency response of the recorder. All data were recorded on videotape for later analysis.

2.3. Immunohistochemical staining

To reveal the morphology of the impaled neurons, the neuronal tracer biocytin was injected into the neurons through the recording electrodes by the passage of hyperpolarizing current (0.5 nA for 10–30 min) at the end of each recording session. The anal end of the preparations was marked and the tissue was transferred into a disposable chamber filled with fixative (2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) and kept at 4 °C overnight. After fixation, the preparations were washed (3 × 10 min) in dimethyl sulfoxide, followed by 3 × 10 min washes in phosphate-buffered saline (PBS). The preparations were then incubated with fluorescein streptavidin (1:100) in hypertonic PBS (containing 10% normal donkey serum, 0.3% Triton X-100 and 0.1% sodium azide) for 1 h to reveal the morphology of the biocytin-labeled neuron. Preparations were then washed for 3 × 10 min with PBS, mounted on glass slides with fluorescence mounting medium and examined under a Nikon Eclipse E600 fluorescent microscope (Nikon, Melville, NY) that was equipped with appropriate filters and a SPOT-RT cooled CCD digital camera (Diagnostic Instruments, Sterling Heights, MI).

Neurochemical coding of the neurons that responded to cysteinyl leukotrienes was determined by first reacting the preparations with streptavidin coupled to fluorescein to reveal biocytin fluorescence. They were then processed for immunohistochemical demonstration of calbindin, calretinin, neuronal nitric oxide synthase, substance P, vasoactive intestinal peptide, or choline acetyltransferase. The primary antisera (see Table 1) were diluted in hypertonic PBS (containing 10% normal donkey serum, 0.3% Triton X-100 and 0.1% sodium azide). The preparations were incubated with any one of the primary antibodies for 18 h at room temperature. After that, the tissues were washed (3 × 10 min) in PBS and incubated with the secondary antibodies labeled with Cy3 at room temperature for 1 h. The tissue was then rinsed in PBS and cover-slipped with fluorescence mounting medium. All preparations were examined under a Nikon Eclipse E-600 fluorescent microscope and analyzed by using filter combinations that enabled separate visualization of multiple fluorophores. Digital images were obtained with the SPOT digital camera, stored on disk, and analyzed with SPOT II software. Contrast in the digital images was sometimes enhanced before either converting to JPEG file interchange format (*:jpg) for electronic transfer or printing as photomicrographs with Hewlett-Packard ink jet printers.

Table 1
Codes and sources of the primary and secondary antibodies used in the study

Antigen	Host	Code	Dilution	Sources
Calbindin	Mouse	C9848	1:2500	Sigma
Calretinin	Goat	AB1550	1:2500	Chemicon
ChAT	Goat	AB144P	1:50	Chemicon
nNOS	Sheep	AB1529	1:500	Chemicon
SP	Rat	MAB356	1:200	Chemicon
VIP	Rabbit	IHC7161	1:200	Peninsula
Goat IgG	Donkey Cy3	705-165-147	1:500	Jackson
Mouse IgG	Donkey Cy3	715-165-150	1:500	Jackson
Rabbit IgG	Donkey Cy3	711-165-152	1:500	Jackson
Rat IgG	Donkey Cy3	AP189C	1:200	Chemicon
Sheep IgG	Donkey Cy3	713-165-147	1:500	Jackson

ChAT, choline acetyltransferase; nNOS, neural nitric oxide synthase; SP, substance P; VIP; vasoactive intestinal peptide; IgG, immunoglobulin G.

2.4. Drug application

Drugs were applied by either addition to the superfusion solution or by pressure microejection from a micropipette (10–20 μm diameter) manipulated with the tip close to the impaled neurons. Pressure pulses of nitrogen with predetermined force and duration were applied to the micropipettes through electronically controlled solenoid valves. *S*-hexyl glutathione (100 μM) and L-cysteine (5 mM) were co-applied with leukotriene C_4 and leukotriene D_4, respectively, to prevent the metabolism of leukotriene C_4 to leukotriene D_4 and on to leukotriene E_4.

2.5. Chemicals

Leukotriene C_4, leukotriene D_4, leukotriene E_4 and α-pentyl-3-(2-quinolinylmethoxy)-benzenemethanol (REV 5901) were purchased from Cayman Chemical (Ann Arbor, MI). Product information from Cayman Chemical states that each of the three leukotrienes as supplied are stable for a minimum of 1 year when stored at −80 °C. Samples used in the present study were stored at −80 °C for no longer than 60 days. Samples of leukotriene C_4 and leukotriene E_4, which when delivered consisted of 50 μg in 500 μl ethanol, were subdivided into 50 vials each containing 1 μg for storage at −80 °C. Leukotriene D_4 (50 μl) was delivered in 500 μl methanol. Frozen samples were never thawed and refrozen. For use, the 1 μg samples were thawed and the alcohol solvent evaporated under nitrogen prior to dissolving the sample in dimethyl sulfoxide. Neither *S*-hexyl glutathione (100 μM), L-cysteine (5 mM) nor dimethyl sulfoxide alone altered the resting membrane potential or synaptic responses in the enteric neurons. (*E*)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid (MK 571) and BAY u9773 came from BIOMOL Research Laboratories (Plymouth Meeting, PA). Biocytin, piroxicam, indomethacin, tetrodotoxin, 1-[2-hydroxy-3-propyl-4-[4-(1*H*-tetrazol-5-yl)butoxy]phenyl}-ethanone (LY 171883), *S*-hexyl

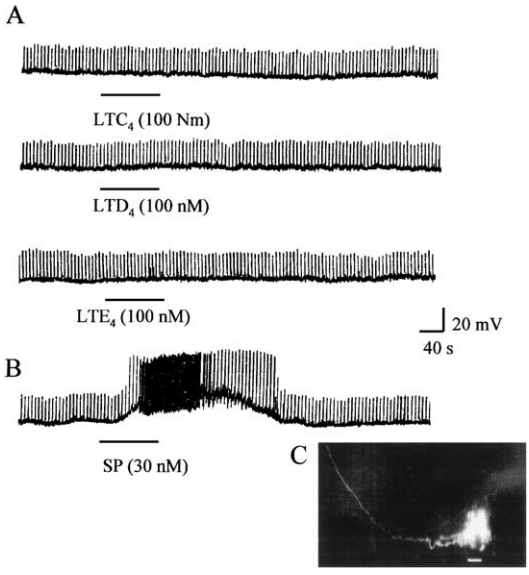

Fig. 1. The cysteinyl leukotrienes had no effect on the membrane potential and excitability of gastric myenteric neurons. (A) Application of LTC_4, LTD_4 and LTE_4 (100 nM) did not alter the membrane potential or input resistance of a Gastric type I neuron. Upward deflections are electrotonic potentials evoked by repetitive intraneuronal injection of constant-current depolarizing pulses. (B) Application of substance P (30 nM) evoked a slowly activating, depolarizing response associated with enhanced excitability in the same neuron. (C) Morphology of the Dogiel type 1 neuron from which the records were obtained. Scale bar = 20 μm.

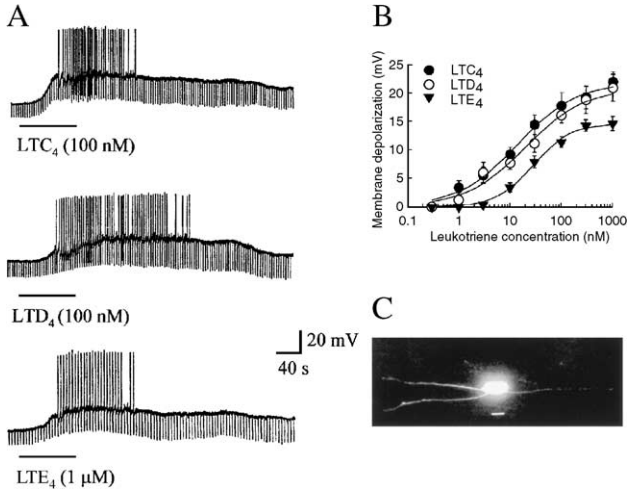

Fig. 2. Excitatory actions of cysteinyl leukotrienes on myenteric neurons in the guinea-pig small intestine. (A) Application of LTC_4, LTD_4 or LTE_4 evoked slowly activating depolarizing responses in an AH type myenteric neuron. Downward deflections are electrotonic potentials evoked by intraneuronal injection of constant current hyperpolarizing pulses. Increased amplitude of the electrotonic potentials reflects increased input resistance. The depolarizing responses were associated with increased input resistance and enhanced excitability as indicated by the occurrence of anodal-break excitation at the offset of hyperpolarizing current pulses. (B) Concentration–response curves for LTC_4, LTD_4 and LTE_4. The EC_{50}s for LTC_4, LTD_4 and LTE_4 were 13.9 ± 1.5, 19.9 ± 3.2 and 28.4 ± 1.9 nM, respectively. Each data point represents the mean ± S.E. for 4–20 neurons for LTC_4, 4–26 neurons for LTD_4, and 5–20 neurons for LTE_4. (C) Morphology of the Dogiel type II neuron from which the records in (A) were obtained. Scale bar = 20 μm.

glutathione and L-cysteine were obtained from Sigma (St. Louis, MO). The codes and sources of the primary and secondary antibodies used are given in Table 1.

2.6. Data analysis

All data were expressed as means ± standard error; *n*-values refer to the number of neurons. The concentration–response curves for drug-induced responses were constructed using the following least-squares fitting routine: $V = V_{max}/[1+(EC_{50}/C)^{n_H}]$, where V is the observed response, EC_{50} is the concentration which induces the half-maximal response, and n_H is apparent Hill coefficient. Student's *t*-test and one-way analysis of variance were used to determine the statistical significance. P values <0.05 were considered statistically significant.

3. Results

Results were analyzed for 30 gastric myenteric neurons and 317 small intestinal (249 myenteric and 68 submucosal) neurons from 156 guinea-pigs. All neurons had resting membrane potentials greater than −40 mV with impalements lasting from 20 min to 8 h. The gastric neurons were classified as Gastric I, Gastric II, Gastric III or AH types, and the small intestinal neurons were classified into S and AH types according to their electrical and synaptic behavior as previously described (Wood, 1989; 1994).

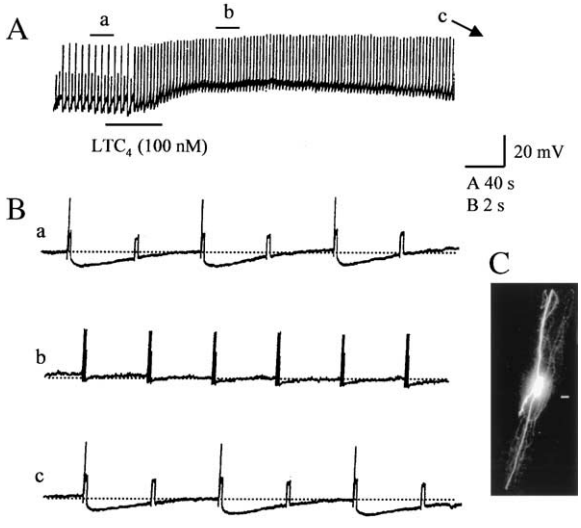

Fig. 3. Cysteinyl leukotriene-evoked depolarizing responses in AH type small intestinal neurons were associated with enhanced excitability and suppression of hyperpolarizing after-potentials. (A) Application of LTC_4 (100 nM) evoked membrane depolarization and enhanced excitability in an AH type myenteric neuron in the guinea pig small intestine. (B) Traces from the record in (A) recorded with an expanded time base to show the suppression of hyperpolarizing after-potentials by LTC_4. (Ba) In the absence of LTC_4, large amplitude after hyperpolarizing potential followed each action potential that was evoked by intraneuronal injection of a depolarizing current pulse. (Bb) At the peak of the depolarizing response induced by LTC_4, the hyperpolarizing potentials were suppressed. The neuron discharged repetitively during intraneuronal injection of the same depolarizing current pulses. (Bc) Action of LTC_4 was reversed after washout. Lower case letters refer to corresponding points on the traces in (A) and (B). (C) Dogiel type II neuron from which the records were obtained. Scale bar = 20 μm.

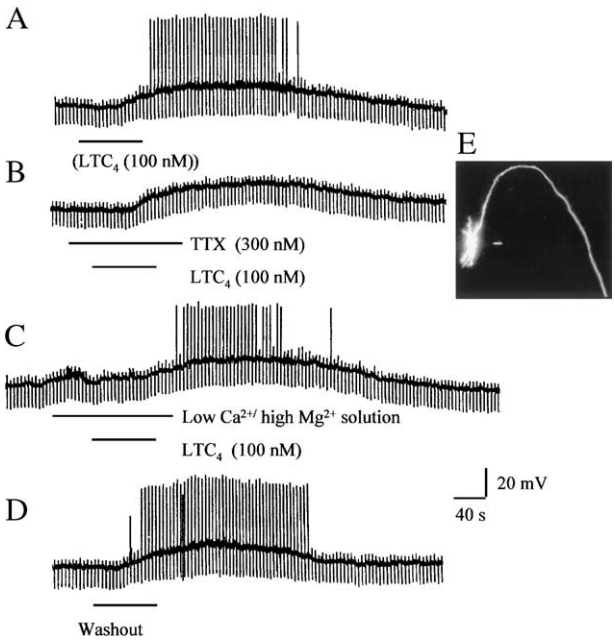

Fig. 4. The depolarizing action of cysteinyl leukotrienes was resistant to TTX and low Ca^{2+}/high Mg^{2+} solutions. (A) Application of LTC_4 evoked membrane depolarization and enhanced excitability in an S type myenteric neuron in the guinea-pig small intestine. (B) Tetrodotoxin (300 nM) did not suppress depolarizing responses to 100 nM LTC_4. Action potentials that occurred at the offset of hyperpolarizing current pulses were suppressed by TTX. (C) Low Ca^{2+} (0.5 mM)/high Mg^{2+} (12 mM) in the bathing solution did not suppress depolarizing responses to 100 nM LTC_4. (D) Reversal of TTX action after washout. Downward deflections on all records are electrotonic potentials evoked by repetitive intraneuronal injection of constant-current hyperpolarizing pulses. (E) Morphology of the Dogiel type I neurons from which the records were obtained. Scale bar = 20 μm.

3.1. Actions of cysteinyl leukotrienes on enteric neurons

Application of leukotriene C_4, leukotriene D_4 and leukotriene E_4 either by superfusion or by micropressure ejection from fine-tipped pipettes had no effect on membrane potential or excitability of 30 gastric myenteric neurons when applied in concentrations of 1 nM to 1 μM (Fig. 1). The same

Table 2
Association of morphology, electrophysiology and cysteinyl leucotriene actions on enteric neurons

Morphology	*n*	Electrical behavior	CysLT excitation
Dogiel II	96	96 AH type	89
Dogiel I	80	80 S type	38
Filamentous	43	40 S type	25
		3 AH type	3

cysteinyl leukotrienes evoked slowly activating depolarizing responses in most of the myenteric and submucosal neurons in the small intestine (see Figs. 2, 3, 4, 8 and 10A for examples). The depolarizing responses evoked by cysteinyl leukotrienes were associated with enhanced excitability as indicated by an increased number of action potentials evoked by intracellular injection of depolarizing current pulses (Fig. 3Bb), by spontaneous action potential discharge (Fig. 10A), and by the occurrence of anodal-break excitation at the offset of hyperpolarizing current pulses (Fig. 2A). Suppression of hyperpolarizing after-potentials occurred in AH type neurons (Fig. 3). These effects began within 30–45 s after entry of the cysteinyl leukotrienes into the tissue chamber and reached their peak after 2.5–3.5 min. Recovery of the membrane potential to control levels required 15–20 min after washout. In 30% (26/85) of the intestinal neurons, the membrane potential did not repolarize to the baseline and subsequent applications of cysteinyl leukotrienes at the same

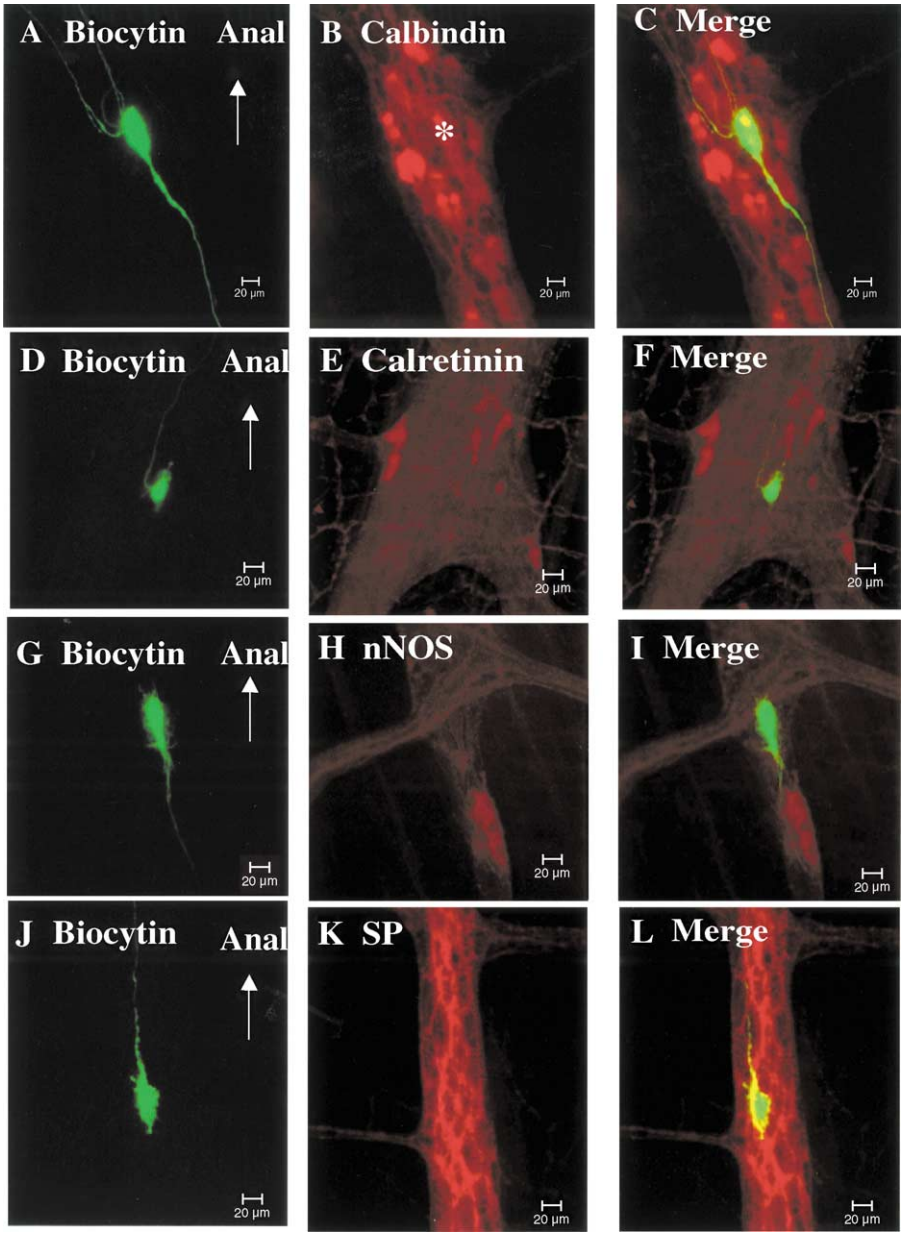

Fig. 5. Morphology and neurochemical coding of myenteric neurons that were excited by cysteinyl leukotrienes. (A–C) An AH type neuron with Dogiel type II morphology was immunoreactive for calbindin. (D–F) An anally projecting S type neuron with Dogiel type I morphology was not immunoreactive for calretinin. (G–I) An anally projecting S type neuron with Dogiel type I morphology was not immunoreactive for nNOS. (J–L) An S type neuron with Dogiel type I morphology and a single long process running in the circumferential direction was immunoreactive for SP. For each series, the right panel is the digital merger of the preceding two images.

concentration evoked weaker responses, presumably due to receptor desensitization phenomena. In neurons without apparent desensitization, the effects of cysteinyl leukotrienes were concentration-dependent (Fig. 2B). The EC_{50}s for leukotriene C_4, leukotriene D_4 and leukotriene E_4 were 13.9 ± 1.5, 19.9 ± 3.2 and 28.4 ± 1.9 nM, respectively, with a potency order of leukotriene C_4>leukotriene D_4>leukotriene E_4. Leukotriene C_4 and leukotriene D_4 produced comparable maximum depolarization (22.0 ± 1.8 and 21.0 ± 2.4 mV; $n = 4$, respectively). At a concentration of 1 μM, leukotriene E_4 evoked significantly smaller responses than either leukotriene C_4 or leukotriene D_4 (14.7 ± 1.2 mV; $n = 6$; $p < 0.05$) at the same concentration.

In order to explore whether the cysteinyl leukotrienes evoked responses were due to secondary synaptic release of other excitatory neurotransmitters, tetrodotoxin (300 nM) or low Ca^{2+} (0.5 nM)/high Mg^{2+} (12 mM) solutions were used to suppress neurotransmitter release. The depolarizing actions of cysteinyl leukotrienes persisted in 300 nM tetrodotoxin or low Ca^{2+}/high Mg^{2+} solutions in all of the 15 neurons tested (Fig. 4), indicating a direct action of cysteinyl leukotrienes on the neuron from which the recording was made rather than activation of neurons synaptically connected with the impaled cell.

Both AH and S type neurons in the small intestine were depolarized by cysteinyl leukotrienes. However, the responses to these leukotrienes were quantitatively different for the two cell populations with the greatest proportion of responses occurring in AH type neurons. Leukotriene C_4 was effective in 94% (87/93) of AH type and 52% (36/69) of S type neurons. Leukotriene D_4 was effective in 96% (106/110) of AH type and 46% (36/78) of S type neurons. Leukotriene E_4 was effective in 87% (55/63) of AH type and 33% (10/30) of S type neurons.

3.2. Morphology and neurochemical coding of the neurons excited by cysteinyl leukotrienes

The morphology of the neurons exposed to cysteinyl leukotrienes was identified in 219 of 317 neurons that were

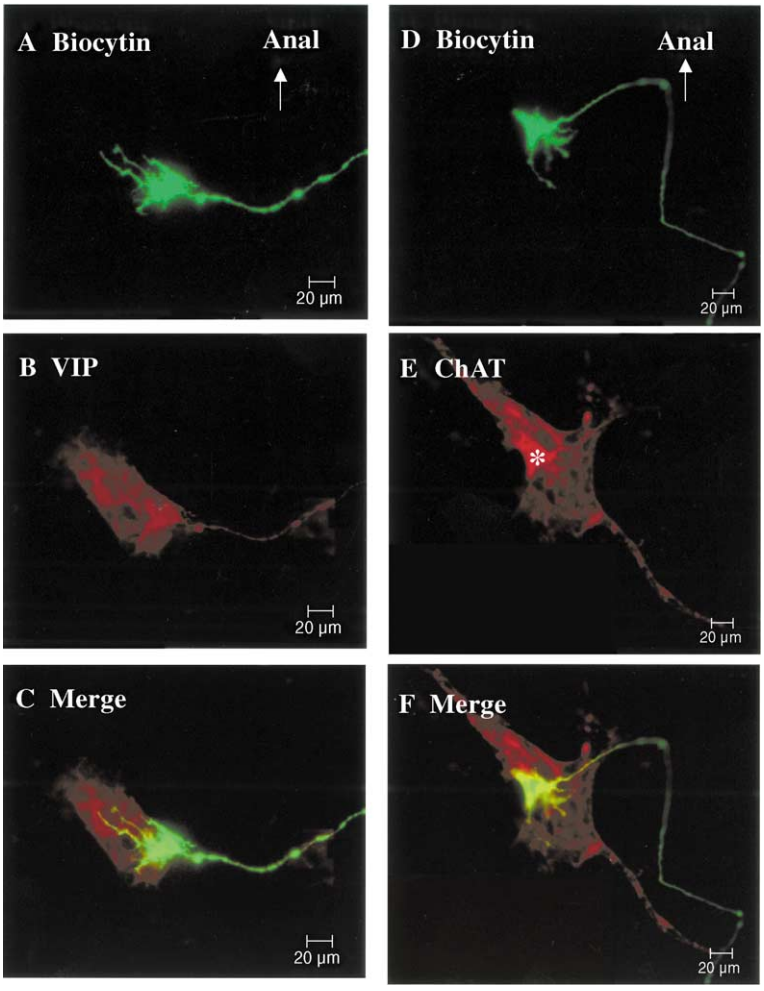

Fig. 6. Morphology and neurochemical coding of submucosal neurons that were excited by cysteinyl leukotrienes. (A–C) An S type neuron with filamentous morphology was immunoreactive for VIP. (D–F) An S type neuron with filamentous morphology was immunoreactive for ChAT. For each series, the bottom panel is the digital merger of the preceding two images.

impaled for electrophysiological recording. They were classified according to their shape (Furness et al., 1988). Cysteinyl leukotrienes excited multipolar Dogiel type II neurons (93%; 89/96), uniaxonal Dogiel type I neurons (48%; 38/80), and uniaxonal neurons with numerous filamentous dendrites (65%; 28/43). All the cysteinyl leukotriene-responsive Dogiel type II neurons had AH (after hyperpolarization) type electrophysiological behavior, and all the Dogiel type I neurons had S (synaptic) type electrophysiological behavior. Neurons with filamentous dendrites belonged to either the AH or S type (Table 2).

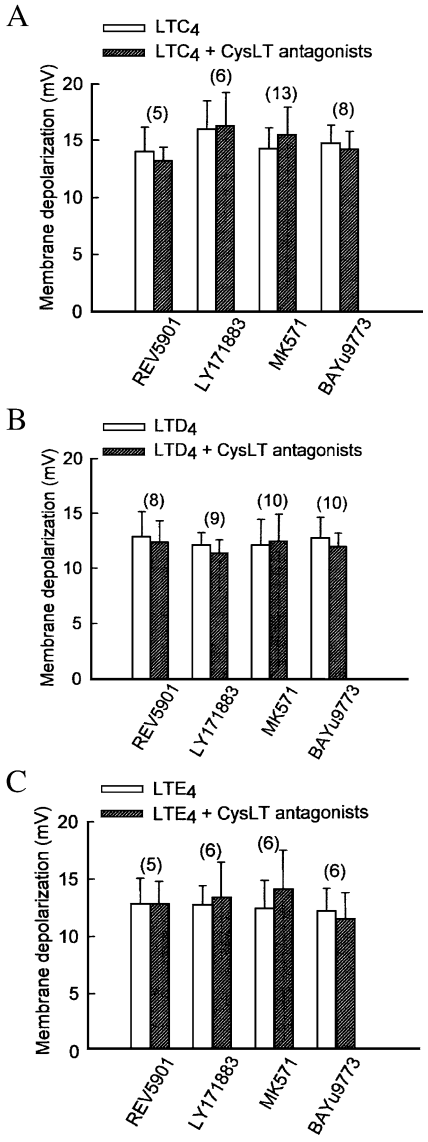

Fig. 7. Effect of cysteinyl leukotriene receptor antagonists on the depolarizing action of LTC_4 (30 nM), LTD_4 (30 nM) and LTE_4 (30 nM). (A) The $CysLT_1$ receptor antagonists REV 5901 (10 μM), LY 171883 (10 μM) and MK 571 (10 μM) or the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (10 μM) did not significantly alter the depolarizing action of LTC_4. (B) The same four antagonists did not suppress depolarizing responses to LTD_4. (C) The same four antagonists did not suppress depolarizing responses to LTE_4. Numbers in parenthesis represent the number of neurons tested.

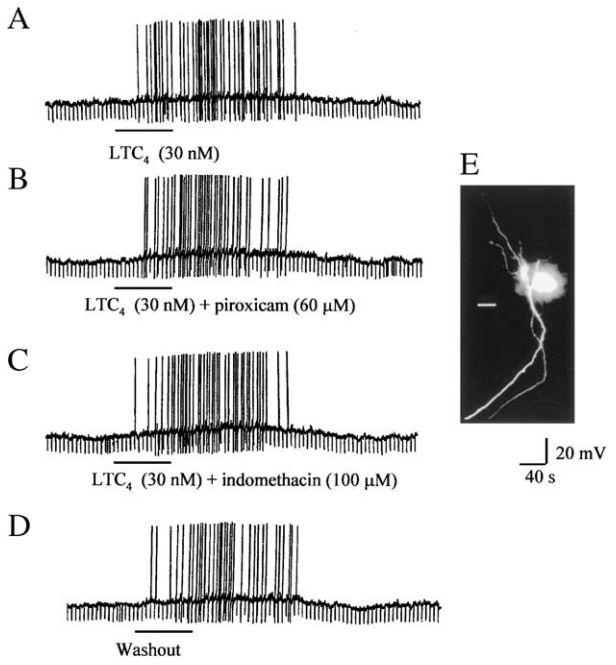

Fig. 8. Effect of cyclooxygenase inhibitors on depolarizing responses to cysteinyl leukotrienes. (A) Application of LTC_4 (30 nM) evoked membrane depolarization and enhanced excitability in an AH type myenteric neuron in the guinea-pig small intestine. (B) Piroxicam (60 μM) did not suppress the excitatory action of LTC_4. (C) Indomethacin (100 μM) did not suppress the excitatory action of LTC_4. (D) After washout of indomethacin. Downward deflections on each record are electrotonic potentials evoked by repetitive intraneuronal injection of constant-current hyperpolarizing pulses. (E) Morphology of the Dogiel type II neuron from which the records were obtained. Scale bar = 20 μm.

Data on immunoreactivity for calbindin, calretinin, nitric oxide synthase, and substance P were obtained from 48 myenteric neurons of the guinea pig small intestine that were excited by cysteinyl leukotrienes. Eighteen AH type neurons with Dogiel type II morphology were tested for calbindin immunoreactivity; of these, 14 neurons (78%) were immunoreactive (Fig. 5A–C). Ten S type neurons with Dogiel type I morphology were tested for calretinin immunoreactivity; none was immunoreactive (Fig. 5D–F). Eight S type neurons with Dogiel type I morphology were tested for nitric oxide synthase immunoreactivity; none was immunoreactive (Fig. 5G–I). Twelve S type neurons with Dogiel type I morphology were tested for substance P immunoreactivity; of these, five neurons (42%) were immunoreactive (Fig. 5J–L).

Immunoreactivity for vasoactive intestine peptide and choline acetyltransferase was obtained from 21 submucosal neurons that were excited by cysteinyl leukotrienes. Ten S type neurons with Dogiel type I or filamentous morphology were tested for vasoactive intestinal peptide immunoreactivity; of these, five neurons (50%) were immunoreactive (Fig. 6A–C). Eleven S type neurons with Dogiel type I or filamentous morphology were tested for choline acetyltransferase immunoreactivity; of these, six neurons (54%) were immunoreactive (Fig. 6D–F).

3.3. Pharmacological properties of the cysteinyl leukotriene responses

To obtain full recovery of cysteinyl leukotriene responses during pharmacological experiments, the concentration of cysteinyl leukotrienes was reduced to 30 nM. The $CysLT_1$ receptor antagonists MK 571 (10 μM), LY 171883 (10 μM) and REV 5901 (10 μM) did not significantly alter the depolarizing action of leukotriene C_4, leukotriene D_4 and leukotriene E_4, neither did the dual $CysLT_1/CysLT_2$ receptor antagonist BAY u9773 (10 μM) (Fig. 7). Application of the antagonists alone had no membrane depolarizing effect.

To determine whether the effects of the cysteinyl leukotrienes were mediated by cyclooxygenase metabolites of arachidonic acid, indomethacin or piroxicam was added to the superfusion solution before and during cysteinyl leukotrienes application. Pretreatment with 60 μM piroxicam or 100 μM indomethacin did not alter the amplitude of depolarization evoked by leukotriene C_4 in seven enteric neurons (Fig. 8). Similar results were observed for leukotriene D_4 and leukotriene E_4 (data not shown). These results suggest that prostaglandins such as prostaglandin E_2, prostaglandin D_2 or thromboxane A_2 were not involved in the cysteinyl leukotriene-evoked excitatory effects in enteric neurons.

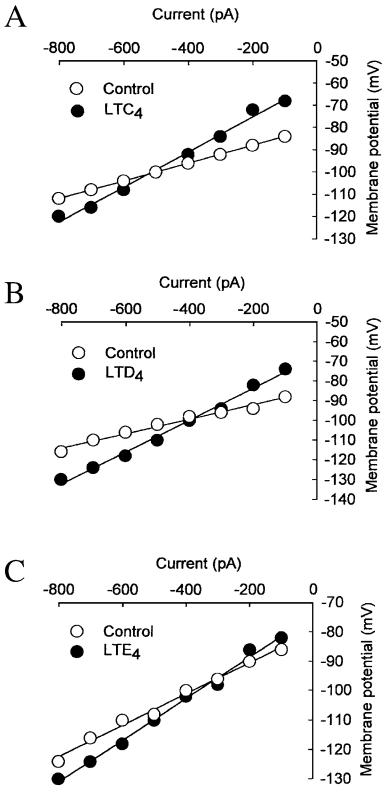

Fig. 9. Current–voltage ($I–V$) relations in the presence and absence of cysteinyl leukotrienes. (A) The slope of $I–V$ curves was increased relative to control when $I–V$ data were obtained in the presence of LTC_4. (B) The slope of $I–V$ curves was increased relative to control when $I–V$ data were obtained in the presence of LTD_4. (C) The slope of $I–V$ curves was increased relative to control when $I–V$ data were obtained in the presence of LTE_4. An increased slope reflects increased input resistance. The $I–V$ curves intersect near a membrane potential of −90 mV, which is close to the value for the estimated K^+ equilibrium potential. The concentration for LTC_4, LTD_4 and LTE_4 was 100 nM.

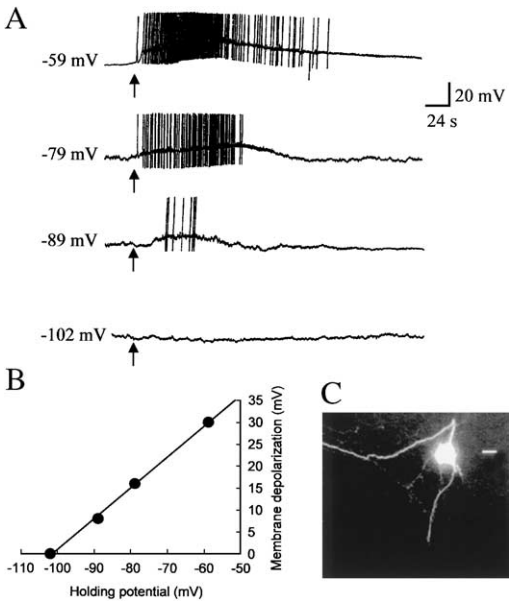

Fig. 10. The reversal potential for the depolarizing responses to the cysteinyl leukotrienes was near a membrane potential of −100 mV. (A) Amplitude of the depolarizing responses to 40 ms micropressure pulses of 1 μM LTC_4 progressively decreased as the resting membrane potential was current-clamped in the depolarizing direction starting at −59 mV and continuing in steps that stopped at −102 mV. (B) The data from (A) extrapolate to a reversal potential near −100 mV. (C) Morphology of the Dogiel type II neuron from which the results in (A) were obtained. Scale bar = 20 μm.

3.4. Current–voltage relationship for cysteinyl leukotrienes-evoked depolarization

Effects of cysteinyl leukotrienes on neuronal input resistance were evaluated by comparing the amplitudes of electronic potentials evoked by intraneuronal injection of hyperpolarizing current pulses before and after application of cysteinyl leukotrienes. Most of the neurons (92%, 77/84) showed an increase in input resistance during leukotriene C_4 application, while others (8%, 8/84) exhibited no measurable change. Similar results were observed for leukotriene D_4 and leukotriene E_4. Plots of current–voltage relations also revealed increased input resistance in a majority of the neurons during the cysteinyl leukotriene-evoked depolarizing responses, which was reflected by an increased slope relative to control (Fig. 9). The current–voltage curves obtained in the presence and absence of cysteinyl leukotrienes intersected at membrane potentials between −85 and −110 mV, with an average of -100.4 ± 2.4 mV ($n = 5$) for 100 nM leukotriene C_4 (Fig. 9A), -98.8 ± 2.9 mV ($n = 5$) for 100 nM leukotriene D_4 (Fig. 9B) and -98.6 ± 3.2 mV ($n = 5$) for 100 nM leukotriene E_4 (Fig. 9C). These results suggested that the reversal potential for

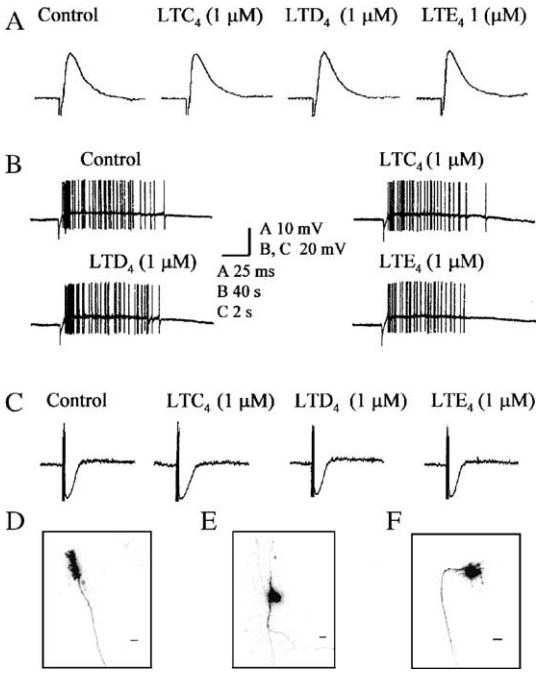

Fig. 11. Exposure to cysteinyl leukotrienes did not alter neurotransmission in the myenteric or submucous plexuses of guinea-pig small intestine. (A) Fast nicotinic EPSPs evoked in an S type myenteric neuron were unaffected by LTC_4, LTD_4 or LTE_4. (B) Slow EPSPs evoked in an AH type myenteric neuron were unaffected by LTC_4, LTD_4 or LTE_4. (C) Noradrenergic IPSPs evoked in an S type submucosal neuron were unaffected by LTC_4, LTD_4 or LTE_4. (D) Morphology of the neuron from which the results in (A) were obtained. (E) Morphology of the neuron from which the results in (B) were obtained. (F) Morphology of the neuron from which the results in (C) were obtained. Scale bars = 20 μM.

the conductance change was near the estimated K^+ equilibrium potential for the neurons (Wood, 1989, 1994). This was reinforced by observations that progressively current-clamping the membrane potential to potentials more negative than the resting membrane potential was accompanied by a decrease in the amplitude of the depolarizing responses to leukotriene C_4 (Fig. 10A and B). Current clamping the membrane potential to levels more positive than the resting membrane potential resulted in an increase in the amplitude of depolarization to leukotriene C_4 (Fig. 10A and B). The reversal potential obtained in this manner was between -87 and -102 mV with an average of -96.4 ± 2.6 mV ($n = 5$), which was also close to the estimated K^+ equilibrium potential for enteric neurons (Wood, 1989, 1994).

3.5. Effects of cysteinyl leukotrienes on synaptic transmission

Focal electrical stimulation applied to nerve fibers in interganglionic fiber tracts is well known as an effective method for the study of synaptic transmission in the enteric nervous system. Fast EPSPs, slow EPSPs and slow IPSPs are the principal synaptic potentials evoked by fiber tract stimulation in the enteric nervous system (Wood, 1989, 1994). Cysteinyl leukotrienes had no effects on any of the three categories of synaptic potentials in either the stomach or small intestine even at supramaximal concentrations of 1 μM (Fig. 11 and Table 3). This suggests that cysteinyl leukotrienes do not influence neurotransmission in the enteric nervous system.

4. Discussion

The results show a potent excitatory action of the cysteinyl leukotrienes, leukotriene C_4, leukotriene D_4 and leukotriene E_4 in both myenteric and submucosal neurons of the guinea-pig small intestine. On the contrary, no action of an excitatory or inhibitory nature was found in gastric myenteric neurons. Lack of action in the gastric ENS may be explained by absence of expression of the appropriate receptor/s for cysteinyl leukotrienes, by absence of necessary post-receptor signal transduction cascades or a combination of the two.

Electrophysiological responses to cysteinyl leukotrienes in small intestinal neurons mimicked the slow synaptic excitation that is characterized by membrane depolarization, enhancement of excitability and suppression of hyperpolarizing after-potentials in AH type neurons in the enteric nervous system. Persistence of the excitatory action after blockade of synaptic transmission by tetrodotoxin or low Ca^{2+}/high Mg^{2+} solutions was indicative of a direct action at receptors on the neuronal cell bodies.

Table 3
Effects of cysteinyl leukotrienes on synaptic transmission

	LTC_4	LTD_4	LTE_4
Fast EPSP	Control 16.3 ± 3.1 mV LTC_4 16.7 ± 3.0 mV ($n = 6$)	Control 17.0 ± 3.5 mV LTD_4 16.7 ± 3.4 mV ($n = 6$)	Control 17.3 ± 3.4 mV LTE_4 17.3 ± 4.3 mV ($n = 6$)
Slow EPSP	Control 10.8 ± 2.5 mV LTC_4 11.6 ± 3.2 mV ($n = 5$)	Control 11.7 ± 2.2 mV LTD_4 11.0 ± 2.0 mV ($n = 6$)	Control 11.2 ± 1.5 mV LTE_4 10.8 ± 1.2 mV ($n = 5$)
Slow IPSP	Control 18.8 ± 3.4 mV LTC_4 18.4 ± 3.5 mV ($n = 5$)	Control 21.6 ± 3.0 mV LTD_4 21.2 ± 3.2 mV ($n = 5$)	Control 19.2 ± 3.4 mV LTE_4 18.4 ± 3.5 mV ($n = 5$)

All values are means ± S.E.M. Concentrations used were 1 μM for LTC_4, LTD_4 and LTE_4, respectively. The n values in parentheses refer to the number of neurons tested. For all paired comparisons, $p > 0.05$.

No effect on neurotransmission was found at the fast nicotinic excitatory synapses, the slow excitatory synapses or the slow noradrenergic inhibitory synapses in the synaptic microcircuits of either stomach or small intestine. Lack of effects of the cysteinyl leukotrienes on neurotransmission contrasts with the actions of other putative inflammatory mediators, such as histamine, interleukin-1β and bradykinin, which suppress neurotransmission (Hu et al., 1998; Xia et al., 1999; Liu et al., 2000).

The $CysLT_1$ receptor antagonists REV 5901, LY 171883 and MK 571 did not suppress the cysteinyl leukotriene-evoked excitation of either myenteric or submucosal neurons. These antagonists have previously been shown to be potent and selective at the $CysLT_1$ receptor (Van Inwegen et al., 1987; Krausz et al., 1988; Lynch et al., 1999). According to the current IUPHAR definition of CysLT receptors, the receptor responsible for a functional response resistant to $CysLT_1$ receptor antagonists is termed "$CysLT_2$" (Coleman et al., 1995). The leukotriene E_4 analogue BAY u9773 has been shown to inhibit some of the responses that are resistant to $CysLT_1$ receptor antagonists, and so far is the only known dual antagonist that has activity at both $CysLT_1$ and $CysLT_2$ receptors (Coleman et al., 1995; Back et al., 1996). Excitation evoked by cysteinyl leukotrienes in enteric neurons was also resistant to BAY u9773, suggesting that this effect was mediated by a CysLT receptor with properties distinct from those of previously described $CysLT_1$ and $CysLT_2$ receptor subtypes. In fact, the existence of new receptor subtype(s) for cysteinyl leukotrienes has been suggested by previous studies in guinea-pig lung parenchymal strips and in porcine and human pulmonary artery where a substantial residual contraction evoked by leukotriene C_4 and leukotriene D_4 was not blocked by either the classical CysLT receptor antagonists or BAY u9773 (Jonsson et al., 1998; Back et al., 2000a,b). Further delineation of the nature of this new CysLT receptor subtype will require availability of new selective agonists and antagonists.

Arachidonic acid metabolites (e.g. prostaglandins) were proposed to be mediators of cysteinyl leukotriene-evoked intestinal smooth muscle contraction and ion secretion as determined by suppression of these actions by the cyclooxygenase inhibitor indomethacin (Smith et al., 1988; Percy et al., 1990b; Back et al., 2000b). Such a proposal was reasonable in view of the importance of prostaglandins as signal substances in neuroimmune interactions in the gastrointestinal tract (Lawson and Powell, 1987). Our findings are inconsistent with the proposal for involvement of prostaglandins or thromboxanes as intermediates in the actions of cysteinyl leukotrienes because the neuronal actions of the cysteinyl leukotrienes were unaffected by cyclooxygenase inhibition with indomethacin or piroxicam.

Input resistance was increased in most of the neurons during the excitatory responses to cysteinyl leukotrienes. Analysis of current–voltage relations suggested that reversal potentials for depolarizing responses would be near the value estimated for the K^+ equilibrium potential in enteric neurons (Wood, 1989, 1994). Increased input resistance during a depolarizing response with a reversal potential near the K^+ equilibrium potential suggests that inactivation and closure of K^+ channels was involved in the actions of cysteinyl leukotrienes. Exposure to the cysteinyl leukotrienes, like other inflammatory mediators, suppressed the hyperpolarizing after-potentials in AH type neurons. Considering that hyperpolarizing after-potentials in AH type neurons reflect opening of Ca^{2+}-activated K^+ channels suggests that inactivation of Ca^{2+}-activated K^+ channels is also an action of cysteinyl leukotrienes (Wood, 1989, 1994).

The cysteinyl leukotrienes evoked depolarizing responses in neurons with Dogiel type I, Dogiel type II and filamentous morphology. The percentage of responding neurons in each of the three populations differed, with the Dogiel type II population having by far the greatest proportion of neurons expressing the receptors. The order of percentage of neurons with CysLT responses for each population, ranging from highest to lowest, was Dogiel type II>filamentous>Dogiel type I neurons. All CysLT responsive Dogiel type II neurons had AH type electrophysiological properties and most filamentous and all Dogiel type I neurons had S type electrophysiological properties. These neuronal subtypes are known to subserve specific functions when synaptically connected into ENS microcircuits.

In the myenteric plexus, only a small number of Dogiel type I neurons with S type electrophysiological properties and SP immunoreactivity responded to the cysteinyl leukotrienes, whereas most of the Dogiel type II neurons with calbindin immunoreactivity and AH type electrophysiological properties responded. The cysteinyl leukotrienes evoked depolarizing responses in both VIPergic and cholinergic neurons in the submucosal plexus. The available evidence suggests that S type neurons with Dogiel type I morphology and SP immunoreactivity in the myenteric plexus are excitatory motor neurons that project to the intestinal musculature, whereas the AH type neurons with Dogiel type II morphology and calbindin immunoreactivity are interneurons in feed-forward synaptic circuits that are responsible for excitatory drive and coordination of the discharge of motor neurons to intestinal effector systems (Furness et al., 1988; Wood, 1989, 1994; Thomas et al., 1999). Vasoactive intestinal peptide-immunopositive neurons in the submucosal plexus are secretomotor neurons that evoke chloride secretion from the intestinal crypts, and the cholinergic neurons are either secretomotor neurons or interneurons (Bornstein and Furness, 1988). Activation of the excitatory motor neurons and interneurons in the myenteric plexus by cysteinyl leukotrienes would be expected to augment contractile responses of the smooth muscle and activation of secretomotor neurons and interneurons in the submucosal plexus would be expected to enhance water and electrolyte secretion. This may be the neural basis for the elevated contractile activity of the intestinal musculature and enhanced mucosal secretory activity seen during exposure to the cysteinyl leukotrienes (Sirois et al., 1981; Goldenberg and Subers, 1983; Smith et al., 1988).

The slow EPSP-like excitatory effects of cysteinyl leukotrienes on enteric neurons are strikingly similar to the effects of other inflammatory mediators such as histamine, prostaglandin E_2, bradykinin and cytokines (Nemeth et al., 1984; Hu et al., 1998, 1999; Xia et al., 1999). Nevertheless, unlike the other inflammatory mediators, cysteinyl leukotrienes have no effect on synaptic transmissions in the ENS, while histamine, prostaglandin E_2, bradykinin and cytokines act at presynaptic inhibitory receptors to suppress synaptic transmission (Tamura et al., 1988; Hu et al., 1998, 1999; Xia et al., 1999; Liu et al., 2000). Absence of any effects of cysteinyl leukotrienes on synaptic transmission suggests that the presynaptic nerve terminals do not express receptors for cysteinyl leukotrienes.

The physiological significance of cysteinyl leukotriene actions in the ENS remains to be established. A neurotransmitter role for the cysteinyl leukotrienes is unlikely because there is no evidence that cysteinyl leukotrienes are expressed and released by enteric neurons. The cysteinyl leukotrienes are more likely to be paracrine mediators with potential pathophysiological significance in immuno-neural signaling. Cysteinyl leukotriene levels are reported to be elevated in inflammatory bowel disease (Peskar et al., 1986; Fox et al., 1990). Like other better known paracrine mediators in the gut, cysteinyl leukotrienes may be released from inflammatory/immune cells and form a modulatory overlay on integrated circuits of the enteric nervous system that acts selectively on elements of the microcircuits to reconfigure the output of the circuit. Histamine is an example of such a mediator. Histamine is released from intestinal mast cells during allergic reactions and forms a modulatory overlay on the enteric microcircuits that reconfigures the output of the circuits for a specific program of defensive behavior in the bowel. The defensive program called-up from the program library in the enteric nervous system by the paracrine signal consists of an enhanced secretory response followed in sequence by powerful propulsive motility (Wood, 1993, 2000, 2002). Operation of the neural program rapidly expels threats, such as foreign antigens and noxious substances, from the intestinal lumen and is accompanied by symptoms of diarrhea and cramping abdominal pain.

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