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RESEARCH PAPER

Modulation of mouse gastrointestinal motility by allyl isothiocyanate, a constituent of cruciferous vegetables (*Brassicaceae*): evidence for TRPA1-independent effects

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BACKGROUND AND PURPOSE

Allyl isothiocyanate (AITC, mustard oil), a constituent of many common cruciferous vegetables (*Brassicaceae*), activates transient receptor potential of ankyrin type-1 (TRPA1) channels, claimed to regulate gastrointestinal contractility. In this study, we have investigated the effect of AITC on intestinal motility.

EXPERIMENTAL APPROACH

Effects of AITC were investigated *in vivo* on upper gastrointestinal transit in mice and in mouse isolated ileum [contractions induced by electrical field stimulation (EFS), acetylcholine and spontaneous contractility]. The contractor activity of AITC was studied in mouse isolated colon. The ability of TRPA1 channel antagonists to block AITC-induced elevation of intracellular Ca^{2+} $[Ca^{2+}]_i$ was assessed in HEK293 cells transfected with rat TRPA1 channels.

KEY RESULTS

AITC increased $[Ca^{2+}]_i$ in HEK293 cells, reduced ileal contractility (acetylcholine-, EFS-induced contractions and spontaneous contractility), but contracted the isolated colon. Gentamicin and camphor (non-selective TRPA1 channel antagonists), HC-030031 and AP18 (selective TRPA1 channel agonists) inhibited AITC-induced effects in HEK293 cells but not in the ileum or colon. AITC-induced contractions were reduced by tetrodotoxin and strongly reduced by nifedipine, cyclopiazonic acid and ryanodine. *In vivo*, AITC reduced (following i.p. administration) or increased (following intragastric administration) upper gastrointestinal transit in mice These effects were not affected by HC-030031.

CONCLUSION AND IMPLICATIONS

AITC, depending, *in vitro*, on the regions of gut examined and, *in vivo*, on the route of administration, exerted both stimulatory and inhibitory effects on intestinal motility, which were not sensitive to TRPA1 channel antagonists. The proposition that TRPA1 channels are the primary targets for AITC to induce contraction should be revised.

Abbreviations

AITC, allyl isothiocyanate; AP18, 4-(4-chlorophenyl)-3-methyl-3-buten-2-one oxime; EFS, electrical field stimulation; GC, geometric centre; HC-030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl) acetamide; i.g., intragastric; TRPA1, transient receptor potential ankyrin 1

Introduction

Allyl isothiocyanate (AITC), also known as mustard oil, is a colourless organo-sulphur compound responsible for the pungent taste of many common cruciferous vegetables, such as black mustard (*Brassica nigra*), horseradish (*Armoracia rusticana*) and wasabi (*Eutrema japonica*). AITC is derived from the glucosinolate sinigrin (a thio-sugar) (Kushad *et al.*, 1999; Fahey *et al.*, 2001; Assayed and Abd El-Aty, 2009; Björkman *et al.*, 2011). Whenever sinigrin-containing plant tissue is crushed, the enzyme myrosinase hydrolyses sinigrin to AITC (Appendino *et al.*, 2008; Björkman *et al.*, 2011). AITC has been shown to exert a number of pharmacological actions, including anticancer (Bhattacharya *et al.*, 2010; Chen *et al.*, 2010), antioxidant (Hasegawa *et al.*, 2010), analgesic, anti-inflammatory (Woo *et al.*, 2007), gastric antiulcer (Matsuda *et al.*, 2007) and chemopreventive (Assayed and Abd El-Aty, 2009; Zhang, 2010) effects. Many of the pharmacological actions of AITC are believed to be due to activation of the transient receptor potential (TRP) ankyrin 1 (TRPA1) channel, a member of the TRP family of ion channels (Jordt *et al.*, 2004; Bautista *et al.*, 2005; McNamara *et al.*, 2007; Viana and Ferrer-Montiel, 2009; Baraldi *et al.*, 2010; Nassini *et al.*, 2010; channel nomenclature follows Alexander *et al.*, 2011).

TRPA1 channels, originally characterized as noxious, cold-activated, ion channels (Story *et al.*, 2003), were later shown to be activated by a number of structurally different plant – mainly pungent – compounds, including AITC (Jordt *et al.*, 2004; Bautista *et al.*, 2005; Cortright and Szallasi, 2009; Viana and Ferrer-Montiel, 2009; Wu *et al.*, 2010). TRPA1 channels are present in a large proportion of mammalian somatic and visceral sensory neurons and their activation was found to cause neurogenic inflammation (Trevisani *et al.*, 2007; Kondo *et al.*, 2010), bronchoconstriction (Andrè *et al.*, 2009) and somatic and visceral hyperalgesia (Bautista *et al.*, 2005; Petrus *et al.*, 2007; Cattaruzza *et al.*, 2010; Mitrovic *et al.*, 2010). Recent evidence also suggests the presence of TRPA1 channels in non-neuronal cells, including the mucosa of the gastrointestinal tract (Stokes *et al.*, 2006; Purhonen *et al.*, 2008; Boesmans *et al.*, 2011; Holzer, 2011). The mRNA for TRPA1 channels was expressed along all the small and large intestine in mice (Penuelas *et al.*, 2007) and functional studies showed that AITC evoked contraction of the guinea pig ileum and mouse colon (Penuelas *et al.*, 2007; Nozawa *et al.*, 2009). The contractile effects of AITC have been attributed to activation of TRPA1 channels, although in these studies, the effect of selective TRPA1 channel blockade was not evaluated (Penuelas *et al.*, 2007; Nozawa *et al.*, 2009).

Given the presumed importance of TRPA1 channels in modulating intestinal contractility, in the present study we have assessed the effect (and mode of action, by using for the first time in intestinal contractility/motility studies recently developed TRPA1 channel antagonists) of the TRPA1 channel agonist AITC on mouse intestinal motility. AITC was evaluated on spontaneous and electrically (or agonist-) induced contractility in the isolated ileum and we also studied its contractile effect in the isolated mouse colon. *In vivo*, the effects of AITC were evaluated on upper gastrointestinal transit in mice.

Methods

TRPA1 channel assay

HEK293 cells overexpressing the rat TRPA1 channel (De Petrocellis *et al.*, 2008) were grown as monolayers in minimum essential medium, supplemented with non-essential amino acids, fetal calf serum (10 %) and glutamine (2 mM), maintained under CO_2 (5%) at 37°C, and plated on Petri dishes (100 mm diameter). The effect of substances on the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was determined by use of Fluo-4, a selective intracellular fluorescent probe for Ca^{2+}. For this purpose, on the day of the experiment, cells were loaded for 1 h at room temperature with the methyl ester Fluo4-AM (4 μM, Invitrogen, Carlsbad, CA, USA) in minimum essential medium without fetal bovine serum. After the loading, cells were washed twice in Tyrode's buffer [NaCl (145 mM), KCl (2.5 mM), $CaCl_2$ (1.5 mM), $MgCl_2$ (1.2 mM), D-glucose (10 mM) and HEPES (10 mM), pH 7.4], resuspended in Tyrode's buffer, and transferred (5–6 x 10^4 cells) to the quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B; Perkin-Elmer, Waltham, MA, USA) with continuous stirring. The $[Ca^{2+}]_i$ was determined before and after the addition of various concentrations of AITC by measuring cell fluorescence at 25°C (λ_{EX} = 488 nm, λ_{EM} = 516 nm). In oreder to assess functional antagonism of TRPA1 channels, four TRPA1 channel antagonists, AP18, 2-(1,3-dimethyl-2,6-dioxo-1,2,3, 6-tetrahydro-7*H*-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide (HC-030031), camphor or gentamicin, were pre-incubated with the cells, for 5 min before the addition of AITC.

Animals

All animal care and experimental procedures complied with the Principles of laboratory animal care (NIH publication no.86–23, revised 1985) and the Italian D.L. no.116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). Male ICR mice (Harlan Laboratories, S. Pietro al Natisone, Italy) weighing 20–25 g were used after a 1 week acclimation period (temperature 23 ± 2°C; humidity 60%, free access to water and standard food).

Isolated organ preparations

Mice were killed by asphyxiation with carbon dioxide and the ileum and the colon were removed, flushed free of luminal contents, and placed in Krebs solution (composition: NaCl 119 mM, KCl 4.75 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, $MgSO_4$ 1.5 mM, $CaCl_2$ 2.5 mM and glucose 11 mM). Segments of 1.0–1.5 cm were cut from the distal ileum (or distal colon) and placed in 20 ml thermostatically controlled (37°C) organ baths, containing Krebs solution gassed with 95% O_2 and 5% CO_2. The tissues were connected to an isometric transducer (tension: 5 mN) in such a way to record contractions from the longitudinal axis. Mechanical activity was digitized on an analog-to-digital converter, visualized, recorded and analysed on a personal computer using the PowerLab/400 system (Ugo Basile, Comerio, Italy). All experiments started after equilibration forat least 1 h.

Electrically and agonist-induced contractions in the isolated ileum

These experiments were performed in order to evaluate the effect of AITC on electrically- and agonist-induced contractions. Accordingly, two different sets of experiments were performed. In one set of experiments, contractions were obtained with electrical field stimulation (EFS; 8 Hz for 10 s, 400 mA, 1 ms pulse duration) by a pair of electrodes placed around the ileal tissue; in another set of experiments tissues were stimulated with acetylcholine (1 μM) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 0.2 mM). Both agonists were added to the bath and left in contact with the tissue for 60 s and then washed out. These concentrations of agonists gave a contractile response that was similar in amplitude to that of EFS. The interval between each contraction (EFS or agonists) was 20 min. After stable control contractions evoked by EFS or acetylcholine had been recorded, contractile responses to increasing cumulative concentrations of AITC (10nM–300μM) were obtained. The contact time for each concentration was 20 min. In some experiments, contractions were induced by KCl (65.9 mM). KCl was left in contact with the tissue for 60 s and then washed out.

Contractions were expressed as % of contractions produced by 100 μM acetylcholine; this concentration of acetylcholine produced a maximal contractile response (100% contraction). EFS-induced contractions were performed in the presence of the acetylcholinesterase inhibitor neostigmine (1 μM), to potentiate cholinergic neurotransmission (Capasso *et al.*, 2008b; Baldassano *et al.*, 2009).

Spontaneous contractility in the isolated ileum

In this set of experiments, we evaluated the effect of AITC on spontaneous contractility. After stable control spontaneous contractions had been recorded (at least 1 h recording), relaxation of the ileum was observed in the presence of increasing cumulative concentrations of AITC (10nM–300μM). The incubation time for each concentration was 20 min. Preliminary experiments showed that AITC produced maximal effects within 20 min. The effect of the antispasmodic drug papaverine (100 μM) was evaluated at the end of each experiment. Inhibitory responses of AITC on spontaneous contractility were expressed as % of the maximal relaxant response produced by 100 μM papaverine, which produced a maximal relaxant response within 20–30 min.

AITC (10nM–300μM) was also evaluated after administration in the bath of the neuronal blocker tetrodotoxin (0.3 μM), the muscarinic receptor antagonist atropine (1 μM), L-NAME (300 μM) plus apamin (0.1 μM, a combination of drugs that is known to block enteric inhibitory nerves), the selective TRPA1 channel antagonists HC-030031 (100 μM) and AP18 (100 μM), the non-selective TRPA1 channel antagonists gentamicin (1 mM) and camphor (1 mM). The contact time for each inhibitor/antagonist was 30 min.

Contractions in the isolated distal colon

In this set of experiments, we studied the contractile effect of AITC. At the start of each experiment, colon was stimulated with exogenous acetylcholine (0.1 μM, contact time: 1 min). After stable control contractions evoked by acetylcholine had been recorded, the tissue was stimulated with AITC (10 nM–300 μM), contact time: 3 min for each concentration). Because the second responses to AITC showed clear desensitization in the same preparation (Penuelas *et al.*, 2007), only one response to AITC was recorded for each tissue. Thus, the concentration–response curve for AITC was obtained from different intestinal segments deriving from different mice. In the experiments using antagonists/inhibitors (see the following discussion), comparisons were made between preparations from different mice.

In some experiments, AITC (30μM and 100μM) was also evaluated after administration, in the bath, of the neuronal blocker tetrodotoxin (0.3 μM), the muscarinic receptor antagonist atropine (1 μM), the 5-HT_3 receptor antagonist ondansetron (0.1 μM), the selective TRPA1 channel antagonists HC-030031 (10 μM) and AP18 (100 μM), the non-selective TRPA1 channel antagonists gentamicin (1 mM) and camphor (1 mM), the L-type Ca^{2+} blocker nifedipine (1 μM), the sarcoplasmic reticulum Ca^{2+}ATPase inhibitor cyclopiazonic acid (10 μM), the inhibitor of Ca^{2+} release from the sarcoplasmatic reticulum, ryanodine (10 μM) or the vehicles used to dissolve these inhibitors/antagonists, which were added to Krebs solution before the administration of AITC (contact time for each drug: 30 min, except for cyclopiazonic acid and ryanodine, which were left in contact with the tissue for 90 min).

In order to verify if HC-030031, AP18, gentamicin and camphor exerted non-specific antispasmodic effects, in preliminary experiments we evaluated the effect of these antagonists on the submaximal contractions induced by acetylcholine (0.1 μM). We found that AP18 (up to 100 μM), gentamicin and camphor (both up to 1 mM) did not modify acetylcholine-induced contractions. However, although HC-030031, at 10 μM, had no significant effect, at higher concentrations (30 and 100 μM), it reduced acetylcholine-induced contractions (inhibition at 30 μM: 29 ± 3%; inhibition at 100 μM: 45 ± 4; $P < 0.05$, $n = 6–7$). Therefore, in order to determine if AITC contracted the distal colon via activation of TRPA1 channels, we used 10 μM of HC-030031, 100 μM of AP18 and 1 mM of gentamicin and camphor.

Contractions were expressed as % of contractions produced by 100 μM acetylcholine, administered at the start of each experiment (this response to acetylcholine was comparable with the response at the end of the experiment); this

concentration of acetylcholine produced a maximal contractile response (100% contraction).

Upper gastrointestinal transit in vivo

Transit was measured by evaluating the intestinal location of rhodamine-B-labelled dextran (Izzo *et al.*, 2009). Animals were given fluorescent-labelled dextran (100 mL of 25 mg·mL^{-1} stock solution) via a gastric tube into the stomach. At 20 min after administration, the animals were killed by asphyxiation with CO_2 and the entire small intestine with its content was divided into 10 equal parts.

The intestinal contents of each bowel segment were vigorously mixed with 2 mL of saline solution to obtain a supernatant containing the rhodamine. The supernatant was centrifuged at 35 g to precipitate the intestinal chyme. The fluorescence in duplicate aliquots of the cleared supernatant was read in a multi-well fluorescence plate reader (LS55 Luminescence spectrometer, Perkin-Elmer Instruments; excitation 530 ± 5 nm and emission 590 ± 10 nm) for quantification of the fluorescent signal in each intestinal segment. From the distribution of the fluorescent marker along the intestine, we calculated the geometric centre (GC) of small intestinal transit as follows: GC = Σ (fraction of fluorescence per segment number) GC ranged from 1 (minimal motility) to 10 (maximal motility) (Capasso *et al.*, 2008a).

AITC (1–20 mg·kg^{-1}) or vehicle was given [i.p. or intragastric (i.g.), with the aid of a stomach tube] 30 min before the administration of the fluorescent marker. The effect of AITC (5 mg·kg^{-1} i.p. or 10 mg·kg^{-1} i.g.) was also evaluated in animals pretreated [10 min (i.p.) or 90 min (i.g.) before AITC] with the TRPA1 channel antagonist HC-030031 [30 mg·kg^{-1} (i.p.) or 100 mg·kg^{-1} (orally)]. The 90 min interval between the i.g. administration of the antagonist and AITC was necessary because the vehicle used to dissolve the antagonist (i.e. carboxymethylcellulose) affected the ability of AITC to modify the transit when the interval between the two i.g. administrations was shorter (when i.g. carboxymethylcellulose was given 30 or 60 min before the i.g. administration of AITC). In addition, i.p. administration of HC-030031 (100 mg·kg^{-1}) increased, *per se*, upper gastrointestinal transit and therefore was not used in the experiments aiming at reversing the effect of AITC.

The *in vivo* efficacy of HC-030031 as a TRPA1 channel antagonist, at the doses used in our experiments [30 mg·kg^{-1} (i.p.) or 100 mg·kg^{-1} (i.g.)] was assessed by evaluating this antagonist in the formalin model of pain (McNamara *et al.*, 2007; Aviello *et al.*, 2011). In this preparation, HC-030031 reduced pain (evaluated by measuring the time spent licking the inflamed paw) induced by 30 µL of a 1.25% formalin solution injected into the mouse hind paw.

Statistical analysis

Data are expressed as the mean ± SEM of experiments in *n* mice. To determine statistical significance, Student's *t*-test was used for comparing a single treatment mean with a control mean, and a one-way ANOVA followed by a Tukey–Kramer (or Bonferroni for the TRPA1 assay in cells) multiple comparisons test was used for analysis of more than two group means. P values < 0.05 were considered significant. The IC_{50} value (the concentration of AITC that produced 50% of maximal inhibition of the response) was used to characterize the potency of AITC and was calculated as geometric mean ± 95% confidence limits (CL) with GraphPad Prism® software (La Jolla, CA, USA).

Materials

ACh hydrochloride, AITC, N^G-nitro-L-arginine methyl ester (L-NAME) hydrochloride, apamin, camphor and gentamicin sulphate were purchased from (Sigma, Milan, Italy). AP18 [4-(4-chlorophenyl)-3-methyl-3-buten-2-one oxime], HC-030031 and ondansetron hydrochloride were was purchased from Tocris Cookson (Bristol, UK). AP18, HC-030031 were dissolved in DMSO whereas the other drugs were dissolved in saline. AITC was dissolved in DMSO to yield a 3 M concentration. Dilutions to 10^{-1} M were made in DMSO, with subsequent dilutions (starting from 10^{-2} M) with distilled water. AITC and HC-030031 were suspended in carboxymethylcellulose 1% in the set of experiments in which these drugs were administered by gavage. The drug vehicles had no significant effect on the responses under study, both *in vitro* and *in vivo* (see Results section).

Results

TRPA1 receptor assay

Using a fluorimetric test, we showed that AITC was able to increase $[Ca^{2+}]_i$ in HEK-293 cells stably transfected with cDNA for the rat TRPA1 channels, with a concentration for half-maximal activation of 2.5 ± 0.7 µM. AITC did not increase $[Ca^{2+}]_i$ in non-transfected HEK-293 cells.

AP18, HC-030031, camphor and gentamicin were found to antagonize the agonist effect of AITC (10 µM) on TRPA1-mediated increases of $[Ca^{2+}]_i$ in HEK-293 cells over-expressing rat TRPA1 channels (Figure 1). The antagonists behaved as TRPA1 channel antagonists with IC_{50} values against AITC

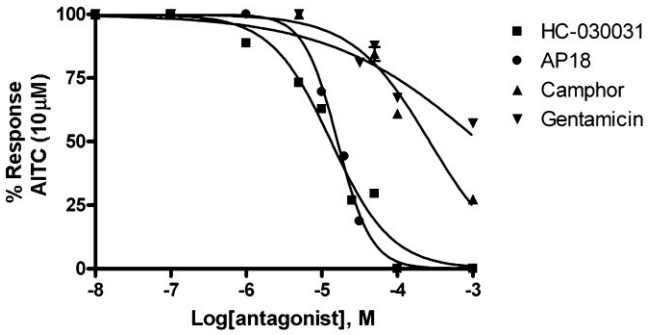

Figure 1

Concentration-related inhibition by gentamicin, camphor, HC-030031 and AP18 on AITC–induced elevation of $[Ca^{2+}]_i$ in HEK-293 cells stably transfected with the rat recombinant TRPA1 channel. AITC elicited no response in nontransfected cells. Data are expressed as percentage of the maximal effect observed with AITC (10 µM). Data are the means of 4 separate determinations. Standard error bars are not shown for the sake of clarity and were never greater than 5% of the means.

(10 μM) of 16.2 ± 0.5 μM (AP18) 13.3 ± 1.5 μM (HC-030031), 1.65 ± 0.33 mM (gentamicin) and 0.55 ± 0.05 mM (camphor).

Effect of AITC on agonist (or EFS)-induced contractions in the isolated ileum

EFS (8 Hz for 10 s, 400 mA, 1 ms pulse duration) of the mouse ileum or administration of agonists [acetylcholine (1 μM) or $PGF_{2\alpha}$ (0.2 μM)] evoked a contractile response that was about 50% of the contraction produced by 100 μM acetylcholine. The 100 μM concentration of acetylcholine produced a maximal contractile response in the ileum (100% contraction). EFS-induced contractions were blocked by tetrodotoxin (0.3 μM) and atropine (1 μM). AITC (10nM–300μM), but not its vehicle, (DMSO, 0.03%), inhibited agonist- and EFS-evoked contractions (Figure 2) with similar potency [IC_{50} (CL.) 3.98 μM (1.33–11.8 μM) vs. acetylcholine; 4.38 μM (1.88–10.1 μM) vs. EFS; 3.71 μM (1.67–8.27μM) vs. $PGF_{2\alpha}$]. The inhibitory effects of AITC were statistically significant at 10 μM and above, irrespective of the stimulus used to evoke contractions.

AITC also inhibited the contractions evoked by KCl (65.9 mM) with a similar IC_{50} value (data not shown).

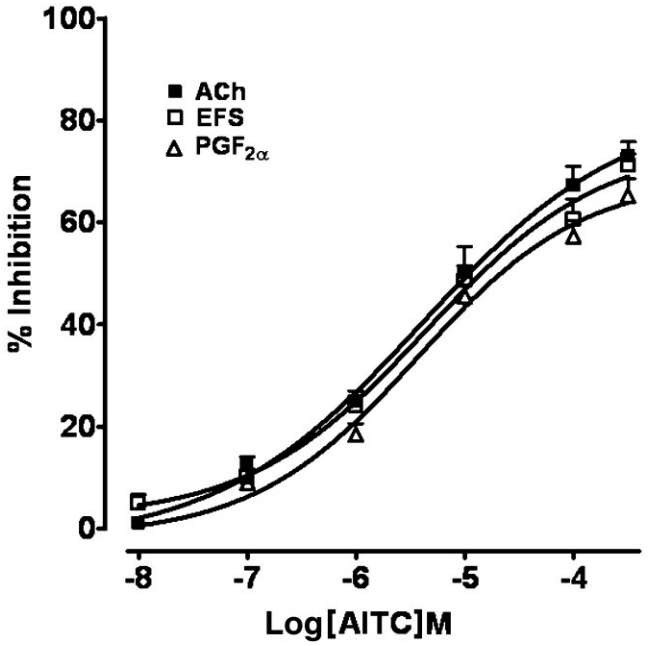

Figure 2

Isolated mouse ileum: inhibitory effect of AITC (10nM–300μM, shown as 10^{-8}–3×10^{-4} M) on the contractions induced by acetylcholine (ACh, 1 μM), $PGF_{2\alpha}$ (0.2 μM) or EFS in the isolated ileum. Each point represents mean ± SEM of 7–10 experiments. AITC significantly reduced contractions (vs. corresponding control) at 10μM and above ($P < 0.05$, at 10μM; $P < 0.01$, at 100 μM and 300 μM). No statistical differences were observed among the curves representing the inhibitory effect of AITC against the three different stimuli (ACh, EFS and $PGF_{2\alpha}$).

Spontaneous contractility in the isolated ileum

Isolated segments of mouse ileum displayed spontaneous activity, characterized by phasic contractions with amplitude corresponding to about 2 mN and frequency of about 30 cycles·min^{-1}. These contractions were not affected by tetrodotoxin (0.3 μM), suggesting that they were myogenic in nature.

AITC 10nM–300μM but not its vehicle (DMSO, 0.03%), caused a concentration-dependent inhibitory effect, consisting in a decrease of both mean amplitude of spontaneous contractions and resting tone (Figure 3A). The threshold concentration of AITC producing a significant effect was 10μM. The effect of AITC was not significantly modified by the TRPA1 channel antagonists gentamicin (1 mM), camphor (1 mM), HC-030031 (100 μM) and AP18 (100 μM) (Figure 3B), by tetrodotoxin (0.3 μM) and atropine (1 μM) (Figure 3C) and by a combination of L-NAME (300 μM) and apamin (0.1 μM) (Figure 3D). The vehicles used to dissolve these antagonist/inhibitors (i.e. DMSO 0.01% or distilled water 0.1%) did not affect the concentration–response curve to AITC. The inhibitory effect of a submaximal concentration of AITC was stable for at least three to four doses; however, after washing the tissues for subsequent AITC administration, the spontaneous contractility (control contractility) gradually reduced. In some experiments, at the higher concentrations of 100μM and 300μM, the inhibitory effect of AITC was preceded by a small contractile effect, 5–8% of the maximal contractile effect caused by acetylcholine (100 μM).

Given alone (i.e. in the absence of AITC), gentamicin (1 mM) and camphor (1 mM) did not modify significantly spontaneous contractions. AP18 (100 μM), HC-030031 (100 μM), and atropine caused a small inhibitory effect (10–15% inhibition for AP18 and HC-030031, about 30% inhibition for atropine), L-NAME plus apamin provoked an increase of spontaneous motility.

AITC-induced contractions in the isolated distal colon

AITC evoked a concentration-dependent contraction of the isolated distal colon, with an effective threshold concentration at 30μM (Figure 4). DMSO (0.01%) the vehicle used to dissolve AITC, did not induce contraction. The contraction induced by AITC was rapid in onset (latency less than 20 s) and reached a maximum within 30–60 s. When the tissue bath was drained and fresh Krebs was added, there was rapid relaxation of the muscle to base-line resting tension. The contractions induced by effective and submaximal concentrations of AITC (30 μM and 100 μM) were not significantly modified by HC-030031 (10 μM) and AP18 (100 μM) (selective TRPA1 channel antagonists) as well as by gentamicin (1 mM) and camphor (1 mM) (non-selective TRPA1 channel antagonist (Figure 5). In addition, the neuronal Na^+ channel blocker tetrodotoxin (0.3 μM) and, to a lesser extent, the 5-HT_3 receptor antagonist ondansetron, significantly reduced AITC (100μM)-induced contractions, whereas the muscarinic receptor antagonist atropine (1 μM) showed a trend toward a reduction, which, however, did not reach a statistical significance (Figure 6A–C). AITC (100 μM)-induced contractions were strongly reduced by the L-type Ca^{2+} channels blocker

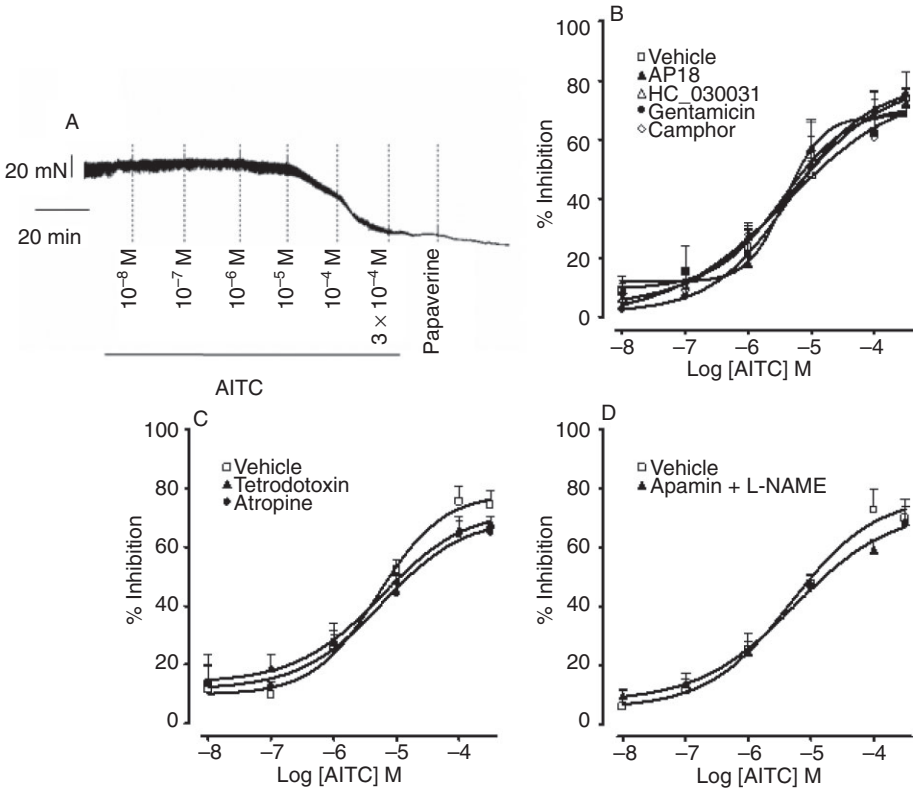

Figure 3

Isolated mouse ileum: inhibitory effect of allyl isothiocyanate (AITC), 10 nM–300 μM, shown as 10^{-8}–3×10^{-4} M, see a typical experimental trace showing the inhibitory effect of cumulative concentrations of AITC with papaverine (100 μM) added at the end of the experiment, (A) on intestinal spontaneous contractility alone (vehicle) or in presence of AP18 (100 μM), HC-030031 (100 μM), gentamicin (1 mM), camphor (1 mM) (B), tetrodotoxin (0.3 μM) or atropine (1 μM) (C) or L-NAME (300 μM) and apamin (0.1 μM) (in combination) (D). Each point represents mean ± SEM of five to seven experiments. AITC significantly reduced spontaneous contractility (vs. control) at 10 μM and above ($P < 0.05$, at 10 μM; $P < 0.01$, at 100 μM and 300 μM). No statistically significant differences were observed among the curves representing the inhibitory effect of AITC alone (vehicle) or in the presence of the various antagonists/inhibitors (i.e. AP18, HC030031, gentamicin, camphor, tetrodotoxin, atropine, apamin plus L-NAME).

nifedipine (1 μM), by cyclopiazonic acid (10 μM, an inhibitor of the sarcoplasmatic reticulum Ca^{2+}ATPase), and by ryanodine (10 μM, an inhibitor of Ca^{2+} release from the sarcoplasmatic reticulum) (Figure 6D–F). When tetrodotoxin was tested together with the Ca^{2+} modulators, the contractile effect was almost abolished (Figure 6D–F). The vehicles used to dissolve these antagonists or inhibitors (0.01% DMSO or 0.1–0.2% distilled water in Krebs solution) did not affect the concentration–response curve to AITC.

Effect of AITC on upper gastrointestinal transit

Injection (i.p.) of AITC (1–20 mg·kg^{-1}) reduced, whereas i.g. administration increased transit (Figure 7A and B). The inhibitory effect was significant starting from the 5 mg·kg^{-1}, whereas the stimulant effect was significant starting from the 10 mg·kg^{-1} dose. The effect of AITC 5 mg·kg^{-1} (i.p.) or AITC 10 mg·kg^{-1} (i.g.) was not modified by the TRPA1 channel antagonist HC-030031 [(30 mg·kg^{-1} (i.p.) or 100 mg·kg^{-1} (i.g.)] (Figure 7C and D). A higher (i.p.) dose of HC-030031 (i.e. 100 mg·kg^{-1}) was not used because, at this dose, the TRPA1 antagonist tended to increase upper gastrointestinal transit *per se* (data not shown). The drug vehicles, [DMSO, 4 μL per mouse (i.p.) or CMC 50 μl per mouse (i.g.)] did not affect intestinal transit (G.C. control 5.1 ± 0.4, DMSO 5.0 ± 0.4, $n = 10$; control 5.3 ± 0.4, carboxymethylcellulose 4.9 ± 0.3, $n = 10$). The vehicles did not affect transit also when they were injected twice (i.e. in the experiments when AITC was evaluated in the presence of the TRPA1 channel antagonists).

Discussion

In the present study, we have shown that AITC exerts both a contractile and an antispasmodic effect in the mouse gastrointestinal tract, and that these effects cannot be necessarily explained on the basis of the well-known ability of AITC to activate TRPA1 channels.

Inhibitory effect of AITC in the isolated ileum

AITC reduced, in a concentration-dependent manner, the contractions induced by acetylcholine, EFS, $PGF_{2\alpha}$ and KCl, with similar potencies. This non-selective profile of inhibition probably indicates that AITC acts directly on the final

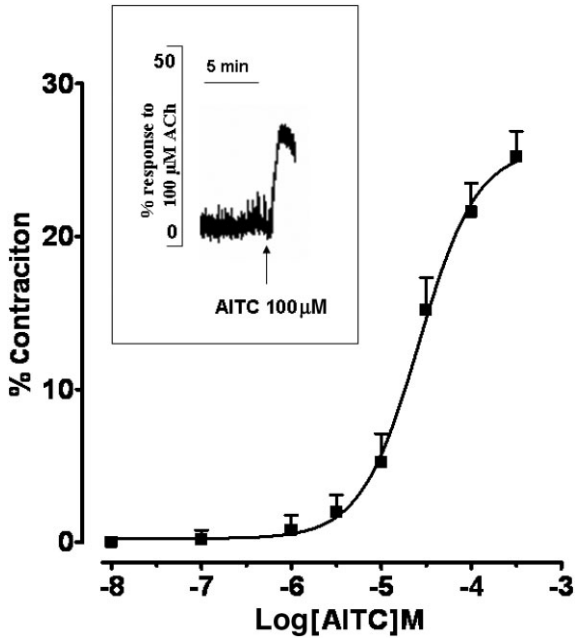

Figure 4

Concentration–response curve for AITC in the isolated mouse colon. The ordinates show the percentage of contraction compared with acetylcholine (ACh 100 μM, a concentration of acetylcholine that produced a maximal contractile response). Data are means ± SEM of 8 experiments. The insert shows a typical trace showing the contractile effect of AITC (100 μM).

effector of the contractile response (i.e. the smooth muscle) rather than on processes involved in neurotransmitter(s) release. The direct antispasmodic effect of AITC was confirmed in the set of experiments in which this plant compound was evaluated against the myogenic spontaneous ileal contractions. Using this experimental set up, we found that the inhibitory response of AITC in the mouse ileum was not modified by a blocker of neuronal conduction, tetrodotoxin, further suggesting a direct antispasmodic action on the smooth muscle. The inhibitory effect of AITC on ileal spontaneous contractility was not blocked by AP18 and HC-030031 (selective TRPA1 channel antagonists), nor by gentamicin and camphor (non-selective TRPA1 channel antagonists), suggesting the lack of involvement of these channels. Further experiments enabled us to exclude inhibition of the release of: (i) acetylcholine from neural or non-neural sources, as atropine did not modify the inhibitory effect of AITC on spontaneous ileal motility; (ii) other mediators from enteric inhibitor nerves, as the effect of AITC on spontaneous contractions was not modified by a combination of drugs, which is known to block enteric inhibitory nerves, that is, apamin (a blocker of Ca^{2+}-activated K^+ channels that blocks the enteric inhibitory component mediated by ATP or a related purine) plus L-NAME (a NO synthase inhibitor) (Costa *et al.*, 1986; Crist *et al.*, 1992).

Contractile effect of AITC in the isolated colon

Two previous reports have shown that AITC was able to contract both the isolated mouse colon (Penuelas *et al.*, 2007) and the guinea pig ileum (Nozawa *et al.*, 2009). In both studies, the effect was postulated to be due to activation of TRPA1 channels, although direct evidence for this assumption, for example through the use of selective TRPA1 channel antagonists – or TRPA1-deficient mice – was not provided. In the present study, we have shown that the contractile effect of AITC is not modified by two non-selective TRPA1 channel antagonists (i.e. gentamicin and camphor) and by two recently-developed selective TRPA1 channel antagonists, HC-030031 and AP18. All four antagonists counteracted the AITC-evoked increase in intracellular Ca^{2+} in HEK-293 cells overexpressing the rat TRPA1 channels. Although the antagonists were evaluated in two different species (cells overexpressing the rat TRPA1 channels and mouse intestine), these data suggest that the TRPA1 channel is not the primary target for AITC to induce contraction.

There is already evidence that these antagonists, at the concentration used in the present study, may effectively antagonize AITC-induced effects *in vitro*. Specifically, camphor (1 mM, the same concentration used here in the colon) and gentamicin (0.1 mM, a concentration that is ten-fold lower than the concentration here used in the colon) were previously shown to reduce the cough elicited by AITC (10 mM) in the guinea pig (Andrè *et al.*, 2009). The concentration of AP18 used in this set of experiments (100 μM) was five-fold higher than the concentration blocking responses to AITC in human lung fibroblasts (Hu *et al.*, 2010). Unfortunately, we could not use concentrations higher than 10 μM of HC-030031, because this antagonist, at 30 and 100 μM, also inhibited acetylcholine-induced contractions (see Results), demonstrating a non-specific antispasmodic effect. Nevertheless, at the concentration used in the present experiments (10 μM) or even at a lower concentration (0.5 μM), HC-030031 antagonized the response to AITC (0.2 mM) in rat isolated nodose neurons (Choi *et al.*, 2011), as well as blocking the TRPA1-mediated response in mouse dorsal root ganglion neurons (Zhang *et al.*, 2011). Overall, by using selective and non-selective TRPA1 channel antagonists, our data do not support the hypothesis that AITC contracts the isolated colon via activation of TRPA1 channels.

We have also shown here that the contractile effect of AITC was only in part blocked by tetrodotoxin. Atropine showed a trend toward the inhibition of AITC-induced contractions, whereas the 5-HT_3 receptor antagonist ondansetron showed a small, but significant, effect. These results suggest that the AITC-induced contractions were in part due to a direct action on the smooth muscle, that acetylcholine did not play an important role and that 5-HT might contribute to the contractile effect of AITC. In line with our results, Nozawa and colleagues found that AITC stimulated 5-HT release from enterochromaffin cells (Nozawa *et al.*, 2009).

We also evaluated the effect of AITC in the presence of drugs that influence Ca^{2+} levels. We found that that the contractile effect of AITC was greatly reduced by nifedipine, an L-type Ca^{2+} channel antagonist, by cyclopiazonic acid, an inhibitor of the sarcoplasmatic reticulum Ca^{2+} -ATPase pump – which thus allows internal Ca^{2+} stores to be depleted – and by ryanodine, which inhibits Ca^{2+} release from the sarcoplasmic reticulum by locking the ryanodine channel in a sub-conductance state (Alexander *et al.*, 2011). Collectively, these results suggest that the contractile effect of AITC depends

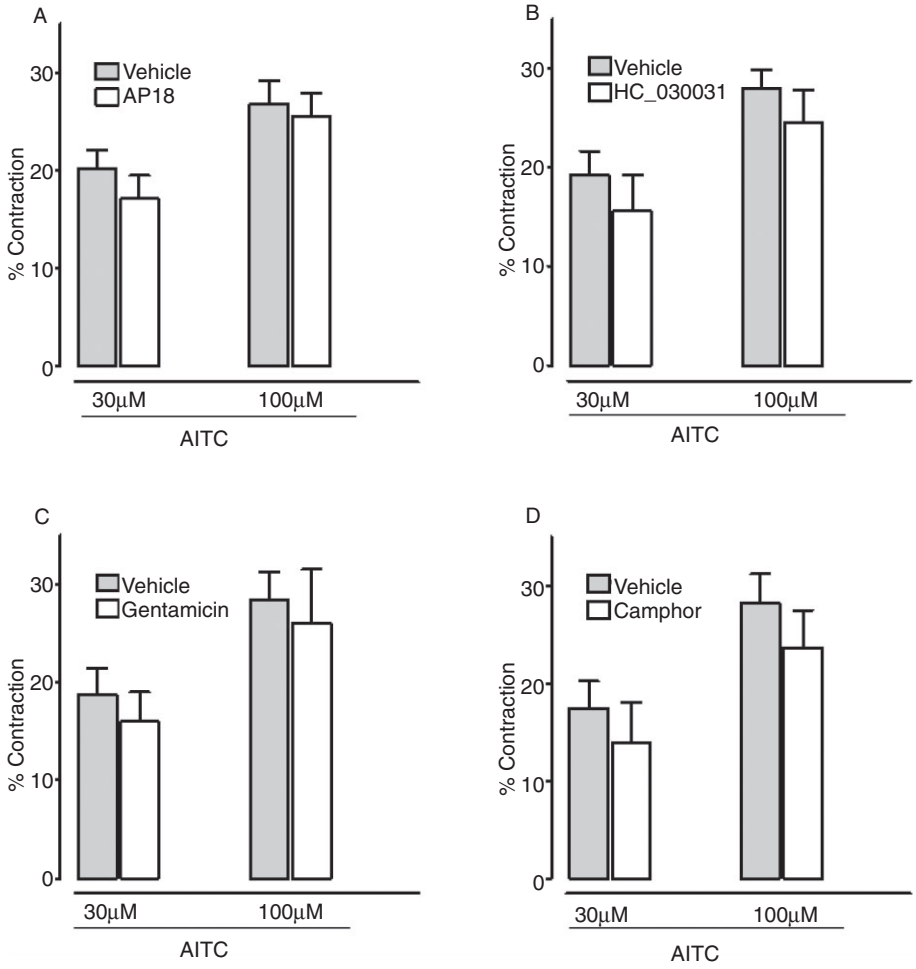

Figure 5

Isolated mouse colon: contractile effect of AITC (30 or 100 μM) alone (vehicle) or in the presence of AP18 (100 μM, A), HC-030031 (10 μM, B), gentamicin (1 mM, C) or camphor (1 mM, D). Each bar represents mean ± SEM of five to eight experiments. No statistical differences were observed.

both on Ca^{2+} entry through L-type Ca^{2+} channel and Ca^{2+} release from the sarcoplasmic reticulum, including ryanodine-sensitive, sarcoplasmic stores. In addition, the contractile effect of AITC was virtually abolished by combination of the Ca^{2+} inhibitors and tetrodotoxin. These results suggest that the tetrodotoxin-resistant (i.e. myogenic) component of the contractile effect of AITC involves Ca^{2+} (specifically L-type Ca^{2+} channels and Ca^{2+} release from the sarcoplasmic reticulum). Previous studies have shown that AITC-mediated contractions of rat urinary bladder are dependent on extracellular Ca^{2+} (Andrade *et al.*, 2006). Likewise, stimulation of mouse intestinal STC-1 cells with AITC increased intracellular Ca^{2+}, which was reduced by the L-type Ca^{2+} antagonist nimodipine (Purhonen *et al.*, 2008). It is worth noting that AITC-induced contractions in the isolated guinea-pig ileum were shown to be prevented by ruthenium red (Nozawa *et al.*, 2009), which, in addition to being a blocker of all TRP channels, is also an inhibitor of Ca^{2+} release from ryanodine-sensitive stores (Alexander *et al.*, 2011).

Effect of AITC on upper gastrointestinal transit in vivo

Few and contrasting results have been thus far reported on the effects of AITC on intestinal motility *in vivo*. Doihara and colleagues reported that AITC (1 $mg \cdot kg^{-1}$, i.g.) stimulated, in a ruthenium red-sensitive manner, gastric antrum and jejunum motility as well as inducing the occurrence of giant migrating contractions in the colon of fasted dogs (Doihara *et al.*, 2009a); on the other hand, the same authors have shown that AITC (1–10 $mg \cdot kg^{-1}$, i.g.) reduced the rate of gastric emptying in the rat (Doihara *et al.*, 2009b). In addition, AITC (1–10 $mg \cdot kg^{-1}$, i.g.) reversed clonidine- and-loperamide-induced constipation in mice (Kojima *et al.*, 2009). In the present study, we have shown that AITC reduced transit after i.p. administration, whereas it stimulated motility after i.g. administration. Although the reasons of these contrasting actions remain to be investigated, some hypotheses can be formulated. For example, the stimulating effect of AITC might be explained by assuming that the high gastrointesti-

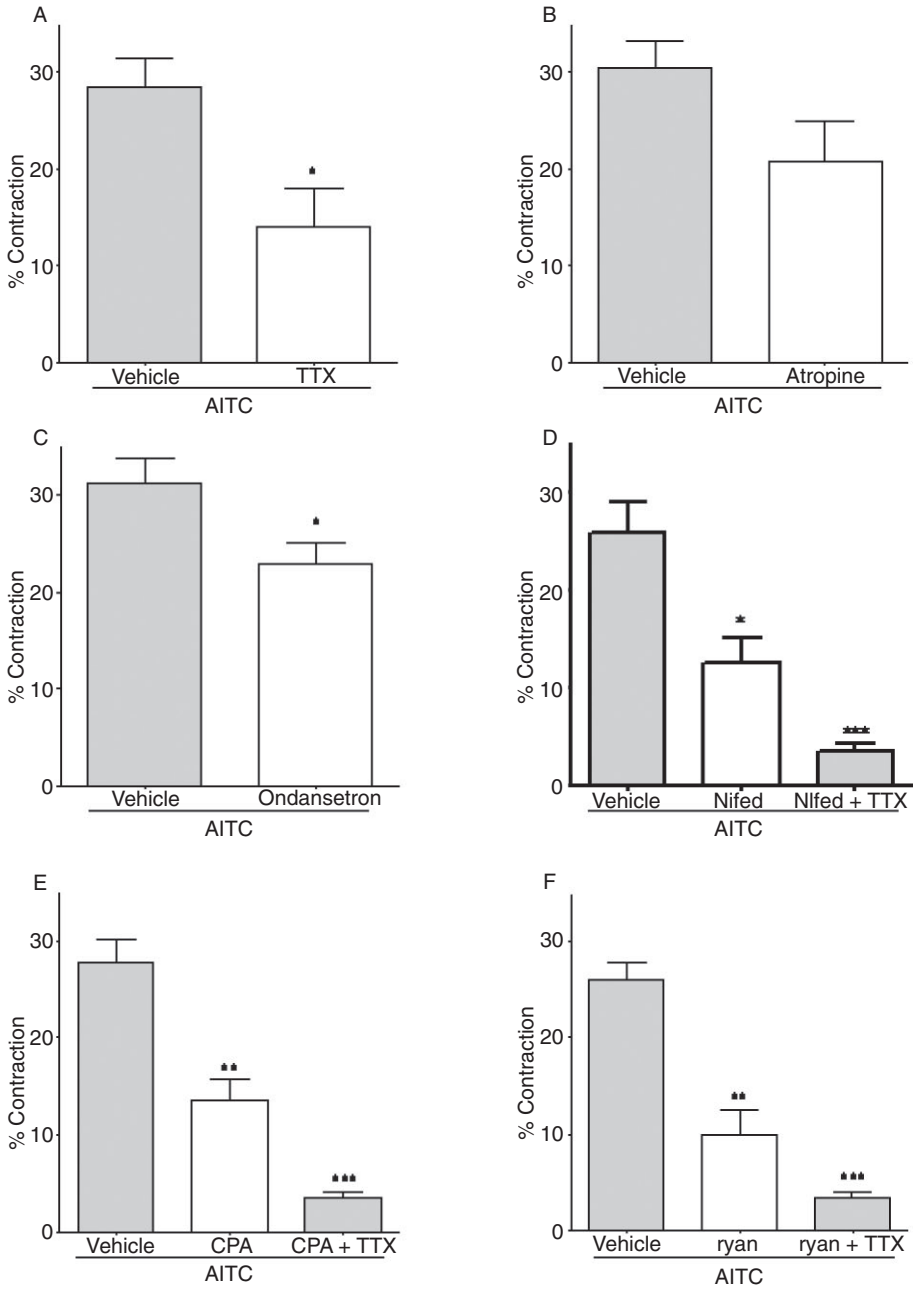

Figure 6

Isolated mouse colon: contractile effect of AITC (100μM) alone (vehicle) or in the presence of tetrodotoxin (TTX 0.3 μM, A), atropine (1 μM, B), ondansetron (0.1 μM, C), nifedipine (nifed, 1 μM, D), cyclopiazonic acid (CPA, 10 μM, E) or ryanodine (ryan, 10 μM, F). In another set of experiments, tetrodotoxin was given in combination with nifedipine, cyclopiazonic acid or ryanodine (D–F). Each bar represents mean ± SEM of 5–10 experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significantly different from vehicle.

nal amounts of AITC – that can be easily reached following i.g. (but not i.p.) administration – cause the release of endogenous stimulant molecules by mucosal cells. Consistent with this hypothesis, AITC has been shown to contract the guinea pig ileum through the release of 5-HT from enterochromaffin cells (Nozawa *et al.*, 2009) and to release cholecystokinin in enteroendocrine STC-1 cells (Purhonen *et al.*, 2008). In addition, in the present study, we have observed, in some ileal preparations, that AITC, at the high concentrations tested, caused a small contractile effect. This observation further supports the view that high amounts of AITC (present in the gastrointestinal tract after oral administration) might stimulate motility. Conversely, the inhibitory effect after i.p. administration could be due, for example, to a direct inhibitory action on muscle cells (more easily accessed from the systemic circulation), which is in line with our *in vitro* results showing the ability of AITC to inhibit contractility via a direct action on smooth muscle cells. Finally, we cannot rule

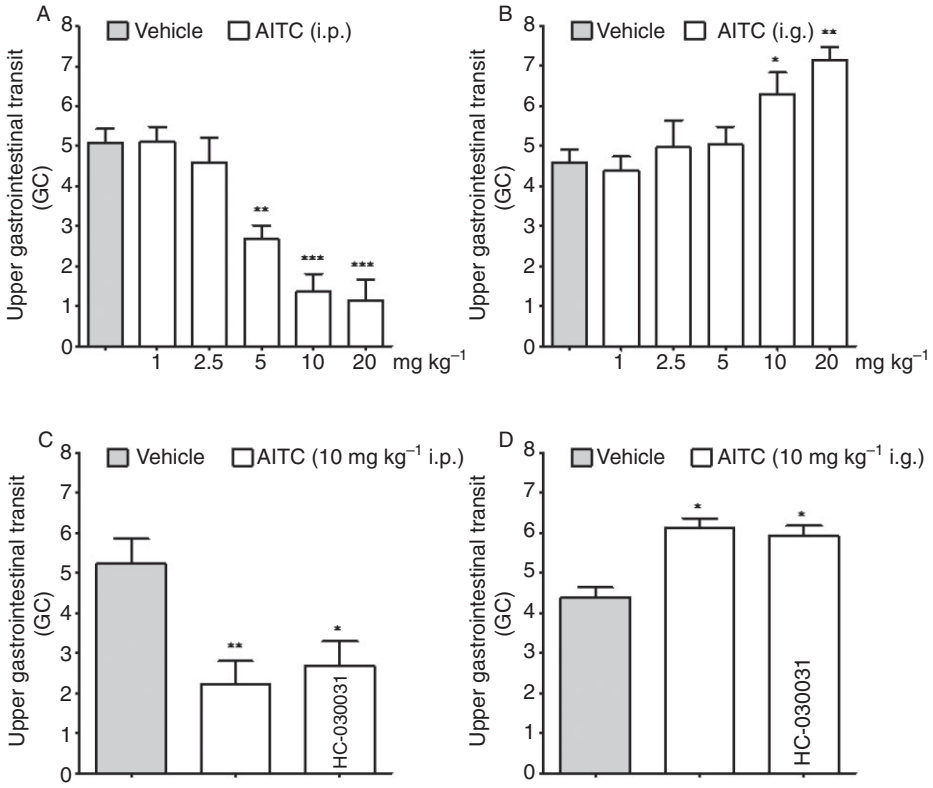

Figure 7

Effect of i.p. (A) or i.g. (B) AITC (1–20 mg·kg^{-1}) on upper gastrointestinal transit in mice *in vivo*. In a set of experiments, AITC [5 mg·kg^{-1} (i.p.) or 10 mg·kg^{-1} (i.g.)] was given in mice pretreated with the TRPA1 antagonist HC-030031 [30 mg·kg^{-1} i.p. (C) or 100 mg·kg^{-1} i.g. (D)]. Transit was expressed as the GC of the distribution of a fluorescent marker along the small intestine. GC ranged from 1 (minimal motility) to 10 (maximal motility) (see Methods). Each bar represents the mean ± SEM from 5–12 mice. *P < 0.05; **P < 0.01; ***P < 0.001, significantly different from control.

out completely the possibility that i.p. administration of AITC affects motility via altered autonomic activity (and not through a direct action in the gut), or that some compound(s) derived from the hepatic metabolism of AITC inhibited motility.

Consistent with *in vitro* studies, both the inhibitory and the stimulating effects of AITC on intestinal motility were not significantly modified by the selective TRPA1 antagonist HC-030031, administered both i.g and i.p. The i.p and i.g. doses of HC-030031 used in motility experiments were found to reduce, in our experimental conditions, formalin-induced pain, which is suggestive of efficacy of the compound as a TRPA1 channel antagonist (McNamara *et al.*, 2007).

Conclusions

We have shown that the plant-derived TRPA1 channel agonist, AITC, exerts *in vitro* both stimulatory and inhibitory effects on intestinal contractility. Specifically, AITC reduced ileal contractility and evoked contractions of the isolated colon. The contractile effect was, at least in part, due to a direct action of AITC on smooth muscle and involved Ca^{2+} entry through the L-type calcium channels as well as intracellular Ca^{2+} released from the sarcoplasmic reticulum. The inhibitory effect was due to a direct action on smooth muscles and did not involve enteric inhibitory nerves. *In vivo*, AITC reduced upper gastrointestinal transit after i.p. administration, whereas it increased motility after i.g. administration. Both *in vitro* and *in vivo*, the effect of AITC on intestinal contractility and motility was insensitive to antagonism of TRPA1 channels. The proposition that AITC contracts the isolated mouse colon via activation of TRPA1 channels, previously put forward on the basis of indirect evidence, should be revised. On the other hand, we cannot exclude the possibility that TRPA1 channels may modulate gastrointestinal contractility in other regions of the gut not investigated in the present study, such as the stomach, duodenum or proximal colon, as well as in other animal species.

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Conflict of interest

The authors state no conflict of interest.

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